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Characterization of feather-degrading bacterial populations from birds' nests – Potential strains for biomass production for animal feed

Maria Saarela*, Mia Berlin, Heli Nygren, Panu Lahtinen, Kaisu Honkapää, Raija Lantto, Johanna Maukonen

VTT Technical Research Centre of Finland Ltd, P.O. Box 1000, FI-02044 VTT, Finland

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ABSTRACT

Feather degrading bacteria from birds' nests were characterized to find safe bacterial strains that could be utilized to convert feathers into soluble form and bacterial biomass for feed purposes. Of all tested 571 isolates 122 were keratinolytic. Partial sequencing of the 16S rRNA gene revealed that the keratinolytic isolates represented *Proteobacteria* (genera *Pseudomonas* and *Stenotrophomonas*) and *Firmicutes* (genera *Bacillus, Exiguobacterium, Paenibacillus, Rummeliibacillus,* and *Sporosarcina*). *Bacillus cereus* group and gram-negative bacterial isolates were not further characterized due to safety concerns related to potential pathogenicity or potential exposure of animals or workers to endotoxins (lipopolysaccharide part of the gram-negative cell wall). Keratinolytic strains showed clear differences in their ability to solubilize feathers with feather weight losses up to 30%. Amino acid composition of feather hydrolysates shifted from feather composition towards more bacterial-like composition. Especially the levels of lysine could be increased in the feather hydrolysate with bacterial fermentation. Feathers can be effectively hydrolyzed with non-pathogenic bacteria without any additional nutrients. The resulting feather hydrolysate is easier to digest than feather material and it has an improved amino acid composition regarding some limiting amino acids. The amino acid content of the final product can be tailored by varying the fermentation time and thus the ratio of bacterial cells to the feather hydrolysate.

1. Introduction

Protein-rich materials such as feathers, hair, nails and horns are abundantly available side-streams of the animal production. The need to better utilize these nutrient rich materials, together with the marked increase in the volume of poultry industry, has created a need for alternative methods for the thermo-mechanical processing to treat poultry feather material. Feather material is a challenging side-stream since it is resistant to mild biotechnical treatments (Brandelli, 2008). Currently in EU most of the slaughter house side streams (including for example bones, blood, hooves, hides and feathers) are either delivered directly from slaughter houses to fur animal farms for feed or they are treated at destruction facilities and afterwards used as e.g. energy sources, fur animal feed or fertilizer. The total amount of poultry feathers from broiler chicken and turkey produced annually in EU is about one million tons (estimation made on the basis of AVEC report 2014, Web ref.). As the amount of this protein-rich side-stream is significant, feathers should be seen as a valuable raw material for protein-based products. Bio-economical alternative uses for feathers are needed to better utilize their nutritional potential and/or technological properties suitable for various non-food applications, such as industrial absorbents (ADURO biopolymers, Web ref.), biocomposite building materials (New composite made from feathers, Web ref.), and various industrial fiber applications (Durham, 2004; Web ref.).

Feathers contain keratin (91%) as a highly stable insoluble structural protein, water (8%) and some lipids (1%) (Shih, 1993). Although they are resistant to various treatments, keratins are recycled in nature and can be degraded by some microbes. Keratinolytic enzymes are produced by some fungi, actinomycetes, and bacteria and have been isolated for example from poultry plants or soil (including polluted soils) (Jeong et al., 2010; Daroit and Brandelli, 2014; De Medeiros et al., 2016). Feather-degrading bacteria and their interaction with the host have been studied in wild bird populations (Burtt and Ichida, 1999, 2004; Gunderson, 2008). In addition, individual microbial strains, representing various

* Corresponding author.
E-mail address: maria.saarela@vtt.fi (M. Saarela).

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Bacillus and other microbial species have been investigated for their ability to utilize feathers as substrates for fermentation (Werlang and Brandelli, 2005; Fakhfakh et al., 2011; Sahoo et al., 2012; Al-Musallam et al., 2013; Demir et al., 2015; Liu et al., 2016).

Keratinases are proteolytic enzymes able to degrade insoluble keratin substrates (Brandelli, 2008). Screening for keratinolytic non-pathogenic microbes that could be incorporated into feed either as biomass or active additives may prevent the need for isolation and purification of the enzymes. In the case of biomass microbes would contribute to the protein and amino acid content of the fermented feather meal. Biomasses obtained from microbial fermentation of various substrates can be used in animal nutrition in EU without authorization, provided that they are safe and that the microbes are not genetically modified (EFSA Journal, 2011). To our knowledge this aspect of feather utilization has not been studied before. To make feather fermentation process economically viable, the fermenting bacteria should be easy to grow: They should have modest nutrient requirements (preferably growth on industrial side-streams) and mesophilic growth in aerobic conditions. To ensure safe working conditions the pathogenic potential of the strains should also be considered. Furthermore, endotoxin (lipopolysaccharide (LPS) part of the gram-negative cell wall) can a pose a health risk to workers when inhaled and potentially also to animals when ingested in high amounts (Cort et al., 1990; Thorn, 2001; Wallace et al., 2016).

The aim of the present study was to isolate and characterize feather degrading bacterial populations from birds' nests and to use them to produce biomass for animal feed. The study was focused on aerobic mesophilic gram-positive spore-forming bacteria since these could then be used directly to ferment feathers into biomass for feed use, without introducing any potential health risk related to lipopolysaccharide (LPS) of gram-negative bacteria.

2. Materials and methods

2.1. Isolation of bacteria from feather containing samples

Bird nest samples (containing mainly feathers but also moss, hay, bark, etc.) were obtained from Southern Finland, from various little birds' nests (including pied flycatcher, redstart, blue tit, great tit) and stored frozen at −20 °C until analyzed. In addition feather samples from wild Finnish grouse and farmed duck were included into the study. Feather containing sample material (1.25 g) was mixed with 50 ml of peptone buffer (0.85% NaCl 0.1% peptone, pH 7.2) and thoroughly mixed with Stomacher for 4 times 5 min. Part of the sample was heat treated (20 min at 75 °C) to destroy the vegetative cells. Both heat treated and non-heat treated samples were serially diluted and inoculated onto milk agar (Jeong et al., 2010) and *Bacillus* agar (Atlas, 1993) and aerobically incubated for 3 d at 30 °C. An optional way to isolate keratinolytic bacteria was first to mix the sample (1 g) with sporulation medium (40 ml) (DSMZ 531) as above. The mixture was then transferred into a Schott glass bottle (Sigma-Aldrich) and incubated for 3 d at 30 °C under agitation (100 rpm/min) in aerobic conditions. Thereafter the heat treatment and the culture were performed as above (for heat treated and non-heat treated samples). Individual bacterial isolates were picked from agar plates and cultured on *Bacillus* agar plates and finally stored at −80 °C in Protect Multipurpose beads (ThermoFisher Scientific). Altogether 571 isolates were frozen for further analysis.

2.2. Pre-processing of feather material for studies on keratinolytic activity

Feather material for the preparation of feather medium was obtained from a home-made feather pillow. Feathers were thoroughly washed under running tap water and dried at 60 °C for 48 h and ground with ultra-fine friction grinder (MKCA6-2, Masuko Sangyo Co. Ltd, Japan). The grinder was equipped with MKC type stainless steel fillings. During grinding the feathers were fed into the hopper and forced through a gap between rotary and stator grinding plates. Dry fibers were first ground with 4 mm gap width at 2000 rpm. The pre-ground feathers were then soaked with excess water and ground further with a decreasing gap width two more times. The second pass was ground with 2 mm and the third pass with 1.5 mm gap width at 2000 rpm. The feather slurry became a relatively fine feather suspension after the mechanical treatment.

2.3. Determination of keratinolytic activity

Casein was used as a substrate for the initial screening of proteolytic strains, since keratinases are not very substrate specific (Gupta and Ramnani, 2006). Using casein instead of feather material was also dictated by the availability of feather material as well as feasibility in testing a large number of isolates. Bacterial pure cultures (N = 571) were inoculated on milk agar plates to check for caseinolytic activity. All caseinolytic isolates were then inoculated on feather agar plates to identify the keratinolytic isolates among the caseinolytic ones. Feather agar (1 l) contained wet ground feathers (180 g (appr. 10 g of dry feather)), Na_2HPO_4 x 2 H_20 (0.376 g), NaH_2PO_4 x 2 H_20 (0.46 g), and agar (15 g). Feather agar plates were incubated 5 d at 30 °C. Keratinolytic isolates were identified based on the clear halo that formed around the bacterial colonies.

The ability of the selected isolates (N = 42) to utilize feather material in liquid culture was measured as a reduction of feather material weight during incubation. For the study bacterial isolates (taken from frozen stock) were grown in *Bacillus* broth overnight, cell density adjusted to McFarland 1.5 (Mättö et al., 2008) and feather broth bottles (70 ml; composition as above) inoculated with 1% bacterial inoculum in triplicate. Bottles were aerobically incubated at 30 °C for 2 days under agitation (170 rpm/min). After incubation the broth was filtered (Whatman 41; pore size 20 μm), washed and then filters were dried at 60 °C and weighed. Feather degradation was also visually evaluated and recorded. Reduction of feather material weight and change in pH were statistically analyzed by Student's t-test.

2.4. Identification of keratinolytic bacterial isolates

The identification of the keratinolytic isolates was based on partial 16S rRNA gene sequencing. The isolates were sequenced as previously described (Maukonen et al., 2012), with primers according to Satokari et al., (2001) (Table S1). Sequences were analyzed with RDP 10.31 (Ribosomal Database Project, RDP release 11, Web ref.), (EMBL-EBI) ClustalW2 (Clustal W2, Web ref.) and DNAMAN (Lynnon Corporation, version 4.1) and deposited into Genebank with accession numbers MF319343-MF319464. Different isolates representing the same species were initially fingerprinted with RAPD (random amplified polymorphic DNA) using different primers for *Bacillales*, *Xanthomonadales* and *Pseudomonadales* to exclude the incorporation of the same strain twice (Alander et al., 2001; Table S1). All isolates with similar RAPD type within a single sample were considered to represent the same strain. Twenty-eight of the best performing strains were deposited into VTT culture collection (Table 1).

2.5. Amino acid composition of feathers after bacterial fermentation

B. methylotrophicus VTT E-133292 was grown in *Bacillus* broth overnight. Moist chicken/broiler feathers (dry weight 30–33% of