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Keratinase production by newly isolated Antarctic actinomycete strains

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Summary

The ability of actinomycete strains newly isolated from Antarctic soils to produce keratinolytic enzymes during growth on sheep wool waste was investigated. The strains which displayed highest keratinase activity and identified as *Streptomyces flavis* 2BG (mesophilic) and *Microbispora aerata* IMBAS-11A (thermophilic) were selected for a more detailed analysis. The addition of starch to the growth medium affected keratinase secretion by both strains. After 5 days of cultivation, a 6-fold increase in keratinase activity of strain 11A was observed in the presence of 11 g starch/l and a 9-fold increase in keratinase activity of the strain 2BG in the presence of 5 g starch/l. The results obtained showed that both newly isolated strains are very promising for effective processing of native keratinous wastes. To our knowledge, this is the first report of Antarctic actinomycete strains that were able to grow on keratin-containing wastes by producing keratinolytic enzymes.

Introduction

Keratin-containing materials (feather, hair, wool, etc.) are abundant in nature but have limited uses in practice since they are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinous wastes represent a source of valuable proteins and amino acids and could find application as a fodder additive for animals or source of nitrogen for plants. For this purpose, destruction of the rigid keratin structure is necessary. Biodegradation by microorganisms possessing keratinolytic activity represents an alternative attractive method for improving the nutritional value of keratin wastes, as it offers cheap and mild reaction conditions for the production of valuable products. There have been some reports on microorganisms capable of degrading keratinous wastes (Santos *et al.* 1996; Onifade *et al.* 1998; Wang & Shih 1999; Gradisar *et al.* 2000; Gessesse *et al.* 2003). Up to now, a limited number of studies have been reported on the isolation of thermophiles, in particular thermophilic actinomycetes with the ability to hydrolyse wool and other keratinous wastes (Kabadjova *et al.* 1996; Ignatova *et al.* 1998, 1999). Thermophilic actinomycetes have some advantages in comparison with mesophilic strains, such as accelerated accumulation of biomass and enzymes (Atlas of Actinomycetes 1997). Actinomycetes have the ability to break down many different varieties of organic compounds and are crucial in the mineralization of organic matter (Ryckeboer *et al.* 2003). As there are keratin-containing wastes in Antarctica, mainly penguin feathers, there seemed to be good prospects for the isolation of Antarctic actinomycetes possessing keratinolytic activity.

There are no studies so far on the ability of Antarctic microorganisms, including actinomycetes to grow on keratin-containing materials. In the present work, a number of actinomycetes were isolated from soil samples of Livingston Island, Antarctica. The aim of this study was to detect organisms with the best ability to degrade wool waste by means of keratinolytic activity. In order to stimulate keratinase secretion by the selected strains, the effect of starch was investigated.

Materials and methods

Isolation of actinomycetes

Antarctic soil samples from Livingston Island were used for isolation of actinomycetes. One gram of soil was suspended in 9 ml of physiological saline and vigorously mixed. Serial dilutions were made and aliquots were streaked on to agar plates containing (g/l): peptone 5; corn steep liquor 5; starch 10; NaCl 5, and 1.5% agar (Kosmachev 1954). The plates were then incubated 48 h at 55 °C or at 28 °C. The visible morphological types of single colonies were isolated, maintained on the above medium at 4 °C and transferred monthly.

Media and growth conditions

Actinomycete strains were cultivated in 500-ml Erlenmeyer flasks with 100 ml of mineral salt (MS) medium containing (g/l): $K_2HPO_4 \cdot 3H_2O$, 1.5; $MgSO_4$, 0.1; $CaCl_2$, 0.1; $FeSO_4$, 0.03; $ZnSO_4$, 0.005, pH 7.2, supplemented with cut wool waste (6 g/l) as a sole source of carbon and nitrogen. As inocula, 10% spore suspensions were used after 18 h of cultivation in the liquid broth of Kosmachev (1954). Flasks were incubated for 5 days at 55 °C or 28 °C with constant shaking (280 rev/min). Culture supernatants obtained after centrifugation ($4000 \times g$ for 20 min) were analysed for keratinase activity and for soluble protein content as a measure for the degree of degradation of the keratin substrate.

Two of the isolates, the mesophilic *Streptomyces flavis* 2BG (Gousterova *et al.* 2003), and the thermophilic *Microbispora aerata* IMBAS-11A (Ivanova *et al.* 2003) were selected for further experiments. They were cultivated for 10 days at 55 °C (*Microbispora*) or at 28 °C (*Streptomyces*) in MS medium with cut wool waste as a sole source of carbon and nitrogen. In another set of flasks, each of two tested isolates was cultivated for 5 days in MS medium supplemented with wool waste (6 g/l) and increasing amounts of starch as carbon source. Control flasks without actinomycetes were incubated in the same conditions to quantify losses due to abiotic processes. Daily, samples were taken from each flask, centrifuged to remove the cells and insoluble residues, and the supernatants were analysed for protein content, concentration of SH-groups and keratinase activity.

Analytical methods

Protein content was determined by the Bradford method. The concentration of SH-groups was estimated using Ellman's reagent (1959). The keratinase activity was determined by the modified method of Cheng *et al.* (1995). The increase in absorbance at 280 nm was converted into keratinase units (1 KU = 0.1 absorbance increase for 1 h).

Statistical analysis

Nonparametric 2-tailed Mann–Whitney *U*-test was used to determine the significance of differences of the measured keratinase activities of both tested strains. Statistical analyses were performed using the Stawin 5.1 software.

Results and discussion

Growth of actinomycete strains on wool waste

Nine thermophilic and ten mesophilic actinomycete strains were isolated from Antarctic soil samples on agar medium. They were screened for keratinase activity in liquid MS medium containing wool waste (6 g/l). Wool utilization was initially assessed by appearance of turbidity and foaming of the culture medium of isolates. After 5 days of cultivation, the levels of soluble protein in the media of two of isolates, the mesophilic *Streptomyces flavis* 2BG and the thermophilic *Microbispora aerata* IMBAS-11A were higher than the levels of the other strains, indicating degradation of the keratin substrate (Figure 1a). These strains were selected for further studies.

Enzyme production and reduction of disulphide bonds in wool substrate

Maximum keratinase activity was observed on day 5 of cultivation for strain 11A, and on day 8 for strain 2BG (Figure 1b). The synthesis of the extracellular keratinase was associated with the increase of soluble protein in the

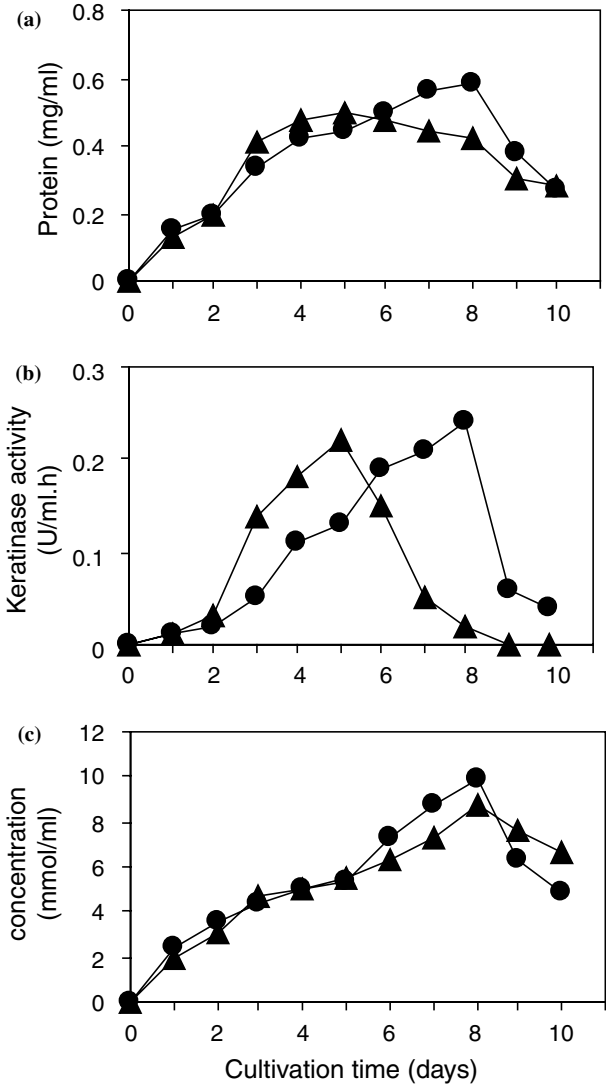

Figure 1. Soluble protein content (a), keratinase activity (b) and concentration of free thiol groups (c) for *Streptomyces flavis* 2BG (●) and *Microbispora aerata* IMBAS-11A (▲). The strains were grown in mineral medium (pH 7.2), supplemented with 6 g wool/l as a sole source of carbon and nitrogen. Mean values from three determinations are given.

culture broth (Figure 1a). At this time, maximum concentration of SH-groups in the broth medium was observed as well (Figure 1c). A simultaneous cleavage of disulphide bonds during microbial growth has been described for *Streptomyces fradiae* (Kunert & Stransky 1988) and *Streptomyces pactum* (Bockle *et al.* 1995). One of the possible mechanisms for microbial reduction of disulphide bonds is by excretion of sulphite. At neutral or alkaline pH sulphite cleaves the disulphide bonds to thiols, thiosulphates, and *S*-thioesters (Kunert & Truper 1986; Kunert & Stransky 1988; Kunert 1989).

Previous studies have shown that the synthesis of extracellular keratinases is constitutive or partially inducible (Malviya *et al.* 1992; Cheng *et al.* 1995). Keratinase activity was not detected during growth of both tested strains in media without keratin. This indicated that the major regulatory mechanism for keratinase synthesis by these strains is substrate induction. Such mechanism was reported for *Thermoactinomyces candidus* (Ignatova *et al.* 1999).

Influence of starch on keratinase secretion by the strains 2BG and 11A

Strains 2BG and 11A were tested for keratinase activity in presence of increasing amounts of starch as carbon source. Since starch is a main component in the media for isolation of actinomycetes (Kosmachev 1954) it was added in order to stimulate the keratinase secretion. Moreover, it is a cheap and widely accessible product. After 5 days of cultivation, an increase in the keratinase activity was observed in presence of starch: 6-fold higher activity in presence of 11 g starch/l for the strain 11A, and 9-fold higher for the strain 2BG in presence of 5 g starch/l (Figure 2). Both strains belong to different genera with different metabolism and have different dependence on the growth temperature. Because of that they did not respond in the same way to increasing starch concentration. The thiol group concentration was found to decrease with increasing starch concentration. Most probably in this case the disulphide bond reduction took place in the cell-bound redox system situated on the surface of the cell. This mechanism requires a close contact between the cell and the insoluble keratinous substrate. Similar observations have been reported for disulphide bond reduction by *Streptomyces pactum* (Bockle & Muller 1997).

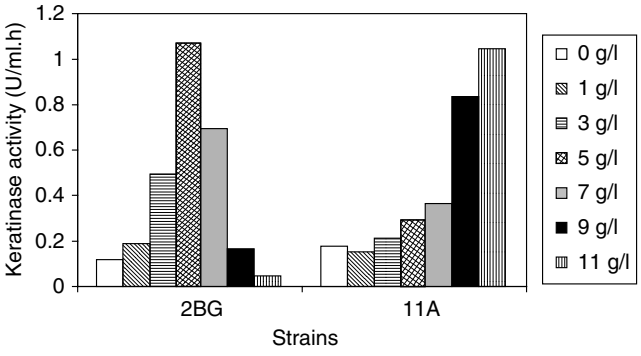

Figure 2. Effect of the addition of different concentrations of starch on keratinase production by *Streptomyces flavis* 2BG and *Microbispora aerata* IMBAS-11A. Concentrations of starch are indicated in the legend. Mean values of three experiments are given.

The microflora of penguin excrement and glaciers in the region of the Livingston Island, Antarctica is characterized by the presence of different ecological types of microorganisms: thermophiles, mesophiles, facultative psychrophiles and typical psychrophiles (Gushterova *et al.* 1999; Noustorova *et al.* 1999). The presence of thermophilic actinomycetes in Antarctica was unexpected for us but is a fact. It could be a result of pollution of this continent (Agre 1986). There are very large deposits of coal in Antarctica, which indicates that rich vegetation existed until glaciation, probably beginning 50 million years ago (Encyclopedia Britannica 2004). So, another hypothesis could be that the thermophilic microorganisms have existed since the Paleozoic and Mesozoic eras when Antarctica was warm, and their spores have survived (in cryoanabiotic state) until now.

This is first report about the ability of Antarctic actinomycetes to grow on keratin-containing wastes by producing keratinolytic enzymes. Increased keratinase activity in the presence of starch in growth media of both Antarctic actinomycete strains makes them very promising for use in softening keratinous wastes, thus helping their degradation.

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References

Agre, N.S. 1986 *Taxonomy of thermophilic actinomycetes*. Ph.D. Thesis, Puschino, Moscow, pp. 5–10 (in Russian).

Atlas of actinomycetes. 1997 ed. Miyadoh, S. Asakura, Japan: Society for Actinomycetes. ISBN4-254-17098-X.

Bockle, B., Galunsky, B. & Muller, R. 1995 Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. *Applied and Environmental Microbiology* **61**, 3705–3710.

Bockle, B. & Muller, R. 1997 Reduction of disulphide bonds by *Streptomyces pactum* during growth on chicken feathers. *Applied and Environmental Microbiology* **63**, 790–792.

Cheng, S.W., Hu, H.M., Shen, S.W., Takagi, H., Asano, A. & Tsai, Y.C. 1995 Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. *Bioscience, Biotechnology and Biochemistry* **59**, 2239–2243.

Ellman, G.L. 1959 Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* **82**, 70–77.

Encyclopedia Britannica 2004 Antarctica. In *Encyclopedia Britannica*, from Deluxe Edition 2004 CD-ROM.

Gessesse, A., Hatti-Kaul, R., Gashe, B.A. & Mattiasson, B. 2003 Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. *Enzyme and Microbial Technology* **32**, 519–524.

Gradisar, H., Kern, S. & Friedrich, J. 2000 Keratinase of *Doratomyces microsporus*. *Applied Microbiology and Biotechnology* **53**, 196–200.

Gushterova, A., Noustorova, M., Tzvetkova, R., Spassov, G. & Chipeva, V. 1999 *Investigations of the microflora in penguin's excrements in the Antarctic*. Vol. 2, pp. 1–7. Bulgarian Antarctic Research, Life Sciences, Sofia-Moscow: Pensoft Publishers.

Gushterova, A., Ivanova, V., Aleksieva, K., Kolarova, M., Schlegel, B. & Graefe, U. 2003 Taxonomic investigations of the Antarctic mesophilic strain 2BG, producer of secondary metabolites. *Biotechnology and Biotechnological Equipment* **18**, 72–76.

Ignatova, Z., Gousterova, A., Spassov, G. & Nedkov, P. 1998 Decomposition of native keratin by thermophilic actinomycetes. *Comptes Rendus de l'Academie Bulgare des Sciences* **51**, 67–70.

Ignatova, Z., Gousterova, A., Spassov, G. & Nedkov, P. 1999 Isolation and partial characterization of extracellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus*. *Canadian Journal of Microbiology* **45**, 217–222.

Ivanova, V., Graefe, U., Schlegel, R., Schlegel, B., Gousterova, A., Kolarova, M. & Aleksieva, K. 2003 Isolation and structure elucidation of tyramine and indole alkaloids from Antarctic strain *Microbispora aerata* IMBAS-11A. *Biotechnology and Biotechnological Equipment* **17**, 128–133.

Kabadjova, P., Vlahov, S., Dalgalarrondo, M., Dousset, X., Haertle, T., Briand, L. & Chobert, J.-M. 1996 Isolation and distribution of *Streptomyces* populations with heat-resistant collagenlytic activity from two protein-rich areas in Bulgarian soils. *Folia Microbiologica* **41**, 423–429.

Kosmachev, A.E. 1954 *Thermophilic actinomycetes and their antagonistic properties*. Ph.D Thesis, Inst. Mikrobiol., Moscow (in Russian).

Kunert, J. 1989 Biochemical mechanism of keratin degradation by the actinomycete *Streptomyces fradiae* and the fungus *Microsporum gypseum*: a comparison. *Journal of Basic Microbiology* **29**, 597–604.

Kunert, J. & Stransky, Z. 1988 Thiosulfate production from cystine by keratinolytic prokaryote *Streptomyces fradiae*. *Archives of Microbiology* **150**, 600–601.

Kunert, J. & Truper, H.G. 1986 Cystine catabolism in mycelia of *Microsporum gypseum*, a dermatophytic fungus. *Archives of Microbiology* **145**, 181–186.

Malviya, H.K., Rajak, R.C. & Hasija, S.K. 1992 Synthesis and regulation of extracellular keratinase in three fungi isolated from the grounds of a gelatine factory in Jabalpur. *Mycopathology* **120**, 1–4.

Noustorova, M., Gushterova, A., Tzvetkova, R. & Chipeva, V. 1999 *Investigations of the microflora in glaciers from the Antarctic*. Vol. 2. pp. 8–12. Bulgarian Antarctic Research, Life Sciences, Sofia-Moscow: Pensoft Publishers.

Onifade, A.A., Al-Sane, N.A., Al-Mussallam, A.A. & Al-Zarbam, S. 1998 Potential for biotechnological application of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresource Technology* **66**, 1–11.

Ryckeboer, J., Mergaert, J., Coosemans, J., Deprins, K. & Swings, J. 2003 Microbiological aspects of biowaste during composting in monitored compost bin. *Journal of Applied Microbiology* **94**, 127–137.

Santos, R.M.D.B., Firmino, A.A.P., De Sa, C.M. & Felix, C.R. 1996 Keratinolytic activity of *Aspergillus fumigatus* Fresenius. *Current Microbiology* **33**, 364–370.

Wang, J.-J. & Shih, J.C.H. 1999 Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and *B. subtilis* FDB-29. *Journal of Industrial Microbiology and Biotechnology* **22**, 608–616.