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Isolation, Identification, and Keratinolytic Activity of Several Feather-Degrading Bacterial Isolates

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ABSTRACT

Several feather-degrading bacterial isolates were isolated from Egyptian soil. These isolates were able to degrade chicken feather, when grown on basal medium containing 1% native feather as a source of energy, carbon, and nitrogen. Feather waste, generated in large quantities as a byproduct of commercial poultry processing, is nearly pure keratin, which is not easily degradable by common proteolytic enzymes. The isolates were identified according to the morphological characteristics, biochemical tests, and API 50 CHB *Bacillus* system. Proteolytic and keratinolytic activities of these isolates were monitored throughout the cultivation of the bacterial isolates on feather. Resulting soluble proteins, which were released as a result of the biodegradation of feather, were demonstrated by SDS-PAGE.

Index Entries: *Bacillus*; isolation; identification; keratinolytic activity.

INTRODUCTION

Environmental wastes are found in large quantities in many countries. Although some of them contain a considerable amount of protein and various carbon compounds, little attention is given to using them in a technological way. Recently, the authors have focused on the utilization of some environmental wastes, mainly feather waste. Feather waste, generated in large quantities as a byproduct of commercial poultry processing, is nearly pure keratin *(1)*. Because of the high degree of disulfide bonds, hydrophobic interactions, and hydrogen bonds, keratin in its native state is not degradable by common proteolytic enzymes, such as pepsin, trypsin,

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and papain (*2*). Feather waste is utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Generally, the feather is steam-pressure-cooked or chemically treated before use (*3*). Biodegradation of feather by microorganisms represents an alternative method to improve the nutritional value of feather waste.

The present work reports the isolation of several feather-degrading bacterial isolates from soil. These isolates are able to degrade whole chicken feather when grown on basal medium containing 1% native feather. The isolates were classified as members of the genus *Bacillus*. Proteolytic and keratinolytic activities of these bacterial isolates were monitored throughout the biodegradation process.

MATERIALS AND METHODS

Media

Bacterial isolates were activated and grown on PY medium (*4*) (Bacto peptone, 10 g; Difco (East Molesley, Surrey, UK) yeast extract, 5 g; and NaCl, 5 g/L). PA medium is PY supplemented with 1.5% agar agar. Basal medium II (*5*) (NH_4Cl, 0.5 g; NaCl, 0.5 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.4 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; and yeast extract, 0.1 g/L), supplemented with 1% (w/v) whole chicken feather, was used to check the proteolytic and keratinolytic activity of the bacterial isolates. Feather plates were made as follows: 2 g of whole feather were treated with about 5 mL of NaOH (5 *N*), to the point that feather was converted to a soft paste. The volume was adjusted to 100 mL with basal medium II, after which the pH was adjusted to pH 7.0, and 1.5 g agar was added. The medium was autoclaved at 110°C for 10 min.

Isolation of Microorganisms

Several pieces of whole chicken feather were placed into wet soil in plastic containers. The containers were watered every 2 d for 2 wk, after which the partially degraded feather pieces were used to inoculate PY medium. The new culture was allowed to grow at 37°C for 24 h, with shaking. Suitable dilutions of this culture were plated on PA plates. Single colonies were screened for their ability to hydrolyze milk and keratin, by patching them into milk agar plates and feather plates, respectively. Alternatively, 100 mL of PY medium was inoculated with 1% soil suspension, and the culture was allowed to grow overnight, with shaking, at 37°C. Basal medium II, containing 1% whole feather, was inoculated with 1 mL of the above culture, and the new culture was allowed to grow at 37°C for 2, 4, and 6 d. A sample was taken every 2 d, diluted, and plated on PA plates. Single colonies were checked on milk plates and feather plates, as described above.

Determination of Bacterial Viable Count

Colony-forming units (CFU) were determined as described earlier *(6)*.

Identification of Feather-Degrading Isolates

Feather-degrading isolates were identified according to morphological examinations and several biochemical tests *(7,8)*. Identification was mainly based on the use of API 50 Carbohydrates *Bacillus* (CHB) strips (API Laboratory, Basingstoke, Hants, UK). The strips contain 49 *Bacillus*-specific carbohydrates tests. Bacterial isolates were prepared and treated as described earlier *(9)*.

Monitoring Proteolytic and Keratinolytic Activity

Bacterial isolates were activated by growing them overnight on PA plates at 37°C. Fresh colonies of each isolate were then transferred to 10 mL PY medium, and cultures were allowed to grow at 37°C for 2 h. One hundred mL preautoclaved basal medium II, containing 1% untreated whole feather, was inoculated with 5 mL of the above culture. Cells were allowed to grow at 37°C, with shaking, to the indicated time. At the indicated time, 1 mL culture was taken to determine the CFU, as described earlier. Two other mL were taken, centrifuged at 4500*g* for 2 min, using a microcentrifuge, and the supernatants were used as crude solution to determine proteolytic and keratinolytic activity.

Proteolytic Activity

Proteolytic activity was measured as described by Cliffe and Law *(10)*, using hide powder azure (HPA). One unit of enzyme is the amount of enzyme that develops a change of absorbance, against control reaction, at 595 nm/30 min at 37°C. Additionally, the proteolytic activity was determined according to the method of Lin et al. *(11)*, using skim milk agar plate.

Keratinolytic Activity

Keratinolytic activity was determined based on the free amino (-NH_2 groups) that were released as a result of the biodegradation of feather by bacterial cells. Free amino groups were determined as described earlier *(11)*, and a standard curve for leucine (0.01–0.1 μmol) was established. Additionally, the physical appearance of chicken feather in the culture was observed.

Analysis of Soluble Proteins by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell-free supernatant of each feather culture was precipitated with solid ammonium sulfate to reach 70% saturation, and was kept on an ice bath for 2 h. The mixture was centrifuged at 7000*q* for 30 min at 4°C.

The pellet was suspended in a small volume of Tris-HCl buffer, pH 8.0, and dialyzed overnight against this buffer.

A second precipitation by acetone (3 vol) was carried out, and the precipitate was collected by centrifugation at 3500*g* for 5 min using microcentrifuge. The pellet was directly suspended in a small volume of sample application buffer (SAB) and applied to a 10% SDS-polyacrylamide gel. The SDS-PAGE was carried out according to the method of Laemmli *(12)*.

RESULTS AND DISCUSSION

Identification of Feather-Degrading Bacterial Isolates

Feather, in its native state, is not degradable by common proteolytic enzymes such as trypsin, pepsin, and papain. However, feather or keratin-containing compounds do not accumulate in nature (especially in soil) *(5)*. Isolation of the feather-degrading bacterial isolates was carried out using soil sample and/or embedded feather in soil. The identification of these bacterial isolates was based on cell morphology, colony morphology, growth characteristics, several biochemical and supplementary tests, and, finally, the use of API 50 CHB strips that were designed to identify members of the genus *Bacillus*. API 50 CHB system has been shown to be rapid, accurate, and more reproducible than the classical tests. A taxonomy based on API 50 CHB tests is in good agreement with those obtained by other methods *(9)*.

Throughout the identification steps, *B. subtilis* DB100 *his*$^-$ *met*$^-$ strain *(13)* was used as a reference strain. All feather-degrading bacterial isolates were Gram-positive, endospore-forming, rod-shaped bacilli. Bacterial isolate Bio 8/2 was identified as *B. pumilus*, isolates Bio 27/1, Bio 1/2, Bio 23, and Bio 6/2 were identified as *B. cereus*, and the bacterial isolate Bio 23′ was identified as *B. subtilis*.

Monitoring Proteolytic and Keratinolytic Activity

Bacterial isolates were activated by growing them on PY medium for 2 h, to get vegetative active cells. Proteolytic activity was rapidly and accurately determined, using the synthetic substrate HPA, as well as the skim milk agar plate method. Data from the latter method (not shown) were consistent with those of HPA. Figure 1 shows two patterns for the production of extracellular proteases regarding the time of secretion, taking into consideration viable count of each isolate (log CFU). The isolates Bio 8/2, Bio 23, and Bio 23′ secreted their proteases early (early genes), after which the proteolytic activity was decreased with increasing the cultivation time. In contrast, bacterial isolates Bio 27/1 and Bio 1/2 secreted their proteases late (late genes). Since members of the genus *Bacillus* are characterized by the formation of endospores, some genes encoding pro-

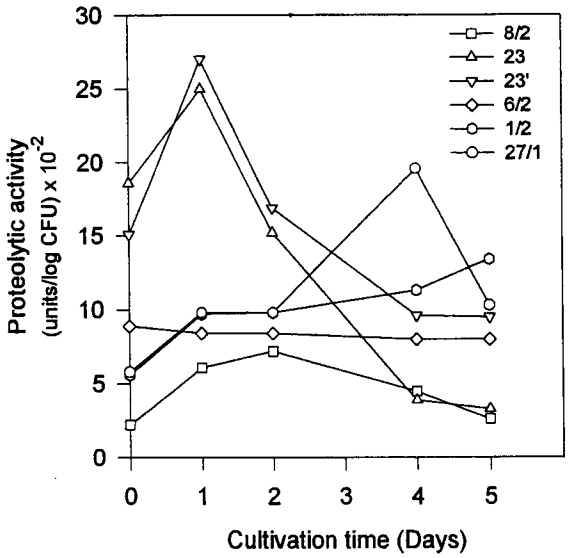

Fig. 1. Monitoring proteolytic activity (U/log CFU) of the bacterial isolates throughout the cultivation time. Isolates were activated on PY medium for 2 h, after which they were grown on basal medium II, supplemented with 1% whole chicken feather.

teases are expressed early during the log phase (i.e., neutral protease of *B. subtilis*), while other protease genes are known to be expressed late, during the stationary phase, or just before sporulation (i.e., the alkaline protease gene of *B. subtilis*) *(14)*. Unlike the above two patterns, the bacterial isolate Bio 6/2 showed constant production of their extracellular protease.

The keratinolytic activity of the bacterial isolates was determined based on measuring the free amino groups that were released in the cultures as a result of the biodegradation of feather. Generally, keratinolytic activity (μmol/mL culture/log CFU) increased with cultivation time (Fig. 2). Bacterial isolates Bio 23 and Bio 23' showed high level of both proteolytic and keratinolytic activities. This would suggest that their proteases may be acting as keratinases, and this is in agreement with some previous reports *(15–17)*. In contrast, the bacterial isolate Bio 27 showed high level of proteolytic activity, but a moderate level of keratinolytic activity.

The authors reported earlier the cloning of the *B. subtilis* alkaline protease (*aprA*) gene on a pUB110-derivative plasmid. *(14)* High levels of expression and gene stability were demonstrated when *B. subtilis* cells were grown on the laboratory medium 2XSG *(14)*. When these cells were grown on basal medium, supplemented with 1% whole chicken feather, high levels of proteolytic and keratinolytic activity were obtained. Data would indicate that the *aprA* gene is acting as a keratinase gene *(17)*. In the present study, *B. subtilis* DB100 (pS1) cells carrying the multicopy *aprA* gene were used to compare the proteolytic and the keratinolytic activity of these cells

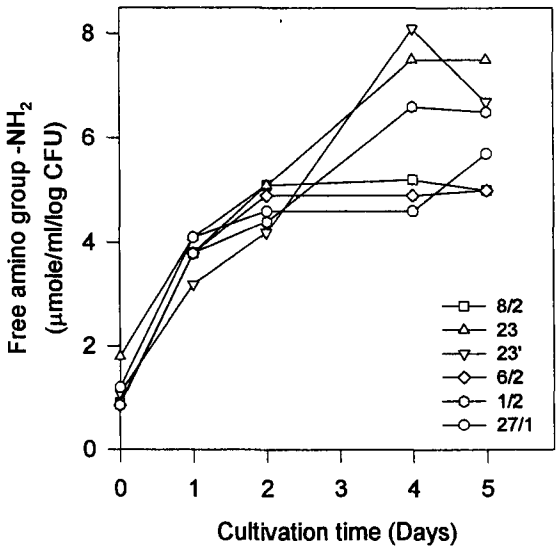

Fig. 2. Monitoring keratinolytic activity of the bacterial isolates throughout the cultivation time. Isolates were activated on PY medium, then grown on basal medium II, supplemented with 1% whole chicken feather. The keratinolytic activity is expressed as µmol of $-NH_2$ groups per mL of cell-free supernatant per log CFU.

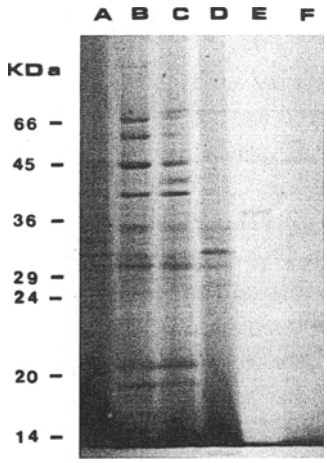

Fig. 3. SDS-polyacrylamide gel to analyze soluble proteins resulted from the biodegradation of whole feather at d 4. Preparation of samples and gel conditions were described in Materials and Methods. Lanes A–F represent 100 µg soluble proteins of bacterial isolates Bio 8/2, 23, 23', 6/2, 1/2, and Bio 27/1, respectively.

with those of the bacterial isolates. The proteolytic activity of *B. subtilis* DB100 (pS1) strain was several-fold higher than that of any of the bacterial isolates; the keratinolytic activity (specific activity) was at the level of isolates Bio 23, Bio 23′ and Bio 1/2 (6.7 μmol free NH_2 group/mL/log CFU) *(17)*.

Analysis of Soluble Proteins

On the biodegradation of feather using the bacterial isolates, considerable amounts of soluble proteins were obtained. These proteins were analyzed by SDS-polyacrylamide gel as described in Materials and Methods (Fig. 3). A zymogram technique, using gelatin in the polyacrylamide gel, is needed to know which protein band(s) might have a proteolytic activity.

The biodegradation of feather waste using these bacterial isolates represents an alternative way to improve the nutritional value of feather, since feather waste is currently utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Research is being carried out to improve the ability of some of these isolates to degrade feather by transforming these bacterial isolates with the multicopy (pS1) plasmid that carries the *aprA* gene *(14)*.

REFERENCES

1. Goddard, D. R. and Michaelis, L. (1934), *J. Biol. Chem.* **106,** 604–614.
2. Harrap, B. S. and Woods, E. F. (1964), *Biochem. J.* **92,** 19–26.
3. Steiner, R. J., Kellems, R. O., and Church, D. C. (1983), *J. Anim. Sci.* **57,** 495–502.
4. Bernhard, K., Schrempf, H., and Goebel, W. (1978), *J. Bacteriol.* **133,** 897–903.
5. Williams, C. M., Richter, C. S., Mackenzie, J. M., and Shih, J. C. (1990), *Appl. Environ. Microbiol.* **56,** 1509–1515.
6. Pelczar, M. J. and Chan, E. C. (1977), *Laboratory Exercises in Microbiology,* 4th ed., McGraw-Hill, New York.
7. Claus, D. and Berkeley, R. C. W. (1986), in *Bergey's Manual of Systematic Bacteriology,* vol. 2 Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. (eds). Williams and Wilkins, Baltimore, MD pp. 1105–1138.
8. Cheesbrough, M. (1985), *Medical Laboratory Manual for Tropical Countries,* vol. 2, ELBS.
9. Logan, N. A. and Berkeley, R. C. W. (1984), *J. Gen. Microbiol.* **130,** 1871–1882.
10. Cliffe, A. J. and Law, B. A. (1982), *J. Dairy Res.* **49,** 209–219.
11. Lin, X., Lee, C. G., Casale, E. S., and Shih, J. C. (1992), *Appl. Environ. Microbiol.* **58,** 3271–3275.
12. Laemmli, U. K. (1970), *Nature (London)* **227,** 680–685.
13. Sadaie, Y. and Kada, T. (1983), *J. Bacteriol.* **153,** 813–821.
14. Zaghloul, T., Abdel Aziz, A., and Mostafa, M. (1994), *Enzyme Micro. Technol.* **16,** 534–537.
15. Lin, X., Kelemen, D. W., Miller, E. S., and Shih, J. C. (1995), *Appl. Environ. Microbiol.* **61,** 1469–1474.
16. Takami, H., Akiba, T., and Horikoshi, K. (1990), *Appl. Microbiol. Biotechnol.* **33,** 519–523.
17. Zaghloul, T. (1998), *19th Symposium on Biotechnology for Fuels and Chemicals,* ABAB paper no. 2-08.