



Isolation, Identification, and Characterization of a Keratin-degrading Bacterium *Chryseobacterium* sp. P1-3

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Abstract In this study, a keratin-degrading bacterium was isolated from soil contaminated with feather waste. The isolated strain was identified as *Chryseobacterium* sp. P1-3 on the basis of the 16S rRNA gene sequence alignment. *Chryseobacterium* sp. P1-3 is currently used in various biotechnological applications (e.g., in the hydrolysis of poultry feathers). It hydrolyzed the feather meal within 2 days and possesses a high level of keratinase activity (98 U/mL). The keratinase, partially purified from this strain, prefers casein as a substrate and shows optimal activity at a temperature of 30°C and at a pH of 8.0.

Keywords *Chryseobacterium* sp. · isolation · keratin

Introduction

Keratins are ubiquitously present in animal bodies. The livestock industry is a key producer of keratinous wastes, the disposal of which is exceedingly difficult (Joshi et al., 2007). Several million tons of these wastes are generated annually around the world. At present, various physicochemical and bacteriological methods are being used to convert feathers into feather meal, which is then used as animal feed. Therefore, it would help livestock producers greatly, if this feedstuff were enriched in proteins and essential

amino acids. However, the processing methods necessary for producing enriched livestock feedstuff necessitate the investment of large amounts of energy. Additionally, the finished product lacks digestibility and contains components of low quality (Wang and Parsons, 1997; Nam et al., 2002).

Keratin is the most abundant protein present in feathers, horns, skin, hair, hooves, and wool. It harbors large amounts of the sulfur-containing amino acid cysteine and exhibits α -helical or β -sheet structures (Bockle et al., 1995). Keratin, in its native state, cannot be easily degraded by commonly occurring proteases such as trypsin, papain, and pepsin. However, it can be efficiently degraded by the keratinase produced by microorganisms (Onifade et al., 1998). In recent years, several keratinases from various microorganisms such as *Chryseobacterium* (Riffel et al., 2007), *Bacillus* (Macedo et al., 2005), thermophilic bacteria (Riessen and Antranikian, 2001), and fungi (Gradišar et al., 2000) have been purified and characterized. Thus, keratinase has an important role in the bioremediation of keratinous wastes produced by the livestock, textile, and leather industries. Such a large-scale bioremediation can be achieved through the development of environment-friendly processes (SHIH, 1993). The keratinase-mediated enzymatic hydrolysis can be used to convert the insoluble keratin into livestock feedstuff, fertilizers, films, and glues (Gupta and Ramnani, 2006). In addition, keratinase has also been found to be incredibly useful to the cosmetics and pharmaceutical industries (Brandelli, 2008).

Members of the genus *Chryseobacterium* produce enzymes with strong proteolytic activities (Vandamme et al., 1994). We have isolated a new keratin-degrading bacterium, *Chryseobacterium* sp. P1-3. The isolated strain is able to degrade animal feathers as well as other keratin-containing materials. The extracellular keratinase produced by this bacterium, could potentially benefit the environment and the industry (Riffel et al., 2007). In addition, the keratinolytic product generated by this enzyme could be utilized for producing organic fertilizers and amino acids (Riffel et al., 2003).

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Materials and Methods

Isolation of the keratin-degrading bacterium. The keratin-degrading bacterium was isolated from soil contaminated with feather waste at a poultry processing facility in Korea. For the first screening, we used 100 mL of the feather meal medium (1% (w/v) feather meal, 0.05% NaCl, 0.05% NH₄Cl, 0.03% KH₂PO₄, 0.03% K₂HPO₄, 0.01% MgCl₂·6H₂O, 0.01% yeast extract, pH 7.2). Five grams of the soil was suspended in the feather meal medium. After an incubation period of 7 days, a loopful of the suspension was streaked onto a skim milk agar plate (1.0% skim milk, 0.03% K₂HPO₄, 0.03% KH₂PO₄, 0.01% Na₂CO₃, 1.5% agar, pH 7.2) in order to obtain isolated colonies. The isolates that produced clear zones on this medium were selected (Zerdani et al., 2004). This method was used as an initial screening for the isolation of keratin-degrading strains.

Identification of the keratin-degrading bacterium. The isolated strain was grown overnight in 3 mL of the nutrient broth medium at 30°C at 200 rpm. Genomic DNA was extracted from the cells by using the Genomic DNA prep kit (Bioneer, Korea), according to the manufacturer's instruction. Polymerase chain reaction (PCR) amplification was performed using the Biometra TGradient PCR (Biometra, Germany), which was followed by the DNA sequencing of the corresponding 16S rRNA gene. The universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGC TAC CTT GTT ACG ACT T-3') were used (Weisburg et al., 1991) during this procedure. The PCR mixture contained 5 µL of 5X PrimeSTAR buffer, 4 µL of 2.5 mM dNTP mix, 1 µL of 10 pM primers, 1 µL of genomic DNA template (10 ng/µL), and 1.25 U of PrimeSTAR HS DNA Polymerase (TaKaRa, Japan) in a final reaction volume of 50 µL. The PCR conditions were as follows: initial denaturation at 98°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 90 sec. The amplified PCR products were analyzed by 0.8% (w/v) agarose gel electrophoresis and purified using the AxyPrep™ PCR cleanup kit (Axygen, USA), according to the manufacturer's instructions. The sequencing of the amplified DNA fragments was performed by Solgent Co. (Korea). The 16S rRNA gene sequence was compared with the existing EzTaxon database to identify the corresponding species (Kim et al., 2012).

Phylogenetic analysis. A phylogenetic tree was constructed on the basis of the 16S rRNA gene sequence by using the neighbor-joining algorithm and the p-distance model (Saitou and Nei, 1987) in MEGA ver. 5.2 (Tamura et al., 2011). The level of support for the phylogenies derived from the neighbor-joining algorithm was gauged by 1,000 bootstrap replicates. The percentage of replicates in which the associated taxa clustered together in the bootstrap analysis was shown near the sides of the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the data sheet.

Degradation of chicken feathers. The degradation ratio of

chicken feathers (1% (w/v)) was calculated by determining the dry weight of chicken feathers remaining in the culture medium at the end of the experiment. An outstanding media were removed by using a Whatman no. 2 filter paper and dried at 70°C for 24 h to analysis the degradation of that. The results were expressed as a function of the initial weight (100%) and were calculated by comparing the dry weight of the residual chicken feathers before and after degradation (Cortezi et al., 2008).

Assay for crude keratinase. The protease assay was performed as per the method adopted by Kim et al. (2012). To evaluate the crude keratinase, the supernatant of cells grown in the feather meal medium was used. The reaction mixture contained 0.5 mL of the crude extracellular enzyme and 2.5 mL of a casein solution (0.6% (w/v) casein in 50 mM Tris-Cl buffer, pH 7.0). This mixture was allowed to stand at 37°C for 20 min, and the enzyme reaction was quenched with 2.5 mL of trichloroacetic acid (50% (v/v)). After centrifuging the solution at 12,000 rpm for 20 min, it was filtered with a Whatman no. 2 filter paper. To 1 mL of the filtrate (enzyme-substrate mixture), we added 2.5 mL of 0.55 M Na₂CO₃. To this mixture, we added 0.5 mL of the Folin-Ciocalteu phenol reagent and mixed the solution rapidly. After 30 min, 6 mL of distilled water was added, and the contents were again mixed thoroughly. The mixture was measured spectrophotometrically at 660 nm using UV 1800 spectrophotometer (Shimadzu, Japan) (Lowry et al., 1951). The presence of the liberated amino acids was detected using a blank control at 660 nm, and the corresponding quantities were estimated with a standard L-tyrosine solution (10–100 µM), using a spectrophotometer (Shimadzu, Japan). One protease unit (U) was defined as the amount of enzyme that caused an increase of 0.01 unit in the absorbance of the sample at 660 nm, relative to the control assessed under the same conditions.

Influence of pH and temperature on enzyme activity. The influence of pH and temperature on the activity of the crude extracellular enzyme was assessed by varying one factor at a time while keeping the remaining factors constant. The optimal pH of this enzyme was in the range of 4.0–10.0 at 30°C. Citrate-NaOH, sodium phosphate, and glycine-NaOH buffers were used in the pH ranges 4.0–6.0, 7.0–8.0, and 9.0–10.0, respectively. Likewise, the optimal temperature for the activity of the crude extracellular enzyme was determined by varying the temperature in the range of 20–60°C at a constant pH (pH 8.0).

Statistical analysis. The means and standard errors were calculated for at least three different replicates. The statistical analysis was performed using GraphPad Prism ver. 5.0.

Results and Discussion

Isolation and identification of the keratin-degrading bacterium.

Among the samples collected from the different sources, three samples yielded colonies on the agar plates containing minimal salt medium with skim milk as the only source of carbon and nitrogen. Evaluation of the keratinolytic activity in the culture supernatants of these colonies revealed one strain showing the

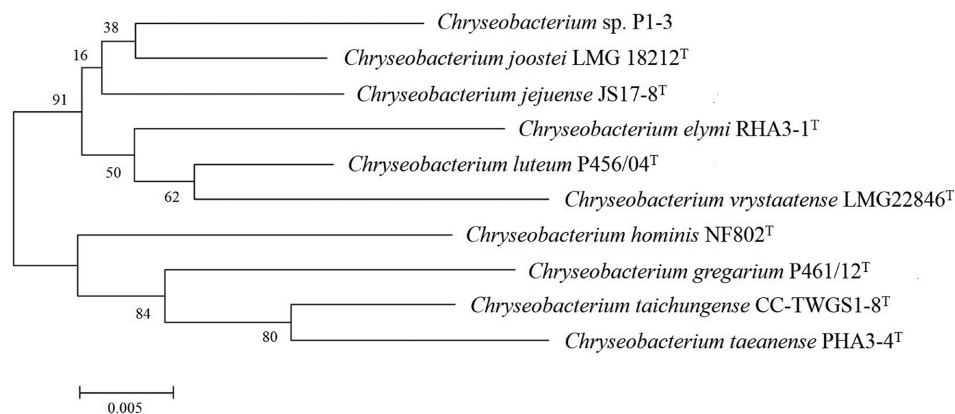


Fig. 1 A phylogenetic tree showing the genetic relationship between *Chryseobacterium* sp. P1-3 strain and the reference strains based on the 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbor-joining method. The number on each branch indicates the bootstrap values based on 1,000 replicates.

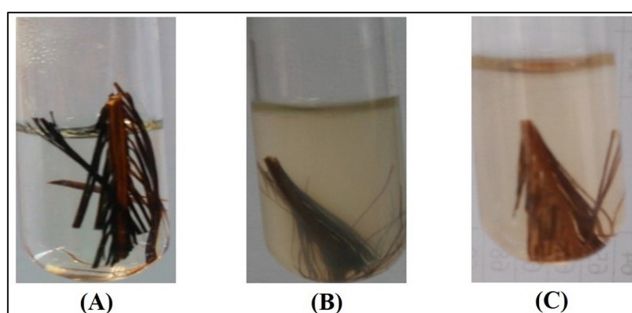


Fig. 2 Feather degradation by the *Chryseobacterium* sp. P1-3 after (A) 1 h and after (B) 7 days at a temperature of 30°C, and (C) feather after 7 days at 30°C without *Chryseobacterium* sp. P1-3.

highest keratinolytic activity. This strain was isolated from soil contaminated with feather waste and was named as P1-3.

The 16S rRNA gene sequence of the isolated microorganism was sequence aligned and compared using the EzTaxon program. The isolated strain was identified as *Chryseobacterium* sp. P1-3. A phylogenetic tree of the isolated strain P1-3 was constructed (Fig. 1). The digits adjacent to the nodes correspond to the statistical frequency of the indicated species. The results of the homology assay showed that *Chryseobacterium* sp. P1-3 in the phylogenetic branch showed maximal similarity of 97.3% with the *C. joostei* strain LMG 18212 (Hugo et al., 2003) and a similarity of 96% with the *C. jejuense* strain JS17-8 (Weon et al., 2008). This strain was established as a *Chryseobacterium* species by the EzTaxon database. It is already known that the degradation of keratin is efficiently performed by gram-positive bacteria (Gupta and Ramnani, 2006). Moreover, some isolates of the Gram-negative *Chryseobacterium* sp. have already been described as good feather-degrading bacteria (Wang et al., 2008). The isolated strain showing high keratinolytic activity could be potentially used for various industrial processes.

Degradation of chicken feathers by using *Chryseobacterium* sp. P1-3. Degradation of chicken feathers by the isolated strain P1-3 was assessed by determining the percentage loss in the

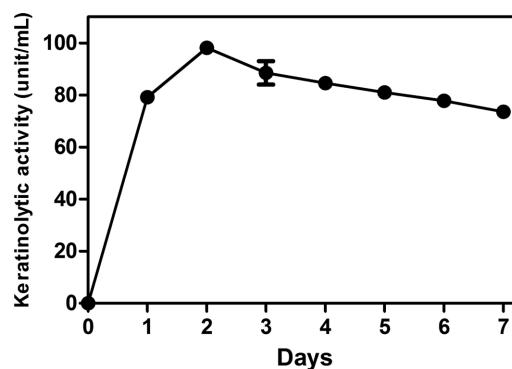


Fig. 3 Extracellular protease activity during the growth of the keratinolytic bacterium *Chryseobacterium* sp. P1-3 strain in chicken feather meal broth. Enzyme activity was measured using casein as the substrate. Each point represents the mean of three independent experiments.

weight of the feathers during the enzymatic treatment. After removing the bacteria from the feather strips, different microscopic changes were observed (data not shown). The percentage loss in the weight of the feathers increased significantly after 2 days of cultivation and went up to 65% after 7 days of cultivation (Fig. 2). However, the chicken feathers were not degraded completely by the isolated strain P1-3 owing to a relatively slower enzymatic reaction. (Corrêa et al., 2010; Jeong et al., 2010). In addition, incomplete degradation of chicken feathers by many other bacterial strains has been previously reported (Bach et al., 2011). **Production of keratinase by using chicken feathers.** The keratinase-producing *Chryseobacterium* sp. P1-3 strain was grown in a liquid minimal medium with 1% (w/v) chicken feather serving as the major source of carbon and nitrogen. The production of keratinase was observed for 7 days. The highest quantity of keratinase was produced within 2 days of culture. The optimal enzymatic activity was 98 U/mL, which decreased 2 days later (Fig. 3). *Chryseobacterium* strains often produce keratinases at mesophilic temperatures (Venter et al., 1999; Chaudhari et al., 2013). Although these conditions were considered satisfactory for

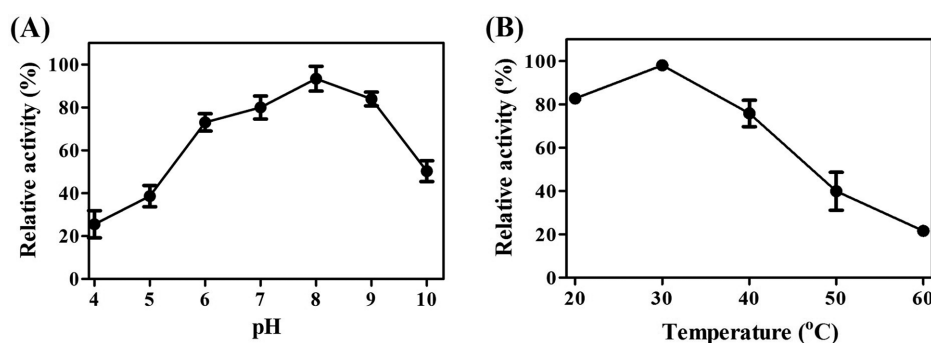


Fig. 4 Influence of pH (A) and temperature (B) on the activity of the crude enzyme isolated from *Chryseobacterium* sp. P1-3 strain.

Table 1 Optimum pH and temperature of other keratin-degrading strains

Strain	Optimum temperature (°C)	Optimum pH	Reference
<i>B. subtilis</i> MTCC(9102)	40	6.0	(Balaji et al., 2008)
<i>B. subtilis</i> KS-1	60	7.0	(Suh and Lee, 2001)
<i>Streptomyces</i> sp. S.K ₁₋₀₂	70	10.0	(Letourneau et al., 1998)
<i>Microbacterium</i> sp. kr10	50	7.5	(Thys and Brandelli, 2006)
<i>Chryseobacterium</i> sp. kr6	55	7.5	(Riffel et al., 2003)
<i>Chryseobacterium</i> sp. RBT	50	8.6	(Gurav and Jadhav, 2013)
<i>Chryseobacterium</i> sp. nov. L99	40	9.0	(Lv et al., 2010)

production of keratinase, the other variables were not investigated. The enzymatic activity at 30°C was sufficient for the degradation of keratin. In future studies, we will perform next-generation sequencing by using the isolated P1-3 strain. We plan to identify the gene encoding keratinase gene in the near future.

Optimum pH and temperature. The influence of pH on the crude enzyme activity was investigated (Fig. 4A). The optimal pH for keratinase activity was 8.0, and more than 80% of the enzymatic activity was retained in the pH range 7–9. However, the enzyme activity decreased significantly in predominantly acidic and basic environments. The optimal temperature for the enzymatic activity was 30°C (Fig. 4B). At 40°C, approximately 80% of the enzyme activity was retained, and it reduced drastically at temperatures above 50°C. Thus, the microbial keratinase showed optimal activity in the temperature range 30–80°C and in the pH range 7–9 (Gupta and Ramnani, 2006). Keratinase isolated from *Bacillus subtilis* MTCC (9102) showed optimal activity at a temperature of 40°C and pH 6.0, while keratinase isolated from *B. subtilis* KS-1 showed optimal activity at a temperature of 60°C and pH 7.0. The keratinase derived from *Streptomyces* sp. S.K₁₋₀₂ showed optimal activity at a temperature of 70°C and pH 10.0; the keratinase derived from *Microbacterium* sp. kr10 showed optimal activity at a temperature of 50°C and pH 7.5; and the keratinase isolated from the *Chryseobacterium* species showed optimal activity in the temperature range 40–55°C and in the pH range 7.5–9.0 (Table 1). Upon comparing the reference strains with the isolated P1-3 strain, it was evident that the optimum pH was quite similar. However, the optimal temperature was higher in the reference strains than in the isolated P1-3 strain. Using mesophilic enzymes and microbes to the industrial process is more beneficial

than the corresponding thermophilic enzymes and microbes owing to the low energy consumption of the former (Kumar et al., 2008). This enzyme could be considered as industrially and commercially important biomolecule, because it has advantages of reproducibility under normal laboratory conditions and mild enzymatic reaction conditions of pH and temperature.

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