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Selection and characterization of feather-degrading bacteria from canola meal compost

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Canola meal that contains a high level of protein (~40% crude protein) was used as compost material for the isolation of feather-degrading bacteria. After 7 and 14 days, bacteria were isolated from compost amended and unamended with soil. Eighty bacterial isolates from canola meal compost were then grown on milk-agar and isolates that produced proteolytic enzymes were identified by the formation of clear haloes around the colonies. A feather medium was chosen for a secondary selection of feather-degrading isolates. Of the eight isolates that hydrolyzed milk protein, five isolates hydrolyzed feathers. Their keratinolytic activities were subsequently confirmed by an assay using azo-keratin as substrate. Seven of the eight bacteria that hydrolyzed milk protein were *Bacillus* spp, and all five isolates that hydrolyzed feathers were strains of *Bacillus licheniformis*. Protease inhibition studies indicated that serine proteases are the predominant proteolytic enzymes produced by these feather-degrading isolates.

Keywords: *Bacillus*; feathers; keratinase; canola meal; compost

Introduction

The major component of feathers is β-keratin [5,8]. Because of the high degree of cross-linking by disulfide bonds, hydrogen bonding, and hydrophobic interactions [6,7], keratin is insoluble and difficult to digest by humans and animals. Feathers represent a large amount of protein that is currently under-utilized or wasted by the livestock industry. For more than half a century, studies have been done to convert feathers into digestible dietary protein for animals [2,25]. At present, feathers generated as a by-product of the poultry industry are usually converted to feather meal using physical and chemical treatments. Feather meal, however, is a poorly digestible feed ingredient [12,13].

To improve feather digestibility, research has focused on microbial proteolytic systems. A number of keratinolytic microorganisms have been reported to degrade feathers. Noval and Nickerson [15] and Nickerson *et al* [14] reported that a strain of *Streptomyces fradiae* digested keratins from wool, hoof and feathers. A strain of *Trichophyton gallinae* was found to possess a high affinity for chicken feathers [21]. Williams and Shih [23,24] isolated a feather-degrading bacterium, *Bacillus licheniformis* PWD-1, from a poultry waste digester. They subsequently used the PWD-1 strain to convert feathers into a digestible protein, feather-lysate [22]. Feather-lysate was digestible by chicken, with a nutritional value comparable to soybean meal to support the growth of chicks for the first 3 weeks. When cell-free extract or a crude preparation of keratinase of *B. licheniformis* PWD-1 was used as an additive to chicken feed with ground feather, a positive growth response was also observed [19]. These results suggested that feather-degrading microorganisms and their keratinolytic enzymes could be used to enhance the digestibility of feather keratin.

Although bacterial keratinolytic proteases show a potential to be utilized in the application of feather bioconversion, enzyme activities and yields have to be improved in order to make it possible for industrial applications. In addition, the mechanism regarding enzymatic hydrolysis of insoluble feather keratin remains unknown. It is important to isolate a variety of different microorganisms and their enzymes for the studies of keratin hydrolysis. In this way, we can probe the process of feather degradation under variable biological and enzymatic conditions. Furthermore, continuing study on DNA and amino acid sequences of the keratinases may provide information that leads us to more active keratinase using site-directed mutagenesis.

In this report, we detail the isolation and characterization of feather-degrading *Bacillus licheniformis* strains from canola meal compost. Some features, such as growth conditions, enzyme productions, and protease profiles, were also determined.

Materials and methods

Canola meal compost

A compost was prepared by mixing canola meal (40% crude protein, Lethbridge Research Centre, AB, Canada) and vermiculite (Aldrich Chemical Company Inc, Milwaukee, WI, USA) in a ratio of 2:1 (w/w). The mixture was then adjusted to a final moisture content of 50% with water and divided into two equal portions (3.5 kg each). One was used as a control to test the pre-existed microorganisms in canola meal and the other was inoculated with a soil sample (100 g). A dark brown Chernozemic soil sample collected from a field of crested wheatgrass (*Agropyron cristatum*) near Lethbridge, Alberta was used as a source of microorganisms. The soil was a sandy-loam texture consisting, by weight, of 54% sand, 18% silt, 29% clay and

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2% organic matter at pH 7.7. Compost was placed in a composter constructed from a 25-litre plastic pail. Holes (4-cm diameter) were drilled on the sides and bottom to facilitate air exchange and to allow drainage of excess moisture. A wire mesh was placed in the pail to contain the materials. The compost was placed at 20°C. A thermometer was inserted into the compost at a depth of approximately 20 cm to monitor the internal temperature. At 7 days, samples (~50 g each) were taken at a depth of 20–30 cm using a soil core sampler; 25 g was added into 100 ml of distilled H_2O at room temperature for pH determination and 10 g was used for the isolation of bacteria.

Isolation of bacteria from canola meal compost

For bacterial isolation, each 10-g sample was added to 90 ml of autoclaved potassium phosphate buffer (70 mM, pH 7.0). The solution was agitated for 1 min with a Stomacher blender (Seward Medical Ltd, London, UK) at the highest setting. The suspension was diluted four times in a 10-fold dilution series, and 100-μl aliquots from each dilution were spread on nutrient agar (NA) (Difco, Detroit, MI, USA) and a casein-peptone agar (CP) (per liter of solution: casein, 5 g; Bacto peptone, 5 g; NaCl, 4 g; K_2HPO_4, 1 g; Dehority's vitamin mix 10 ml; agar, 20 g; pH 6.5). Dehority's vitamin mix contains (per liter): pyridoxine HCl, 0.2 g; riboflavin, 0.2 g; thiamine HCl, 0.2 g; nicotinamide, 0.2 g; Ca-d-pantothenate, 0.2 g; *p*-aminobenzoic acid, 0.01 g; folic acid, 5 mg; biotin, 5 mg; and cobalamine, 0.5 mg. The plates were incubated at 37°C, the number of colonies were counted after 48, 72, 96 and 120 h of incubation.

Representative bacterial colonies were subcultured on NA plates. Catalase, oxidase and Gram-stain reactions were determined for all bacteria after 24-h growth. Bacteria were then grouped on the basis of their fatty acid profiles using gas-liquid chromatography (MIDI Inc, Newark, DE, USA) [17]. Representative taxa were further characterized using standard physiological tests [3,4,11,18]. All purified bacteria were lyophilized for long-term storage.

Identification of feather-degrading bacteria

A milk-agar plate was prepared for primary screening of bacteria that produced proteases. Bacterial isolates were grown on milk agar (per liter of solution: NaCl, 0.5 g; $MgCl_2{\cdot}6H_2O$, 0.1 g; $CaCl_2$, 0.02 g; KH_2PO_4, 0.7 g; K_2HPO_4, 1.4 g; Bacto skim milk, 50 g; agar, 18 g; pH 7.0) at 37°C. Protease-producing bacteria, as indicated by the formation of clear haloes, were selected. These bacteria were subsequently grown in a feather medium (per liter of solution: NaCl, 0.5 g; $MgCl_2{\cdot}6H_2O$, 0.1 g; $CaCl_2$, 0.06 g; KH_2PO_4, 0.7 g; K_2HPO_4, 1.4 g; feather, 10 g; pH 7.0) [9], in which feathers were the only sources of carbon and nitrogen. Cultures (25-ml feather medium in a 125-ml flask or a 15 × 2.8 cm test tube) were grown at 37°C and 50°C at 150 rpm for 2–5 days. Isolates that completely broke down feathers in the medium were selected.

Effect of temperature on growth and proteolytic activity

Selected feather-degrading isolates were grown in nutrient broth at 37°C for 7–8 h to reach a cell density of approximately 10^7 cells ml^{-1}. After dilution, about 100 cells of each isolate were spread on NA plates in triplicate and incubated at 37, 45, 50, 55, 60, 65 or 70°C overnight and growth was recorded. The colony formations were observed on NA plates. To determine the optimal temperature for protease production (OTpp), feather-degrading isolates were also placed on milk-agar in triplicate, and incubated at various temperatures. After 24, 36 and 48 h the diameters of clear haloes were measured. Based on the OTpp determined by plate assay, each isolate was subsequently grown in the feather medium in the temperature range of OTpp ± 10°C at 5°C intervals. Samples taken at 12-h intervals were centrifuged at $12\,800 \times g$ for 5 min, and the supernatants were used for proteolytic activity assay.

Keratinolytic activity was measured by the hydrolysis of azo-keratin, a keratin derivative. Azo-keratin was prepared by reacting ball-milled feather powder with sulfanilic acid and $NaNO_2$ using the method described by Tomarelli *et al* [20] for the preparation of azo-albumin. Five milligrams of azo-keratin were used to assay keratinolytic activity as described by Lin *et al* [10]. The optimum temperature for proteolytic activity was determined by using the samples taken at the times of peak activity. One unit of keratinolytic activity was defined as an increase of 0.01 absorbance units at 450 nm per minute at the optimum temperature.

Azo-casein (Sigma Chemical Co, St Louis, MO, USA)

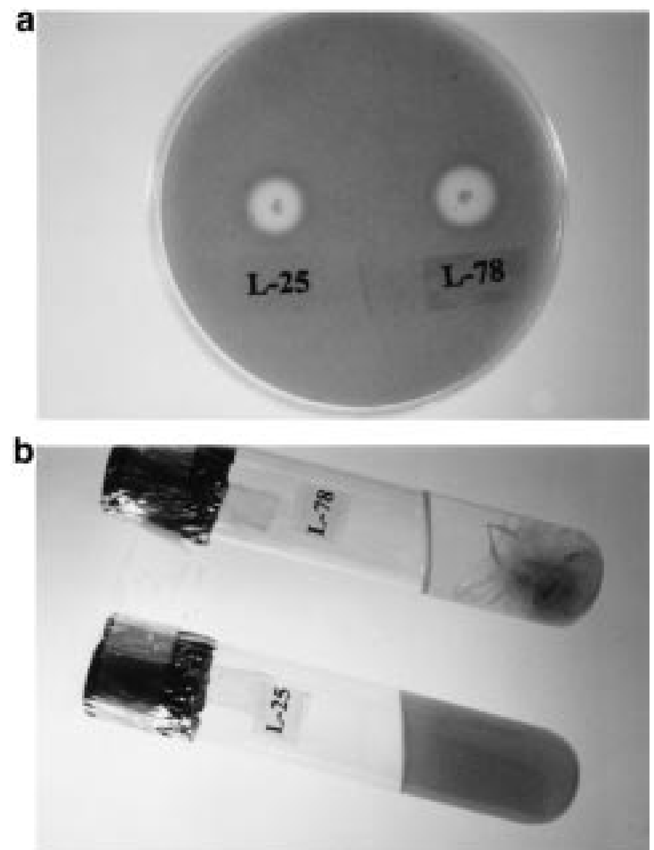

Figure 1 Comparison of a feather-degrading strain (L-25) and a non-feather-degrading strain (L-78) of *Bacillus licheniformis*. (a) On milk-agar plate. (b) In feather medium. L-25 completely hydrolyzed feathers in the medium in 48 h (turbid), whereas L-78 was unable to degrade feathers and the medium remained clear with intact feathers.

hydrolysis was used as an alternative to the azo-keratin hydrolysis. This assay was carried out in a tube containing 0.1 ml of culture supernatant and 0.4 ml of azo-casein solution (5 mg ml^{-1} in 50 mM potassium phosphate buffer, pH 7.5) for 15 min. The reaction temperature was the same as that used in azo-keratin hydrolysis. The reaction was then terminated by the addition of 0.5 ml of 4% trichloroacetic acid solution (w/v). After centrifugation for 5 min, the absorbance of supernatant was read at 440 nm. One unit of caseinolytic activity was defined as an increase of 0.01 absorbance units per minute under the given conditions.

Inhibition of proteolytic activity

The effects of protease inhibitors on the proteolytic enzyme activities of feather-degrading bacteria were measured by azo-casein hydrolysis. Culture supernatants taken at peak activity were used. Phenylmethylsulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), iodoacetic acid, and pepstatin were used to inhibit serine, metallo-, cysteine and aspartic proteases, respectively. The inhibitor working concentrations were used as recommended: PMSF, 1 mM; EDTA, 5 mM; iodoacetic acid, 10 mM; and pepstatin, 1 μM [1,16]. The control treatment consisted of azo-casein without inhibitors. The percentage of each type of protease was calculated as follows:

$$\% \text{ of specific protease} = \frac{A - Ai}{A} \times 100$$

Where A is the total activity measured by azo-casein hydrolysis without any inhibitors, and Ai is caseinolytic activity determined when a specific inhibitor was present.

Results

Canola meal compost

At day 7, the temperatures of the control and soil-amended composts were 50–55°C, and decreased to slightly above ambient by day 14. The pH of the compost was 7.4 and 7.8 at day 7 for the control and soil-amended composts, respectively. At day 14, the pH of both composts was 8.0. Numerous bacteria were recovered from both canola meal composts. At day 7, more than 500 bacterial isolates were taken from soil-amended compost, which was approximately two-fold more than those from control compost. At day 14, some 200 varieties of bacteria were recovered from each compost, no significant differences in number of isolates were observed. The predominant bacterial taxa isolated were *Bacillus licheniformis*, *B. sphaericus*, *B. circulans*, and *Pediococus pentosacens.* There were no conspicuous differences in species compositions between the soil-amended and the control treatments.

Identification of bacteria from Canola meal compost

Of the 80 isolates tested, eight demonstrated proteolytic activity on milk-agar. Five of them (L-9, -10, -25, -26, and -35) degraded feathers and were all *B. licheniformis* strains. Isolates L-9, -25, -26 were recovered from non-amended Canola meal compost, whereas L-10 and -35 were isolated from soil-amended compost. An additional *B. licheniformis* strain from soil-amended compost, L-78, exhibited a lower keratinolytic activity (Figure 1b) even though it rapidly hydrolyzed milk proteins (Figure 1a). Strain L-52 was identified as *Bacillus brevis*, but it demonstrated a minimal activity against feathers (Table 1). Strain L-56 did not degrade feathers and was identified as a *Staphylococcus* strain.

Effect of temperature on growth and proteolytic activity

Seven protease-producing isolates were thermophiles (Table 1). Strain L-58 was able to grow at 50°C, but its optimal protease production occurred at 37–40°C. For all bacilli, proteolytic activities estimated on milk agar were higher at 50–55°C and keratinolytic activity in feather medium was higher at 40–45°C. Keratinolytic activity in feather medium increased over time for all five *B. licheniformis* isolates tested (Figure 2). Maximum activity asymptoted between 48 and 60 h, and keratinolytic activity ranged from 5.5 to 11.1 U ml^{-1} (Figure 2). Strain L-25 produced the highest keratinolytic activity, 11.1 U ml^{-1}. The pH of the medium increased with time for isolates able to degrade feathers. After 3 days of growth, the pHs were elevated from 7.0 to 8.3–8.4 for all five feather-degrading isolates.

Table 1 Characteristics of protease-producing bacteria

Isolate No.	Organism	Growth temp. range[a] (°C)	OTpp[b] (°C)	Proteolytic activity[c]	Keratinolytic activity[d]
L-9	*B. licheniformis*	37–65	40–45	+++	++
L-10	*B. licheniformis*	37–65	40–45	++++	+++
L-25	*B. licheniformis*	37–65	40–45	++++	++++
L-26	*B. licheniformis*	37–65	40–45	++	++
L-35	*B. licheniformis*	37–65	40–45	+++	+++
L-52	*B. brevis*	37–65	40–45	++	–
L-58	*Staphylococcus*	37–50	37–40	++	–
L-78	*B. licheniformis*	50–70	40–50	+++	–

[a]Growth temperature range was determined on NA plate.
[b]Optimal temperature for protease production in feather medium.
[c]Proteolytic activity was estimated by the diameter of hydrolysis haloes on milk agar after 48-h incubation at 50°C, except for L-58 at 40°C. The definitions of proteolytic activity were: 1–4 mm, +; 4–8 mm, ++; 8–12 mm, +++; >12, ++++.
[d]Keratinolytic activity was determined by azo-keratin hydrolysis after growth at 50°C in feather medium for 60 h. The definitions of keratinolytic activity (U ml^{-1}) were: '++++' $\geq$ 10; '+++' $<$ 10 $\geq$ 7.0; '++' $<$ 7 $\geq$ 4.0; '+' $<$ 4.0 $\geq$ 1.0; '–' $<$ 1.0.

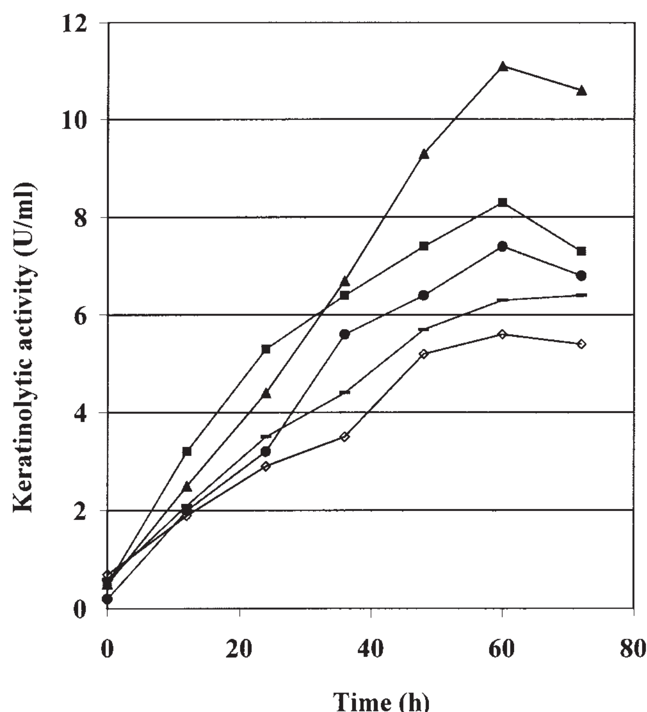

Figure 2 Keratinolytic activity produced by feather-degrading *Bacillus licheniformis*. The activity was measured using an azokeratin assay. One unit of keratinolytic activity was defined as an increase of 0.01 absorbance unit at 450 nm per minute at the optimum temperature. —▪— L-9; —●— L-10; —▲— L-25; —◇— L-26; —■— L-35.

Isolates that exhibited reduced keratinolytic activity, L-52 and L-56, had final pHs of 8.0 and 7.4, respectively.

Inhibition of proteolytic activity

Proteolytic activity of each isolate was also measured using the azo-casein hydrolysis method in the presence of four protease inhibitors (Table 2). Feather-degrading isolates (L-9, -10, -25, -26, -35) possessed a high percentage of serine protease activity (~75% of total caseinolytic activity). Isolates L-52 and L-78 exhibited weak keratinolytic activity, but they demonstrated elevated caseinolytic activity (Table 2), which was predominantly contributed by neutral protease (Table 2). No cysteine protease activity was observed for any of the isolates and aspartic protease activity was reduced (0–11%).

Discussion

Canola meal which contains a high level of protein (~40%), was chosen as compost material to culture protease-producing bacteria. We hypothesized that bacteria possessing the capacity to break down the crude protein in canola meal would be selected. However, of the 80 bacterial isolates collected from canola meal compost and subsequently grown on milk-agar, only eight demonstrated proteolytic activity. Seven of the eight protease-producing bacteria were bacilli; one isolate of *Staphylococcus* also hydrolyzed casein. The feather medium contains keratin as the only source of carbon and nitrogen, and eliminated non-keratinolytic isolates such as L-52, -56 and -78 (Figure 1a and b). Five of the eight protease producers isolated degraded feathers and azo-keratin, they were all *Bacillus lichenformis*.

During composting, the interior temperature of the composts rose 50–55°C. The elevated temperature is suitable for thermophiles and themotolerant bacteria. Seven out of the eight protease-producing isolates were thermophilic. The increases in pH of the medium during feather hydrolysis may be due to the release of excess nitrogen in the form of ammonia.

In feather medium, the best temperature range for production of keratinase by feather-degrading bacilli was between 40 and 45°C, which was lower than the best temperature range for proteolysis on milk plates (50–55°C). Perhaps it was due to severe proteolytic autolysis in liquid medium at higher temperature. For all five feather-degrading bacteria (L-9, -10, -25, -26, -35), serine protease activity accounted for approximately 75% of their total proteolytic activity. In contrast, the two isolates (L-52 and L-78) that did not degrade feathers possessed high levels of neutral protease activity; the sums of neutral and serine protease activities exceeded 100%. To verify these results, double and triple amounts of PMSF and EDTA were utilized for the activity assays, and no significant changes of readings were obtained. It is possible that there are proteases insensitive to PMSF and EDTA produced by these two isolates.

Previous results indicated that mammalian serine protease [10], such as trypsin, was not able to break down feather keratin efficiently. However, as demonstrated, serine proteases produced by the *B. licheniformis* strains expressed significant activity toward feather keratin. So far,

Table 2 Extracellular protease profiles of *Bacillus* spp

Isolate No.	Total activity[a] (U ml^{-1})	Serine protease[b] (%)	Neutral protease (%)	Cysteine protease (%)	Aspartic protease (%)
L-9	29.0	72.7	11.9	2.3	8.2
L-10	41.6	74.0	17.1	3.0	0.0
L-25	42.6	75.9	17.2	0.0	11.0
L-26	22.3	78.4	6.6	0.0	5.4
L-35	32.1	74.6	18.4	0.0	6.3
L-52	31.2	14.3	97.7	0.0	6.6
L-78	29.1	55.6	67.8	0.0	6.6

[a]Total activity was determined by azo-casein hydrolysis at 50°C without inhibitors.
[b]See Materials and methods for calculations.

it is uncertain whether serine protease (keratinase) works on keratin alone or is associated with other types of proteases, and what the appropriate ratios are between different types of proteases.

By the use of the two-step procedure, we have selected five isolates that are feather-degrading bacteria. With certain modifications, this procedure can be applied to the isolation of other feather-degrading microorganisms, such as fungi. We anticipate that more feather-degrading microorganisms and their keratinases will be isolated using different environmental conditions. These could lead to the discovery of the enzymatic process for keratinolysis.

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