Molecular Biology Research Communications 2026;15(4):197-204 DOI:10.22099/mbrc.2026.55909.2288

Original Article MBRC Open Access

Keratinolytic activity of *Stenotrophomonas rhizophila* and *Bacillus* spp. as keratin waste biodegradation

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ABSTRACT

Waste feathers pose a significant environmental challenge due to their resistance and the large amounts produced by the poultry industry. In this study, we isolated and characterized a novel keratinolytic strain, *Stenotrophomonas rhizophila* SMT, from horse feces and evaluated its feather-degrading ability and production of soluble keratin hydrolysates. Additionally, its keratinolytic ability was assessed along with both *Bacillus subtilis* and *Bacillus cereus*. *S. rhizophila* SMT was identified by biochemical profiling and confirmed by 16S rRNA gene sequencing, which showed 99% similarity to *S. rhizophila*. Primary screening on skim-milk agar and egg-yolk agar showed their keratinolytic activity. Subsequent cultivation in minimal salt media with chicken feathers confirmed and quantified their feather degradation efficiency by using the Bradford assay. After 13 days, *S. rhizophila* SMT produced 0.535 mg/mL soluble protein, while *B. subtilis* and *B. cereus* produced 0.615 mg/mL and 1.215 mg/mL, respectively. *S. rhizophila* SMT is a safe and non-pathogenic candidate despite producing less soluble protein than *B. cereus*. This makes it an eco-friendly choice for management of feather wastes, especially when biosafety is a priority.

Keywords: Feather; *Stenotrophomonas rhizophila* SMT; *Bacillus* spp.; Keratinase

INTRODUCTION

Chicken feathers are a major by-product of the poultry industry and contain a highly resistant-to-degradation protein known as keratin. Globally, the processing of approximately 400 million chickens daily produces nearly five million tons of feather waste. Furthermore, owing to the growing population and increasing need for animal-based protein, managing this waste is becoming a significant environmental obstacle by 2050 [1, 2]. Currently, most feather waste is either burned or landfilled, both of which have a negative environmental impact [3]. As a result, there is an urgent need for sustainable and eco-friendly approaches to managing this waste [3, 4].

β-Keratin is the main structural protein found in birds and reptiles, and about 90% of feathers contain β-keratin [3, 5]. The β-helical coils and stable disulfide bonds of β-keratin give it exceptional strength. As a result, feathers are resistant to degradation, even by common enzymes such as pepsin, trypsin, and papain [6, 7]. Keratinases are extracellular enzymes that

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pISSN 2322-181X eISSN 2345-2005

belong to the subgroup of alkaline metalloproteases or serine-like proteases. Although the precise mechanism of keratin degradation by keratinases is not fully understood, it is known to involve the breaking of disulfide bonds (sulfitolysis) along with protein cleavage (proteolysis) [8, 9]. Various keratin-degrading bacteria have been identified in environments such as soil, poultry waste, and feather disposal sites [8, 10]. Among them, feather-degrading bacteria (FDB) mainly include *Bacillus pumilus*, *B. subtilis*, *B. cereus*, *Stenotrophomonas gulbargensis*, and *S. flavus* [11].

Bioconversion using FDBs is an environmentally sustainable method for feather waste management. The keratinases of FDBs have numerous industrial applications, including fertilizers, animal feed production, surfactants, and heavy metal remediation [12]. Traditional methods of degrading keratin to supply animal feed destroy amino acids and reduce protein quality. However, it offers a better alternative to maintaining protein, providing a practical option for generating digestible keratin via peptide production [7, 8, 13]. There is considerable industry interest in microorganisms producing effective extracellular keratinases. However, they are not being used extensively due to the limited effectiveness of keratinolytic protease producers [14].

However, keratinase-producing bacteria beyond the *Bacillus* group remain relatively underexplored. The genus *Stenotrophomonas*, particularly *S. rhizophila*, is known as a plant growth-promoting rhizobacterium (PGPR) that can enhance nutrient availability and provide protection against phytopathogens. In recent years, *S. rhizophila* has drawn more attention as a safe and promising candidate for many practical applications, despite *S. maltophilia*, which is an opportunistic human pathogen [15]. **G**enomic studies have identified two keratinase genes, *KerSMD* and *KerSMF*, with broad substrate specificity [16].

Therefore, this study aimed to isolate and characterize a novel keratinolytic strain of *S. rhizophila* SMT from horse feces and to evaluate keratinolytic properties and feather-degrading capacities of *S. rhizophila* SMT, *B. subtilis*, and *B. cereus*.

MATERIALS AND METHODS

Sample collection and isolation: In the winter of 2024, a fecal sample was obtained from a healthy horse in Bajgah, Shiraz, Fars Province, Iran. The sample was collected under aseptic conditions and stored at 4°C until processing. *B. subtilis* and *B. cereus* were obtained from a laboratory collection at Shiraz University. For bacterial isolation, 0.5 g of the stool specimen was diluted with 4.5 mL of sterile normal saline. Serial dilutions were prepared and cultured on 10% sheep blood agar and MacConkey agar (Merck, Darmstadt, Germany) plates, followed by incubation at 37°C for 48 h. Morphologically distinct colonies were chosen and subcultured on both media to obtain pure isolates. The purified strains were stored in cryovials containing 20% (v/v) glycerol at −70°C for future analysis.

Screening keratolytic bacteria: For primary screening, all isolates were streaked on skim-milk agar and egg yolk agar. Egg yolk agar is composed of nutrient agar (Merck, Darmstadt, Germany) with a 5% egg yolk emulsion. Skim-milk agar contains 1% (w/v) skim-milk powder, 0.1% (w/v) NaCl, 1% (w/v) tryptone, and 1% (w/v) agar. Incubation at 37°C for 24h, the colonies with a clearing zone on this medium, predicting the production of proteolytic enzymes, was selected for transfer to minimal salt (MS) media (NaCl 2.5% (w/v), KCl 0.07% (w/v), $MgSO_4 \cdot 7H_2O$ 0.07% (w/v), NH_4NO_3 0.1% (w/v), KH_2PO_4 0.2% (w/v), and $CaCl_2$ 0.2% (w/v)) with 0.4%(w/v) feathers as an only source of carbon. The pH was adjusted to 7.0-7.3. In parallel, control MS media were cultured for each colony that had glucose (20%) instead of feathers. *B. subtilis, B. cereus, and a selected colony from the* fecal sample *were incubated in* Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) *overnight* at 150 rpm and 37°C. As the inoculum, a 2% (v/v) bacterial suspension in the logarithmic phase was transferred to

500 mL of MS media and control MS media, which were incubated for 13 days at 120 rpm and 37°C.

Soluble protein determination: Every two days, 5 mL of cultured media was taken to precipitate protein with 10 mL of cold acetone at -20°C for 20 minutes. The whole composition was centrifuged at 6000 rpm for ten minutes. Supernatants were allowed to air dry until no residual acetone remained. The soluble protein concentration in both test and control media was measured by using the Bradford assay [17].

Identification of the isolate: Identification of the isolate by the polymerase chain reaction (PCR) method, with the 16S rRNA gene acting as a molecular marker. For this purpose, DNA extraction from the isolate was performed using the boiling method outlined by Derakhshandeh et al. [18]. The DNA was then analyzed for quality and concentration using a NanoDrop spectrophotometer (Spectrum Instrumentation, Grosshansdorf, Germany) by measuring absorbance at 260 and 280 nm. For the PCR reaction, we added 3 µL of template DNA to 12.5 µL of *Taq* DNA Polymerase 2x Master Mix RED (Ampliqon, Odense, Denmark), 1 µL of fD1 and rP2 primer (SinaClon, Tehran, Iran), and 7.5 µL of sterile distilled water. The primer sequence, amplicon sizes, and annealing temperature are summarized in Table S1. The PCR product was run on a 1% (w/v) agarose gel with Safe Stain (Yekta Tajhiz Azma, Tehran, Iran) before it was sent for sequencing analysis. The DNA sequence was aligned and compared using BLAST to identify homologous sequences in the NCBI GenBank database. The sequence was uploaded to the GenBank database as a new strain. The isolate was also examined for its morphological and biochemical characteristics, as outlined in Bergey's Manual of Systematic Bacteriology [19].

Phylogenetic analysis: The phylogenetic study was performed using the neighbor-joining (NJ) method within Molecular Evolutionary Genetics Analysis (MEGA) software version 12.

RESULTS

In this experiment, horse feces, as a rich source of a large number of microorganisms, were used for the isolation of keratinolytic bacteria. In primary screening, *B. cereus* and *B. subtilis* exhibited proteolytic activity by producing clear zones on skim milk agar plates and displaying cloudy, non-transparent appearances that formed around the bacterial colonies on egg yolk agar. The feces isolate produced a zone on skim milk agar and a clear zone on egg yolk agar (Fig. 1). This isolate grew on MS media with feather as the only carbon source, indicating that it could possess keratinolytic activity. Initial morphological identification revealed that the fecal isolate was a Gram-negative, rod-shaped bacterium characterized by a small, smooth, and pale-yellow colony.

The morphological and biochemical characteristics of the fecal isolate are summarized in Table 1. It was identified as a Gram-negative, aerobic, rod-shaped bacterium with smooth, light-yellow-colored colonies.

The PCR sequence results of the fecal isolate confirmed the primary morphological identification. The sequence of the isolate showed high sequence homology to that of other *S. rhizophila*, with 99% similarity. The DNA sequence was submitted to the GenBank database and published in NCBI with accession number PQ896544.1 as *S. rhizophila* SMT.

The taxonomic position of *S. rhizophila* SMT was further confirmed through phylogenetic analysis based on 16S rRNA sequences (Fig. 2). A total of 564 nucleotides from the partial 16S rRNA gene sequence of the isolate showed 99.47–99.82% identity with *S. rhizophila* strains available in the NCBI database. Based on the high sequence similarity, the isolate was identified as *S. rhizophila*.

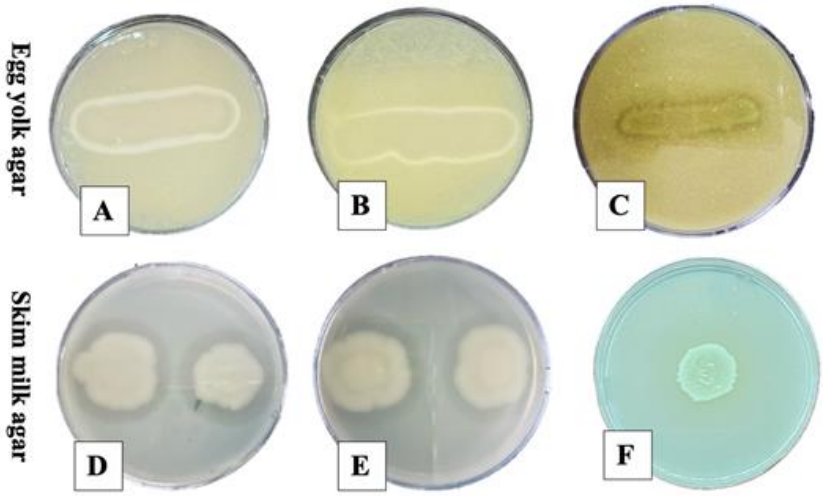

Figure 1: Preliminary screening on skim milk agar and egg yolk agar for three strains (A&D) *B. cereus*; (B&E) *B. subtilis*; (C&F) *S. rhizophila* SMT.

Table 1: Biochemical characteristics used for the identification of *Stenotrophomonas* genes

No.	Biochemical characteristics	Stenotrophomonas rhizophila SMT	S. maltophilia
1	Gram reaction	Negative	Negative
2	O-F test	Fermentative	-
3	Catalase test	Positive	Positive
4	Oxidase test	Negative	Positive
5	Motility	Negative	-
6	Citrate test	Positive	Positive
7	Indole test	Positive	Positive
8	Urease	Negative	Negative
9	Lactose	Positive	Positive
10	Xylose	Negative	-
11	Inositol	Negative	-
12	Maltose	Negative	Positive
13	Rhamnose	Negative	-
14	Sorbitol	Negative	Positive
15	Fructose	Negative	Positive
16	Arabinose	Negative	-

S. rhizophila SMT and two *Bacillus* species were incubated on MS media with whole feathers. *B. cereus* produced a total soluble protein concentration of 1.215 mg/ml, mostly occurring after day 8. *B. subtilis* also steadily increased soluble protein, reaching 0.615 mg/ml by day 13. Additionally, *S. rhizophila* SMT began producing soluble protein after six days, with most activity happening after day 10. On day 13, a total soluble protein concentration of 0.535 mg/mL was reached (Fig. 3). All strains were grown under the same conditions and using the same control media.

DISCUSSION

S. rhizophila has been isolated from diverse ecological niches, from plant to marine habitats [15]. In the present study, we isolated *S. rhizophila* SMT from horse feces and primarily identified it based on its biochemical characteristics. The biochemical profiles of *S. rhizophila* SMT were similar to *S. rhizophila* JC1, especially in the utilization of different carbohydrates and alcohols. However, there were differences in indole production and citrate utilization [20]. Comparison with *S. maltophilia* (Table 1) revealed similarities in urease activity, but differences in oxidase reaction and the pattern of sugar metabolism [21, 22].

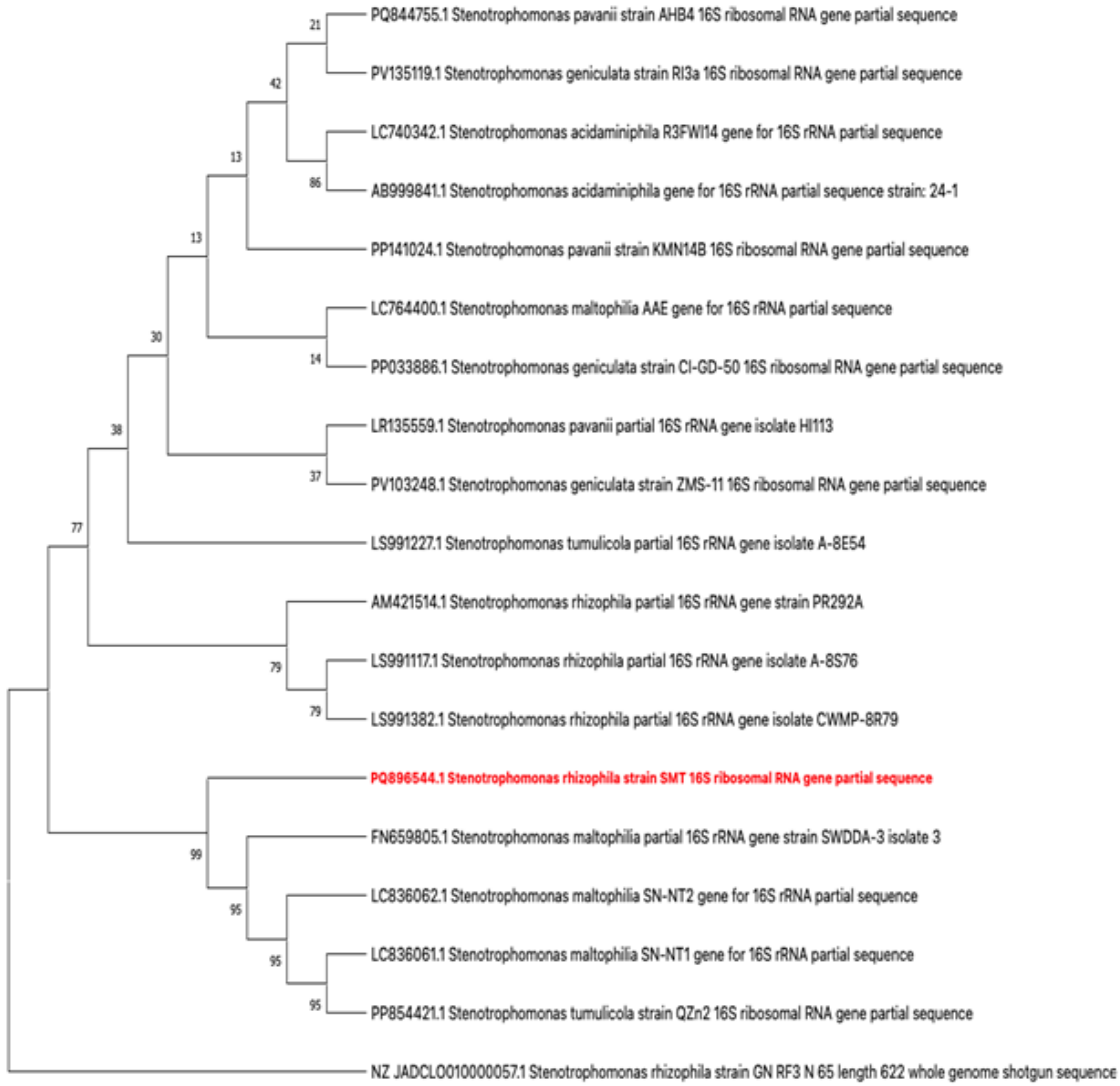

Figure 2: The phylogenetic tree of *Stenotrophomonas rhizophila* SMT was constructed using the neighbor-joining (NJ) method in MEGA-X software. Bootstrap analyses were performed using ultrafast bootstrap approximation with 1000 replicates.

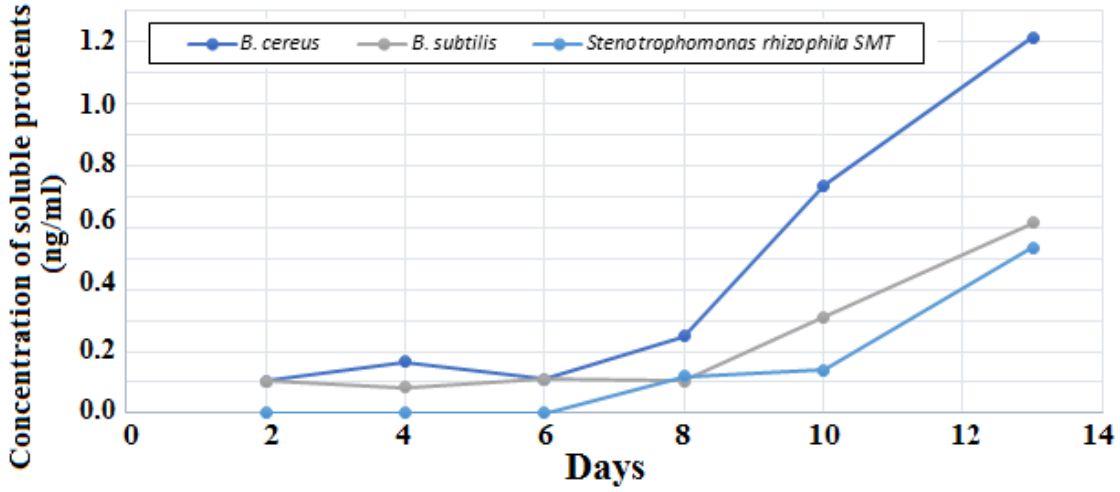

Figure 3: Comparison of the liberation of soluble proteins during the 13-day growth period among the three species.

The isolated *S. rhizophila* SMT showed keratinolytic activity by producing 0.535 mg/mL of soluble protein after 13 days of incubation. Under the same experimental conditions, *B. cereus* and *B. subtilis* produced 1.215 mg/mL and 0.615 mg/mL of soluble protein, respectively. The keratinolytic potential of the genus *Stenotrophomonas* has been documented previously. Herzog et al. reported keratinase production by *S. maltophilia* and *S. rhizophila* [23]. Moreover, Yamamura et al. isolated a *Stenotrophomonas* sp. strain D-1 from deer fur, which produced enzymes that break down proteins (serine protease) and reduce disulfide bonds (disulfide reductase) [24]. Another study isolated a metal-keratinase-producing *Stenotrophomonas* sp. Yang-5 from swan farming soil, which showed the highest keratinase activity at 32.5°C and an initial pH of 7.5 [25]. *S. maltophilia* R13, isolated from reed rhizospheric soil, produces a keratinolytic enzyme at 30°C and an initial pH of 7.0. The highest production of this enzyme was observed with 0.1–0.2% feather [26].

In addition, keratinolytic *Bacillus* spp., including *B. subtilis* and *B. cereus*, have been isolated from diverse environments and examined in several biodegradation studies. [27-30]. Yadav and Khosla isolated the *B. cereus* H16 strain from a poultry farm's waste disposal site, which could degrade 94% of chicken feathers and produce 5.34 mg/mL of soluble protein [31]. Laba and Rodziewicz isolated *B. cereus* B5esz from soil and keratinous wastes, which increased the concentration of soluble protein by 1.2 mg/mL within four days [32]. Similarly, *B. cereus* KB043 produced 1.2 mg/mL soluble protein over six days [33]. The ALICA strain of *B. subtilis* was capable of degrading 86% of the feather weight along with a soluble protein concentration of 0.565 mg/mL after nine days [29]. Mazotto et al. examined the soluble protein concentration of *B. subtilis* LFB-FIOCRUZ 1270, *B. subtilis* LFB-FIOCRUZ 1273, and *B. licheniformis* LFB-FIOCRUZ 1274 and found values ranging from 1.9–3.3 mg/mL for all the *Bacillus* sp. feather samples [13].

Although *S. rhizophila* SMT showed lower keratinolytic efficiency than *B. cereus*, its ability to degrade chicken feathers shows its potential as a keratinolytic microorganism. Moreover, the non-pathogenic characteristic of *S. rhizophila* SMT is an important advantage for future industrial applications. Further studies should focus on optimizing culture conditions, investigating synergistic interactions with other keratinolytic microorganisms, and applying genetic or metabolic engineering approaches to enhance keratinase production and improve feather biodegradation efficiency. These strategies may expand the potential use of *S. rhizophila* SMT in eco-friendly feather waste management and the production of value-added bioproducts.

Funding: None

Acknowledgements: None

Conflict of Interest: The authors declare no conflict of interest

Authors' Contribution: MT: Conceptualization, Funding acquisition, Supervision, Project administration, Methodology, Formal analysis (equal), Validation (equal), Writing – review & editing. ZGF: Investigation (equal), Formal analysis (equal), Writing – original draft (equal). ZR: Investigation (equal), Formal analysis (equal). SHH: Isolation of *S. rhizophila* SMT. All authors read and approved the final version of the manuscript.

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