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ORIGINAL PAPER

Characterization of a multifunctional feather-degrading *Bacillus subtilis* isolated from forest soil

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Abstract In this study, we isolated and characterized a novel feather-degrading bacterium that shows keratinolytic, antifungal and plant growth-promoting activities. A bacterium S8 was isolated from forest soil and confirmed to belong to *Bacillus subtilis* by BIOLOG system and 16S rRNA gene analysis. The improved culture conditions for the production of keratinolytic protease were 0.1% (w/v) sorbitol, 0.3% (w/v) KNO_3, 0.1% (w/v) K_2HPO_4, 0.06% (w/v) KH_2PO_4 and 0.04% (w/v) $MgCl_2 \cdot 6H_2O$ (pH 8.0 and 30°C), respectively. In the improved medium containing 0.1% (w/v) feather, keratinolytic protease production was around 53.3 ± 0.3 U/ml at 4 day; this value was 10-fold higher than the yield in the basal feather medium (5.3 ± 0.1 U/ml). After cultivation for 5 days in the improved medium, intact feather was completely degraded. Feather degradation resulted in free –SH group, soluble protein and amino acids production. The concentration of free –SH group in the culture medium was 15.5 ± 0.2 μM at 4 days. Nineteen amino acids including all essential amino acids were produced in the culture medium; the concentration of total amino acid produced was 3360.4 μM. Proline (2809.9 μM), histidine (371.3 μM) and phenylalanine (172.0 μM) were the major amino acids released in the culture medium. *B. subtilis* S8 showed the properties related to plant growth promotion: hydrolytic enzymes, ammonification, indoleacetic acid (IAA), phosphate solubilization, and broad-spectrum antimicrobial activity. Interestingly, the strain S8 grown in the improved medium produced IAA and antifungal activity, indicating simultaneous production of keratinolytic and antifungal activities and IAA by *B. subtilis* S8. These results suggest that *B. subtilis* S8 could be not only used to improve the nutritional value of feather wastes but also is useful in situ biodegradation of feather wastes. Furthermore, it could also be a potential biofertilizer or biocontrol agent applicable to crop plant soil.

Keywords *Bacillus subtilis* · Feather degradation · Keratinase · Plant growth-promoting activity · Antifungal activity

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Introduction

A huge amount of poultry feathers is generated by poultry processing industries and is crucial industrial

waste over the world. The main component of poultry feathers is keratin, a fibrous and insoluble structural protein rich in β-helical coils linked through disulfide bridges (Gioppo et al. 2009). This renders them resistant to degradation by proteases such as pepsin, papain, and trypsin leading to serious environmental problems. Considering its high protein content, however, feather wastes could have a great potential as a source of protein and amino acids for animal feed and for many other applications (Haddar et al. 2009). Generally they become feather meal used as animal feed after undergoing thermal and chemical treatments. However, these processes require significant energy and also destroy some amino acids (Papadoulos and Ketelaars 1986). Therefore, biodegradation of feather keratin by microorganisms possessing keratinolytic activity represents an alternative attractive method for improving the nutritional value of feather wastes and to prevent environmental pollution. Keratinolytic proteases are used to produce rare amino acids like proline, serine, and cysteine or for the development of nitrogen fertilizers, glues, cosmetic, and biodegradable films (Onifade et al. 1998). It could be also used in newer fields like prion degradation for treatment of the mad cow disease (Langeveld et al. 2003).

Many attempts have been made to isolate feather-degrading microorganisms which have keratinolytic activity (Daroit et al. 2009; Matsui et al. 2009). However, most of these efforts have been directed toward keratinolytic protease used for increasing the digestibility of feathers as an animal feed. At this stage the simplest and most appropriate use is as fertilizers (Hadas and Kautsky 1994). For this purpose, it is necessary to isolate and characterize feather-degrading microorganisms not only with keratinolytic activity but also with plant growth-promoting activity. In addition to traditional use like animal feed or feed additive, culture containing these microbial cells and feather hydrolyzates could be a potential candidate for the development of biofertilizer and biocontrol agent. Even though there have been many researches on characterization of feather-degrading microorganisms, little is known about the isolation and characterization of keratinolytic and plant growth-promoting microorganism for agronomic utilization of feather wastes.

Current trends in agriculture are focused on the reduction of the use of synthetic pesticides and inorganic fertilizers, forcing the search for alternative ways to improve a more sustainable agriculture. In this regard, the use of plant growth-promoting bacteria (PGPB) as biofertilizers and antagonists of phytopathogens has a potential role in developing sustainable systems for crop production (Smit et al. 2001). Plant growth-promoting ability of these bacteria is mainly because of the production of indoleacetic acid (IAA) and antagonistic substances (siderophore, antibiotics or extracellular hydrolytic enzymes), phosphate solubilization, competition for nutrients or induced resistance (Lucy et al. 2004).

Therefore, the aim of this research was to isolate and characterize novel multi-functional feather-degrading microorganism with keratinolytic, antifungal and plant growth-promoting activities.

Materials and methods

Isolation and screening of feather-degrading bacteria

Soil samples were collected from the soil and roots of plants growing in fields at Miryang, Korea. Poultry waste samples were also collected from several sites at a local poultry-processing company in Miryang, Korea. To isolate feather-degrading bacteria, the samples were suspended in sterile saline and were shaken for 4 h on a rotary shaker. Serial dilutions of the sample were then individually inoculated on skim milk agar plate supplemented with 1.5% (w/v) agar. After 2 days of incubation at 30°C, the plates were examined for the presence of colonies developing clear haloes. Colonies with clear haloes resulting from proteolysis were selected for further investigation. The purity of the isolate was confirmed through repeated streaking.

The pure isolates were subsequently grown in basal feather medium containing 0.1% (w/v) whole chicken feathers as the carbon source. Cultures were grown at 30°C and 200 rpm for 5 days. Isolates that completely broke down feathers in the medium were selected. The individual isolates were then examined for their keratinolytic activities. The isolate S8 exhibiting the highest activity was selected and used in the present study.

The skim milk medium used comprised the following: 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and 5.0% (w/v) skim milk

in distilled water (pH 7.5). The basal feather medium used contained the following: 0.03% (w/v) K_2HPO_4, 0.04% (w/v) KH_2PO_4, 0.05% (w/v) NaCl, 0.01% (w/v) $MgCl_2{\cdot}6H_2O$, 0.05% (w/v) urea, and 0.1% (w/v) feather (Lee et al. 2004). The pH was adjusted to 7.5 prior to sterilization. Chicken feathers were obtained from a poultry-processing plant. They were washed extensively with tap water and dried at 60°C for 2 days, and then kept at 4°C until used.

Identification of feather-degrading bacteria

Phenotypic properties of the isolate S8 were investigated according to Manual of methods for general bacteriology (Gerhardt et al. 1981). BIOLOG GP system (Biolog Inc., Haward, CA) microplate for the identification of Gram-positive aerobic bacteria was used as recommended by the manufacturer to obtain the metabolic fingerprint of the isolate S8. Results were analyzed in the statistical databank (MicroLog™ System, Release 4.2, Biolog Inc.). The 16S rRNA gene was sequenced after genomic DNA extraction and polymerase chain reaction amplification as described elsewhere (Ausubel et al. 1988). Databases in GenBank were searched for sequences similar to the 16S rRNA gene sequence. These databases were analyzed using Clustal X, version 1.81 (Thompson et al. 1997). The nucleotide sequence (1517 bp) determined in this study has been deposited in GenBank database with accession number GU576479.

Effect of environmental conditions on keratinolytic protease production

For shaken culture in flasks, 50 ml of the basal feather medium was dispensed into each of 250-ml Erlenmeyer flasks followed by inoculation with 1 ml of the isolate S8 culture (1.8×10^9 cells/ml) grown in nutrient broth at 30°C for 24 h. Cultivations were performed at 30°C and 200 rpm for 3 days in a rotary shaker unless stated otherwise.

The co-carbon sources (glucose, fructose, sucrose, maltose, lactose, galactose, sorbitol, mannitol, glycerol and soluble starch) were provided at a concentration of 0.1% (w/v); sorbitol was added in the range of 0.05–0.3% (w/v). The different nitrogen sources (beef extract, casamino acid, casein, corn steep liquor, gelatin, malt extract, polypeptone, skim milk, tryptone, yeast extract, soytone, urea, $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, KNO_3, $NaNO_2$ and $NaNO_3$) were added separately to the medium at a concentration of 0.05% (w/v). KNO_3 concentrations ranged from 0.05 to 0.4% (w/v). For studying the effect of initial pH and culture temperature, the isolate S8 was grown for 3 days in a medium at different pH values and temperatures, ranging from 4.0 to 10.0 and 20 to 45°C, respectively.

Keratinolytic activity assay and protein determination

The cultures were centrifuged at $17{,}418 \times g$ for 15 min, and the supernatant was used as a crude enzyme preparation. Keratinolytic activity was assayed with soluble keratin as a substrate, according to the method of Wawrzkiewicz et al. (1987). An appropriately diluted enzyme solution (400 µl) was mixed with 600 µl of 0.3% (w/v) soluble keratin in 0.1 M phosphate buffer (pH 7.5) and incubated at 30°C for 3 h with constant agitation at 1,300 rpm by using a Thermomixer (Eppendorf, Hamburg, Germany). The reaction was terminated by adding 500 µl of 10% (w/v) trichloroacetic acid and centrifuged at $17{,}418 \times g$ for 10 min at 4°C. The absorbance of the supernatant was measured at 280 nm. One unit (U) of keratinolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance at 280 nm of 0.01 under the above conditions. The protein concentration was determined by the Bradford (1976) method with bovine serum albumin as the standard.

Amino acid analysis

The isolate S8 was grown in the improved medium containing 0.1% (w/v) feather for 4 days and the culture supernatant was obtained by centrifugation at $17{,}418 \times g$ for 10 min. Amino acid content in the culture supernatant was determined with an amino acid analyzer S433 (Sykam, Germany). Uninoculated medium served as control.

Scanning electron microscopy

In order to observe changes on feather structure during incubation, scanning electron microscopy

(SEM) was accomplished using a JEOL JSM-6390 microscope (JEOL TECHNIC LTD., Japan) with gold-coated feather samples.

Plant growth-promoting activity assay

Proteolytic activity was assayed as described above. Lipase activity was assayed on tributylin plate; lipase activity was indicated by a zone of clearance around the colonies (Sommer et al. 1997). Pectinolytic activity was tested in nutrient agar plate according to the method of Chernin et al. (1995), where clearing zone was detected after 2 days of incubation at 30°C.

IAA production was analyzed in King's B broth with different concentration of L-tryptophan (0, 0.02, and 0.1%, w/v) or in the improved feather medium. Cells were collected by centrifugation at 17,418×*g* for 15 min at 4°C. IAA in the supernatants was measured by the method of Tang and Bonner (1947) with the Salkowski reagent.

Phosphate solubilization was investigated in NBRIP medium containing 0.5% (w/v) $Ca_3(PO_4)_2$ as the insoluble phosphate source (Nautiyal 1999). Soluble phosphate was determined colorimetrically by using the vanadomolybdophosphoric acid colorimetric method (Clesscerl et al. 1998).

Ammonia production was determined according to the method of Trivedi et al. (2008). The isolate S8 was inoculated in peptone water in 30 ml tubes and incubated at 30°C for 24 h. Afterward, 1 ml of Nessler's reagent was added to each tube. Development of a faint yellow color was indicative of weak reaction and deep yellow to brownish color was indicative of strong reaction.

Antagonistic properties of the isolate S8 were tested against phytopathogenic fungi (*Alternaria panax*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora cactorum*, *Phoma* sp., *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) on potato dextrose agar (PDA) plates using a dual culture technique (Trivedi et al. 2008). In order to evaluate antagonistic activity, the isolate S8 was grown in tryptic soy broth or in the improved feather medium. The supernatants (5 µl) of stationary-phase cultures were spotted on the center of PDA plates which had been inoculated previously with a spore suspension (10^6 spores/ml) of test fungus. After the plates were incubated at 28°C for 5 days, the inhibition of fungal growth was observed around the bacterial spots. The control plate was prepared by growing each test fungus without the isolate S8.

Other analytical methods

The free –SH group in the culture medium of the isolate S8 was determined spectrophotometrically according to the method of Ellman (1959). Cell growth was estimated by the measurement of the absorbance at 660 nm. Feather in cultures was harvested by filtration with Whatman number 3 filter paper, washed twice with distilled water and dried at 105°C to constant weight. The percentage of feather degradation was calculated from the differences in residual feather dry weight between a control (feather without bacterial inoculation) and treated sample.

All experiments were repeated three times unless otherwise indicated. The data presented in the tables and figures corresponded to mean values ± standard deviation.

Results

Isolation and identification of feather-degrading bacteria

More than 1,200 microorganisms were isolated from different sources. A total of 156 strains were found to have proteolytic activity when inoculated on the skim milk agar plate by observing the diameter of clear zones. Among them, isolate S8, isolated from forest soil sample, grew well and completely degraded feather in the basal medium containing chicken feather as the sole carbon and energy source (Fig. 1a). SEM was utilized to examine microstructural changes during feather degradation. Undigested intact feather barb with fine barbules was seen in Fig. 1b. The partial degradation of feather barbules after 24 h of incubation was observed (Fig. 1c). Barbules were completely degraded after 3 days of incubation, and the disintegration of the feather rachis structure was observed (Fig. 1d).

The isolate S8 was a Gram-positive, aerobic, catalase-positive, endospore-forming, motile rod-shaped bacterium. It did not produce urease and indole. By determining the 16S rRNA gene sequence (1517 bp) of the isolate S8, a high homology (98%)

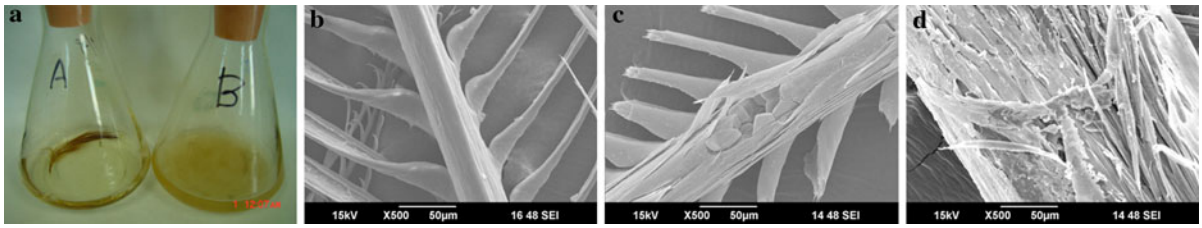

Fig. 1 Degradation of chicken feather by *B. subtilis* S8. **a** visual observation of feather degradation; **b** SEM of untreated feather barbs; **c** SEM of degraded feather barbs after 24 h; **d** SEM of degraded feather rachis after 3 days

with *Bacillus subtilis* DSM10^T was found. The isolate S8 was also confirmed as *B. subtilis* based on metabolic fingerprint analysis by the BIOLOG system, which gave a similarity index value (SIM) of 0.912 (in the BIOLOG system, the SIM must be at least 0.5 to be considered acceptable). Thus, the isolate S8 was named as *B. subtilis* S8.

Improvement of culture condition for keratinolytic protease production

As shown in Table 1, *B. subtilis* S8 produced keratinolytic protease in all co-carbon sources, but the level varied depending on the co-carbon source used. The highest activity was obtained with sorbitol followed by glycerol and mannitol. Galactose reduced the keratinolytic protease production by *B. subtilis* S8. Table 2 shows that keratinolytic protease production was enhanced with increasing amounts of sorbitol up to 0.1% (w/v). Further increases with sorbitol up to 0.3% (w/v) had no effect on the keratinolytic protease production. The influence of the addition of various sorbitol concentrations to the basal medium without feather supplement was also investigated. As shown in Table 2, *B. subtilis* S8 produced a markedly low level of keratinolytic protease as compared with the medium containing feather and sorbitol.

The effects of nitrogen sources on the keratinolytic protease production show that casein, urea or KNO_3 supported similar levels of keratinolytic protease production, considerably higher than the value in the absence of nitrogen source (Table 3). Addition of

Table 1 Effect of co-carbon source on keratinolytic protease production by *B. subtilis* S8 in basal feather medium

Co-carbon source (0.1%, w/v)	Cell growth (A_{660})	Soluble protein (μg/ml)	Keratinolytic activity (U/ml)
None	0.104 ± 0.009	18.6 ± 0.5	5.3 ± 0.2
Glucose	0.625 ± 0.021	53.3 ± 3.6	13.4 ± 0.5
Fructose	0.563 ± 0.011	50.5 ± 2.1	11.8 ± 0.3
Sucrose	1.178 ± 0.015	61.4 ± 3.2	17.1 ± 1.1
Maltose	0.118 ± 0.007	27.2 ± 2.8	5.8 ± 0.4
Lactose	0.418 ± 0.010	52.3 ± 1.5	13.3 ± 0.2
Galactose	0.069 ± 0.003	19.7 ± 1.7	4.5 ± 0.3
Glycerol	0.798 ± 0.016	66.0 ± 2.3	23.5 ± 0.2
Mannitol	0.487 ± 0.013	71.7 ± 2.6	21.9 ± 0.3
Sorbitol	0.857 ± 0.024	78.0 ± 2.0	26.2 ± 0.5
Soluble starch	0.169 ± 0.007	45.6 ± 3.1	6.3 ± 0.4

Values are expressed as mean ± standard deviation of three independent experiments

Table 2 Effect of sorbitol concentration on keratinolytic protease production by *B. subtilis* S8 in basal feather medium and basal medium

Concentration (%, w/v)	Cell growth (A_{660})	Soluble protein (μg/ml)	Keratinolytic activity (U/ml)
	With 0.1% (w/v) feather		
0	0.106 ± 0.019	19.4 ± 1.9	5.2 ± 0.6
0.05	1.028 ± 0.028	55.7 ± 3.1	23.8 ± 0.9
0.1	0.841 ± 0.031	74.5 ± 2.8	27.5 ± 0.4
0.15	0.642 ± 0.008	73.9 ± 2,0	23.5 ± 1.0
0.2	0.501 ± 0.017	75.3 ± 2.3	22.5 ± 0.6
0.3	0.422 ± 0.026	69.6 ± 0.7	22.5 ± 0.5
	Without feather		
0	0.045±	0.05 ± 0.00	0.0 ± 0.0
0.05	0.186±	0.19 ± 0.01	4.4 ± 0.1
0.1	0.202±	0.20 ± 0.02	4.5 ± 0.1
0.15	0.202±	0.20 ± 0.01	4.5 ± 0.2
0.2	0.159±	0.16 ± 0.06	4.6 ± 0.0
0.3	0.293±	0.29 ± 0.02	5.0 ± 0.3

Values are expressed as mean ± standard deviation of three independent experiments

Table 3 Effect of nitrogen source on keratinolytic protease production by *B. subtilis* S8 in basal feather medium containing 0.1% (w/v) sorbitol

Nitrogen source (0.05%, w/v)	Cell growth (A_{660})	Soluble protein (μg/ml)	Keratinolytic activity (U/ml)
None	0.965 ± 0.021	71.8 ± 1.1	9.4 ± 0.2
Beef extract	1.330 ± 0.036	80.3 ± 2.3	13.0 ± 0.5
Casamino acid	1.065 ± 0.027	81.9 ± 1.6	13.7 ± 0.2
Casein	1.875 ± 0.020	95.3 ± 2.6	26.3 ± 0.1
Con steep liquor	1.215 ± 0.055	75.8 ± 4.8	8.7 ± 0.7
Gelatin	1.080 ± 0.025	83.0 ± 1.4	16.8 ± 0.4
Malt extract	0.755 ± 0.047	84.0 ± 2.5	10.3 ± 0.2
Polypeptone	1.520 ± 0.034	89.4 ± 0.4	14.3 ± 0.5
Skim milk	0.905 ± 0.029	95.8 ± 3.3	19.8 ± 0.5
Tryptone	1.205 ± 0.013	85.9 ± 2.7	17.3 ± 0.3
Yeast extract	1.250 ± 0.031	68.6 ± 1.6	14.4 ± 0.4
Soytone	1.580 ± 0.045	61.1 ± 3.7	10.1 ± 1.0
Urea	0.886 ± 0.029	79.3 ± 2.3	25.6 ± 0.5
$(NH_4)_2SO_4$	0.065 ± 0.018	38.6 ± 1.8	3.8 ± 0.4
NH_4Cl	0.195 ± 0.020	38.1 ± 2.9	9.7 ± 0.7
NH_4NO_3	0.110 ± 0.016	40.5 ± 1.6	8.2 ± 0.5
$NH_4H_2PO_4$	0.415 ± 0.025	68.6 ± 1.1	14.0 ± 0.9
$(NH_4)_2HPO_4$	0.465 ± 0.007	58.7 ± 2.4	12.1 ± 0.4
KNO_3	1.010 ± 0.012	76.6 ± 1.8	29.5 ± 0.4
$NaNO_2$	0.785 ± 0.017	36.7 ± 2.3	23.3 ± 0.6
$NaNO_3$	1.225 ± 0.009	79.3 ± 2.8	21.8 ± 1.1

Values are expressed as mean ± standard deviation of three independent experiments

corn steep liquor, $(NH_4)_2SO_4$ or NH_4NO_3 to the medium repressed the keratinolytic protease production. A maximum production of keratinolytic protease (36.5 ± 0.5 U/ml) was observed at 0.3% (w/v) KNO_3 (data not shown). On the other hand, K_2HPO_4 at 0.1% (w/v), KH_2PO_4 at 0.06% (w/v) and $MgCl_2{\cdot}6H_2O$ at 0.04% (w/v) enhanced keratinolytic protease production (data not shown).

The effects of initial pH and temperature on the production of keratinolytic protease were investigated. As shown in Fig. 2, the strain S8 efficiently produced keratinolytic protease over a wide range of initial pH (7.0–10.0). The highest keratinolytic protease production was obtained with an initial pH 8.0 (43.1 ± 0.6 U/ml), whereas the highest cell growth was observed in pH 7.0–9.5 with an optimum at pH 7.5. There were no growth and keratinolytic protease production at pH 4.0. Soluble

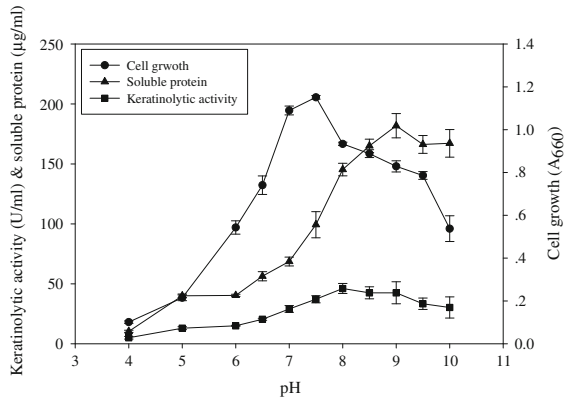

Fig. 2 Effect of initial pH on keratinolytic protease production by *B. subtilis* S8 in basal feather medium. *Error bars* (±SD) are shown when larger than the *symbol*

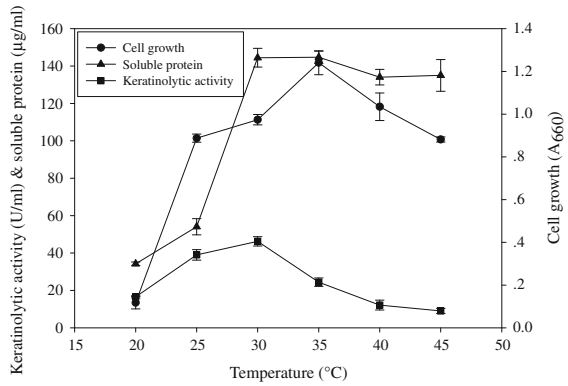

Fig. 3 Effect of temperature on keratinolytic protease production by *B. subtilis* S8 in basal feather medium. *Error bars* (±SD) are shown when larger than the *symbol*

protein concentration was increased with increasing initial pH up to 9.0. High level of keratinolytic protease was observed in the temperature range of 25–35°C with an optimum at 30°C (44.5 ± 0.5 U/ml) (Fig. 3). Cell growth was observed at all temperatures except 20°C, with a maximum at 35°C.

As a result of this study, improved feather medium for the keratinolytic protease production was established. The medium contained 0.1% (w/v) sorbitol, 0.3% (w/v) KNO_3, 0.1% (w/v) K_2HPO_4, 0.06% (w/v) KH_2PO_4, 0.04% (w/v) $MgCl_2{\cdot}6H_2O$ and 0.1% (w/v) feather (pH 8.0). Figure 4 shows typical time courses for cell growth and keratinolytic protease production in the improved feather medium. The keratinolytic activity reached to 53.3 ± 0.3 U/ml at 4 days of the cultivation when the cell growth reached late

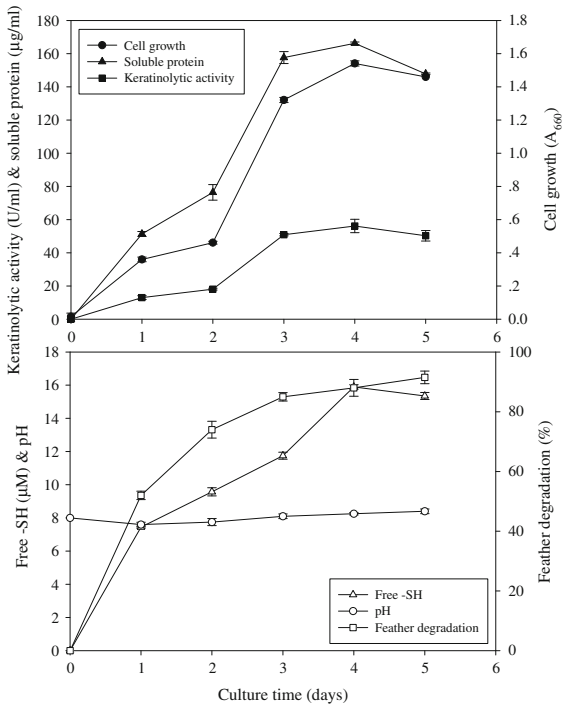

Fig. 4 Time courses of cell growth, keratinolytic protease, feather degradation, soluble protein, free –SH group and pH in an improved feather medium containing 0.1% (w/v) feather. *Error bars* (±SD) are shown when larger than the *symbol*

logarithmic growth phase. This value was approximately 10-fold higher than the yield in the basal feather medium (5.3 ± 0.1 U/ml). The cell growth and soluble protein content had the same tendency as the production of keratinolytic protease. Culture pH increased from 8.0 to 8.4 after 5 days of cultivation. The formation of free –SH groups was observed to increase during cultivation, reaching its maximum after 4 days (15.5 ± 0.2 μM). Feather was degraded about 91% after 4 days of cultivation.

Amino acid contents in culture supernatant

Amino acid composition and concentration of feather hydrolyzate were analyzed from cell-free supernatant of 4-day culture. As shown in Table 4, total free amino acid content in the cell-free supernatant was around 3360.4 μM. All essential amino acids (valine, leucine, isoleucine, methionine, threonine, lysine, phenylalanine and tryptophan) were produced in the culture. Proline was the most abundant (2809.9 μM) followed by histidine (371.3 μM) and phenylalanine (172.0 μM).

Table 4 Composition and concentration of amino acid in cell-free supernatant of *Bacillus subtilis* S8

Amino acid	Concentration (μM)
Glutamic acid	7.5
Aspartic acid	1.9
Asparagine	0.0
Histidine	371.3
Serine	3.8
Arginine	52.8
Glycine	8.2
Threonine	1.3
Alanine	1.1
Cysteine	76.8
Valine	2.1
Methionine	2.2
Proline	2809.9
Isoleucine	5.8
Leucine	2.5
Tryptophan	13.2
Phenylalanine	172.0
Lysine	53.2
Tyrosine	70.6
Total	3360.4

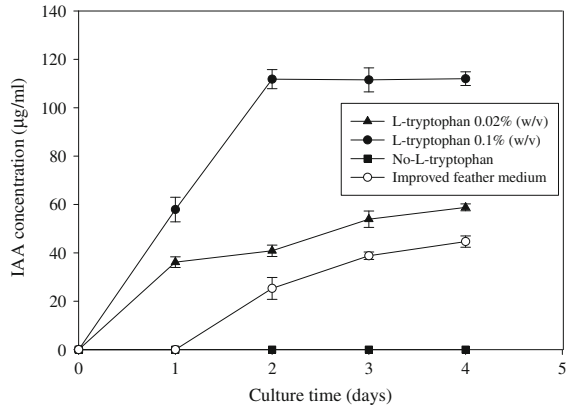

Fig. 5 Production of IAA by *B. subtilis* S8 in the presence of various concentrations of L-tryptophan. *Error bars* (±SD) are shown when larger than the *symbol*

Plant growth-promoting activities of *B. subtilis* S8

Production of IAA was investigated in the presence of different concentrations of L-tryptophan (Fig. 5). With no addition of L-tryptophan, IAA production

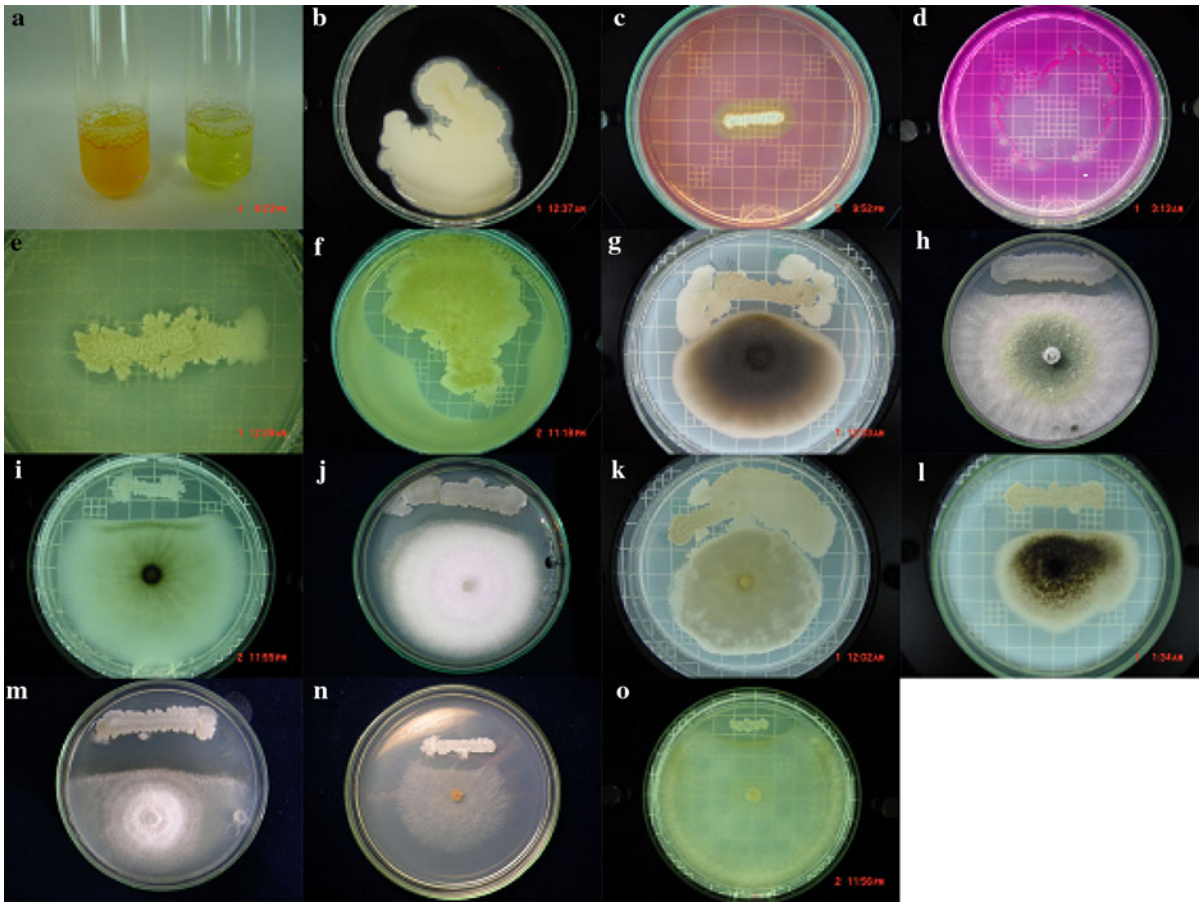

Fig. 6 Biochemical parameters showing biological activities of *B. subtilis* S8. **a** ammonia production in peptone water, **b** amylase production, **c** cellulase production, **d** pectinase production, **e** lipase production, **f** protease production and **g–o** antifungal activities against phytopathogenic fungi (*Alternaria panax*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora cactorum*, *Phoma* sp., *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*) on PDA plates

was not observed. The production of IAA was increased with increasing amounts of L-tryptophan up to 0.1% (w/v). Maximum production of IAA (114.6 ± 4.8 µg/ml) was observed after 2 days of incubation in King's B broth supplemented with 0.1% (w/v) L-tryptophan. In the improved feather medium without L-tryptophan supplementation, IAA was also produced; IAA concentration was increased with increasing culture time, reaching a maximum (42.5 ± 1.0 µg/ml) at 4 days.

As shown in Fig. 6, *B. subtilis* S8 possessed different traits related to plant growth promotion. Ammonia production was recorded when *B. subtilis* S8 changed the color of culture broth to reddish brown after 24 h of incubation (Fig. 6a). Petri dish-based assays carried out for the production of hydrolytic enzymes indicate that *B. subtilis* S8 produced amylase, cellulase, pectinase, lipase and protease (Fig. 6b–f). Production of other metabolites, such as siderophore, β-1,3-glucanase and chitinase, was not observed. *B. subtilis* S8 was also showed a wide range of antifungal activities against *A. panax*, *B. cinerea*, *C. gloeosporioides*, *F. oxysporum*, *P. cactorum*, *Phoma* sp., *P. ultimum*, *R. solani*, and *S. sclerotiorum* by dual culture assay on the PDA plate (Fig. 6g–o). In addition, *B. subtilis* S8 inhibited the growth of pathogenic bacteria including *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes* (data not shown).

Discussion

The scope of present study was to isolate and characterize multi-functional feather-degrading bacteria which could produce keratinolytic, antifungal and plant growth-promoting activities. This is the first report on feather-degrading *Bacillus* species which is able to produce antifungal and IAA-producing activities in the same condition observed for keratinolytic activity.

A novel bacterium from forest soil was identified as *B. subtilis* S8 on the basis of BIOLOG system and 16S rRNA gene sequence analysis. This strain was able to secrete keratinolytic protease, which is known to be responsible for feather keratin degradation in nature. After 5 days of incubation at 30°C, feather residue had an unrecognizable powder-like consistency (Fig. 1a); SEM data (Fig. 1b–d) indicate that *B. subtilis* S8 closely adhere to the barbules and produce keratinolytic protease that diffuses laterally degrading the rachis and barbules.

Although the strain S8 produced keratinolytic protease using chicken feather as the sole carbon source, the addition of co-carbon sources except galactose greatly increased the production of keratinolytic protease (Table 1). In the absence of feather, *B. subtilis* S8 also produced a low level of keratinolytic protease when cultivated in media containing various concentrations of sorbitol. Carbohydrates including glucose are known as a common catabolic repressor for a number of protease in *B. subtilis* (Fisher and Sonenshein 1991). Production of proteases by bacteria is generally regulated by repression and induction (Rao et al. 1998). On the contrary, our results indicate that the synthesis of keratinolytic protease by *B. subtilis* S8 is constitutive and is enhanced by additional carbon sources. This new phenomenon suggests a changed general enzyme-regulatory circuit in the background (Rozs et al. 2001).

Keratinolytic activity was considerably inhibited in the lower pH range, while it was enhanced in the higher pH range (Fig. 2). The optimum pH value for the keratinolytic activity was 8.0. These results are in accordance with previous report showing that the keratinolytic protease produced by *Bacillus* species could be classified as an alkaline protease and was most active under neutral or alkaline conditions (Rai and Mukherjee 2010; Mazzoto et al. 2010). Generally, the keratinolytic microorganisms have higher optimum temperatures of growth and feather degradation (Onifade et al. 1998). *B. subtilis* S8 showed optimal growth and keratinolytic protease production at mesophilic temperatures (Fig. 3). Sangali and Brandelli (2000) pointed that the keratinolytic activity of mesophilic organisms may be an interesting property for biotechnological use because these microorganisms will be less energy consuming than the thermophilic ones. In this respect, *B. subtilis* S8 is another bacterial strain which could be used for the biodegradation of poultry and abattoir wastes.

Lin et al. (1992) reported that the keratinolytic activity by *B. licheniformis* PWD-1 was increased with cultivation time and observed a maximum activity at the late logarithmic growth phase. The same result was reported on *Streptomyces fradiae* (Shama and Berwick 1991). Those results were similar to that observed in *B. subtilis* S8 (Fig. 4).

The high stability of feather keratin against mechanical and enzymatic degradation is due, at least in part, to the occurrence of disulfide bridges (Gioppo et al. 2009). Keratin degradation by keratinolytic protease is still a matter of debate as some research groups showed that keratin degradation was started and facilitated by disulfide reductase enzyme (Ghosh et al. 2008), whereas other groups showed that keratinase alone could cause complete degradation of whole feather (Ignatova et al. 1999). In this study, the formation of free –SH group was observed to increase during cultivation in the improved feather medium, reaching its maximum (15.5 ± 0.2 μM) after 4 days (Fig. 4). Higher feather degradation also resulted in high –SH group formation (data not shown). Therefore, our result indicates that *B. subtilis* S8 possesses disulfide reductase activity along with keratinolytic activity. Reduction of disulfide bridges was observed for *Cryseobacterium* sp. strain kr6 (Riffel et al. 2003) and *Streptomyces pactum* (Böckle and Müller 1997) grown on feathers.

Biotechnological applications consider the use of feather-degrading microorganisms or keratinolytic protease in the production of amino acids and peptides. Moreover, amino acids resulting from the utilization of feather or keratin-containing materials are one important product that can promote several new industries (Haddar et al. 2009). Amino acids released during the degradation of feather by *B. subtilis* S8 were assayed. Any free amino acid was

not produced in uninoculated medium (data not shown), while 19 amino acids including all essential amino acids were detected in the cell-free culture supernatant of the strain S8; proline, histidine and phenylalanine were the major amino acid released (Table 4). It is known that essential amino acids such as tryptophan and lysine are deficient in feather keratin (Harrap and Woods 1964). In this study, cysteine content released was 76.8 μM, which may be due to the high disulfide content of feather keratin. *B. licheniformis* PWD-1 liberated mainly leucine, tryptophan, valine, isoleucine, phenylalanine, and alanine from feather as free amino acids (Williams et al. 1990). This suggests that the keratinolytic protease produced by *B. subtilis* S8 is different from that produced by *B. licheniformis* PWD-1. Based on the above results, the culture broth of the strain S8 could be used as feed additives or nitrogen fertilizers.

The capability to synthesize IAA is an important feature for a strain to be considered a PGPB; it is well known that IAA participates in promotion of plant growth by increasing the radical surface of the inoculated plants (Shoebitz et al. 2009). Phosphorus is one of the major plant nutrients limiting plant growth. Therefore, application of phosphate fertilizers essential for better crop yields. However, availability of phosphate is a serious problem, because it is fixed in the soil and lowers the utilization efficiency of added phosphate fertilizer by plants (Park et al. 2010). Phosphate-solubilizing microorganisms render insoluble phosphate into soluble form through the process of acidification, chelation and exchange reactions. This process not only compensates for higher cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil (Rodriguez and Reynaldo 1999). The role of ammonia in biocontrol has been described; ammonia is the only gas present in sufficient concentrations in soil to inhibit soil fungi (Pavlica et al. 1978). In this study, *B. subtilis* S8 showed potential efficacy not only in antagonizing phytopathogenic fungi (ammonia, antifungal activity and hydrolytic enzyme production) but also in inducing plant growth (IAA production and insoluble phosphate solubilization) (Fig. 6). Especially, the strain S8 produced IAA in the improved feather medium without L-tryptophan supplementation (Fig. 5), indicating L-tryptophan release resulting from feather degradation (Table 4) and simultaneous production of keratinolytic activity and IAA by *B. subtilis* S8. On the other hand, regardless of media used, the strain S8 inhibited mycelial growth of all fungi tested (Table 5). However, the strain S8 grown in the improved feather medium less inhibited the fungal growth than that grown in tryptic soy broth. This result suggests that antifungal activity of the strain S8 could be produced in the same conditions observed for keratinolytic activity. These characteristics of *B. subtilis* S8 have not been described previously in any microorganism. Although *B. subtilis* S8 did not produce any visible clear zone on NBRIP agar plate containing 0.5% $Ca_3(PO_4)_2$ as the insoluble phosphate source, it produced 18 mg/l of soluble phosphate in NBRIP broth after 4 days (data not shown). This may be because of the varying diffusion rates of different organic acids secreted by *B. subtilis* S8 (Gupta et al. 1994). In addition to insoluble phosphate solubilization, substantial production of IAA by *B. subtilis* S8 clearly suggests its inherent plant growth promotion potential. We cannot also exclude the possibility that the culture of *B. subtilis* S8, containing feather hydrolyzate and IAA producing activity, might have an important role in fertilization process of crop soil. Other functional traits such as ammonification, hydrolytic enzyme production and broad-spectrum antifungal activity (Fig. 6) may also enhance its potential as biocontrol agent.

Table 5 Antifungal activity of *B. subtilis* S8 grown in tryptic soy broth and improved feather medium

	Antifungal activity[a]	
	Tryptic soy broth	Improved feather medium
A. panax	+++	++
B. cinerea	++	+
C. gloeosporioides	+++	++
F. oxysporum	++	+
P. cactorum	+++	+
Phoma sp.	+++	++
P. ultimum	+++	++
R. solani	+++	++
S. sclerotiorum	++	+

[a] Antifungal activity was scored as follows: +, weak suppression (0.1–1.0 cm inhibition zones around bacterial spots): ++, medium suppression (1.0–1.5 cm inhibition zones): +++, strong suppression (1.6–2.0 cm inhibition zones)

Conclusions

We isolated and characterized a novel *B. subtilis* S8, which is able to produce simultaneously keratinolytic, antifungal and plant growth-promoting activities. *B. subtilis* S8 could be useful in processes for the conversion of feather or other protein wastes to feedstock additives, and suitable for the in situ microbial degradation of feather wastes (e.g. with composting technology). In addition, because of the innate potential of producing IAA, phosphate solubilizing activity and fungal antibiosis, *B. subtilis* S8 could be also used as a potential biofertilizer as well as a potential biocontrol agent in agricultural environments. We are now investigating the purification and properties of keratinolytic protease from *B. subtilis* S8 and the usefulness of *B. subtilis* S8 on the crop plant growth under greenhouse and field conditions.

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