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

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Received: 10 April 2015 / Accepted: 29 June 2015 / Published Online: 30 September 2015
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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 keratins 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 Rammani, 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).
Materials and Methods

Isolation of the keratin-degrading bacterium. The keratin-
degradating 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 instructions. Polymerase chain reaction
(PCR) amplification was performed using the Biometra TGradient
PCR (Bioneer, Germany), which was followed by the DNA
sequencing of the corresponding 16S RNA 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 1-t-tyrosine solution (10–
100 µM), using a spectrophotometer (Shimadzu, Japan). One
protease unit (U) was defined as the amount of enzyme that
caus a change 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 range 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
highest keratinolytic activity. This strain was isolated from soil contaminated with feather waste and was named as P1-3.

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 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 meal as the major source of carbon and nitrogen. The production of keratinase was observed for 7 days. The highest 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
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.

**Acknowledgments** This research was supported by the Technology Development Program for Bio-industry, Ministry for Agriculture Food and Rural Affairs, Republic of Korea.

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