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Discovery of keratinases using bacteria isolated from marine environments

Bastian Herzog[a,d], David P. Overy[a,b,d], Bradley Haltli[a,c,d], Russell G. Kerr[a,c,d,*]

[a] Department of Chemistry, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI C1A 4P3, Canada
[b] Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI C1A 4P3, Canada
[c] Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI C1A 4P3, Canada
[d] Nautilus Biosciences Canada, Duffy Research Center (NRC-INH), 550 University Avenue, Charlottetown, PEI C1A 4P3, Canada

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ABSTRACT

Bacteria are important for the biodegradation of keratin. Thus, a workflow to isolate keratin-degrading bacteria utilizing an optimized azo-keratin assay was established. Deteriorated feather samples, collected in marine shoreline environments from the intertidal zone, yielded 50 unique bacterial isolates exhibiting keratin degradation when feather meal was supplied as keratin substrate. The majority of isolates, identified by 16S sequencing, belonged to genera previously reported to produce keratinases: *Bacillus* spp. (42%) and *Stenotrophomonas* spp. (40%). The remaining 18% represented the genera *Alcaligenes*, *Chryseobacterium*, *Salinivibrio*, *Delftia*, *Stappia*, and *Microbacterium*, genera not previously been associated with keratinase production. The workflow, also applied to 21 *Bacilli* from our in-house culture collection, additionally revealed four *Bacilli* with remarkable feather degradation potential. The industrial applicability of their associated keratinases was evaluated and the most active keratinase expressed in *E. coli* to confirm keratinase expression. Enriched keratinase fractions demonstrated activity up to 75 °C and retained viability when stored lyophilized at 20 °C for up to 200 d.

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Introduction

Keratinases are a class of proteases able to degrade the insoluble protein keratin, which is the main structural component of feathers, horns, hooves, wool, hair, and fingernails [1]. Keratin is classified into three forms (α-, β-, and γ-keratin), according to the extent and distribution of the internal cross-linking of disulfide linked cysteine units [2], which makes the polymer recalcitrant to degradation [3]. Keratin is further strengthened by hydrogen bonding and hydrophobic interactions within the molecule, creating a densely packed polypeptide that withstands degradation by common proteases such as pepsin and trypsin [4]. Although it is recalcitrant, keratin does not accumulate in the environment due to the activity of specific proteases known as keratinases. A variety of biotechnological applications exist for keratinases such as in the disposal of keratin-containing wastes, enhancing nutrient availability of feather meal as feedstock, in various processes in the leather industry, in the generation of ingredients for human skin-/haircare formulations, and as an additive to laundry and dishwashing detergents [5–7]. As keratinases are often stable over a wide range of temperatures and under alkaline conditions, these enzymes are being evaluated as a replacement for many proteases currently used in various industrial applications [8,9].

In the environment, keratinases produced by filamentous and non-filamentous bacteria degrade feather keratin [1], liberating amino acids that are sequestered by the surrounding microbial community as a source of nitrogen. Several million tons of feather waste are generated annually by the poultry industry [6]. To exploit this protein-rich waste byproduct, feathers are converted into feather meal (an animal feed supplement) by baking at a high temperature and pressure; however, in addition to being expensive, this process results in the destruction of certain essential amino acids yielding a product with poor digestibility and variable nutrient quality [6]. Versazyme is an example of a bacterial keratinase developed to improve the energy efficiency of the process (as a less expensive alternative) while enhancing the nutritional value of the feather meal supplement [10]. The realized potential of bacterial keratinases for various industrial uses has motivated screening programs to isolate and identify microbial strains that produce highly

* Corresponding author at: Department of Chemistry, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI C1A 4P3, Canada.
E-mail address: rkerr@upei.ca (R.G. Kerr).

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active keratinases that remain stable under extreme environment conditions [11]. To date, only a few different genera of bacteria are demonstrated keratinase producers and many of these known organisms belong to the *Bacillus* genus, especially *B. licheniformis* and *B. subtilis* [1]. *Bacillus licheniformis* strains secrete keratinases with molecular weights (MWs) ranging from 26 to 42 kD and highest activities at temperatures ranging from 50 to 70 °C [12,13]. Their catalytic type is either thiol-based (similar to cysteine proteases) or serine-based (similar to serine proteases) which facilitates keratin degradation [14]. *Bacillus subtilis* keratinases range in MW from 20 to 65 kD and have a temperature optima ranging from 40 to 55 °C, a catalytic activity (in most cases) similar to serine proteases, and a high sequence similarity to subtilisins [15].

As *Bacillus* spp. are reported producers of keratinases [1], 21 aquatic *Bacilli* previously isolated from freshwater, marine, and hypersaline environments were screened in this study with an azo-keratin assay, developed for screening environmental isolates for keratinase production. Azo-keratin was generated based on the dying procedure developed for albumin [16]; on the premise that the azo-dyes would bind to amino acids on the surface of feather particles. Azo-keratin (azo-dyed feather meal) is insoluble in water and easily removed from the broth by centrifugation. During incubation with keratinases, smaller, soluble protein particles and free amino acids cleaved from the azo-keratin result in a colored broth. From the increase in absorbance due to the liberation of the azo-dye bound amino acids, the assay can be used to calculate keratinase activity values. It was additionally applied to a large number of environmental samples to identify keratinase producing bacterial species.

Material and methods

Chemicals

All chemicals and media components were purchased from VWR International (Radnor, USA). Feather meal (FM, heat treated and milled) was obtained from Rothsay (Truro, Canada).

Sample collection

Ten feathers showing visible signs of structural deterioration were collected from the intertidal zone along the shore at Brackley Beach, Prince Edward Island (46.430210, -63.197933). The feathers were kept cool and in a sterile plastic bag until arrival in the laboratory. Before further processing, the feathers were gently washed three times with sterile seawater to remove adhering material.

Bacterial isolation

All isolation experiments were performed in feather meal (FM) medium containing 10.0 g L^{-1} NaCl, 0.2 g L^{-1} LB medium, and 10.0 g L^{-1} FM (solid medium was prepared by adding 10.0 g L^{-1} agar). Feather samples were cut with a sterile knife into ca. 1 cm^2 pieces (Fig. 1). Each piece was submerged in 25 mL liquid FM medium and vigorously vortexed. The suspension (200 μL) was used to inoculate 100 mL FM medium in Erlenmeyer flasks, and to inoculate solid FM medium. The remaining feather piece was placed on solid FM medium to screen for bacteria growing on and out of the feather piece. Enrichment cultures were incubated at 22 °C and the liquid enrichment cultures were shaken on an orbital shaker at 200 rpm. Plates were incubated for 7 d, examined daily for bacterial growth and emerging colonies were purified by serial sub-culturing on new FM plates. Liquid enrichment cultures were shaken for 14 d and 1.0 mL was serially diluted from 10^{-1} to 10^{-6}. From each dilution, 200 μL was plated immediately on solid FM medium and screened for bacterial growth over 7 d. The dilution series itself was also incubated for 7 d, then 1 mL was withdrawn, diluted in the same manner, plated on solid FM medium, and screened for bacterial growth over 7 d.

Azo-keratin preparation

Azo-dyed FM (azo-keratin), used to detect and quantify keratinase activity, was prepared according to Refs. [17,18] with the following optimizations (Fig. 1). A single 5 kg batch of commercially available FM served as raw material for all azo-keratin preparations ensuring good reproducibility among different azo-keratin batches. To prepare azo-keratin, thoroughly washed and dried FM (5 g) was suspended in 50 mL ddH_2O, mixed with a magnetic stirrer, and 8 mL of a 10% $NaHCO_3$ (w/v) solution added (solution 1). In a separate tube, 350 mg of sulfanilic acid and 150 mg $NaNO_2$ were dissolved sequentially in 15 mL 0.2 M NaOH. After acidifying with 1.5 mL of 5 M HCl (pH ca. 0-1) and mixing for 2 min, the solution (solution 2) was neutralized by adding 0.8 mL of 5 M NaOH (pH ca. 7-8). This solution was added to solution 1, mixed for 30 min and filtered. The insoluble azo-keratin was rinsed three times with ddH_2O, suspended in ddH_2O and shaken at 50 °C for at least 2 h followed by another filtration step. This procedure was repeated until the pH of the filtrate stabilized at 6.0–7.0 and the absorbance at 450 nm was less than 0.01. The last wash was performed with 50 mM PBS buffer (pH 7.5) and the azo-keratin freeze-dried for storage.

Screening for keratin degradation

Screening for general protease activity was performed on skim milk (SM) agar plates containing 15 g L^{-1} SM and 10 g L^{-1} agar (Fig. 1). All organisms were streaked on SM agar plates, incubated for 7 d at 20 °C, and subsequently monitored for protease activity by measuring the diameter of clearing zones (larger clearing zones indicated greater protease activity). Isolates with clearing zones were inoculated in parallel in 5 mL of two different media in 24 well plates (Seahorse Bioscience, Chicoppe, USA): one containing FM medium and the other containing full strength LB medium (25 g L^{-1}, VWR) without FM as nutrient source. Setups were incubated for 7 d at 20 °C after which the fermentation broth was clarified by filtration (0.2 μm cellulose acetate membrane) and concentrated by freeze-drying (Virtis Freezemobile, SP Scientific, Warminster, USA) at −80 °C, and ≤50 mTorr for about 24–48 h. Before use, the samples were redissolved in 400 μL PBS buffer (pH 7.5).

The azo-keratin assay (modified from Joshi et al., 2007) was used to screen protease positive isolates for keratinase production. Azo-keratin (10 mg) was suspended in 400 μL of 50 mM PBS buffer (pH 7.5) in a 1.5 mL tube. Enzyme solutions (50 μL) were added and the mixture incubated for 60 min at 55 °C. The reaction was terminated by adding 200 μL 10% trichloroacetic acid (TCA). After centrifugation (10 min, 10,000 g), the supernatant absorbance at 450 nm was measured in 96 well plates with a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, USA). Controls for non-enzymatic release of azo-dye already contained TCA before incubation along with azo-keratin and the enzyme solution. One unit (U) of activity was calculated as the amount of keratinase that caused an increase in absorbance of 0.01 with respect to the controls at 450 nm within 60 min in 1 mL (U mL^{-1}) [17].

Cloning and expression of the keratinase gene from Bacillus pumilus *AT16*

B. pumilus AT16 (in-house culture collection) and *E. coli* NEB5α, *E. coli* BL21 (DE3), and the expression vector pET28a (New England Biolabs, Whitby, Canada) were used. The DNA fragment encoding *kerA* from *B. pumilus* KS12 (HM219183) was

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