Novel keratinase from marine *Nocardiopsis dassonvillei* NRC2aza exhibiting remarkable hide dehairing

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Background
The isolation of the locally marine *Nocardiopsis dassonvillei* NRC2aza was characterized by the exceptional dehairing properties of its subtilisin-like keratinase.
Objectives
The aim of this work was to extract keratinase enzyme from the marine *Nocardiopsis dassonvillei* NRC2aza to be an alternative to sodium sulphide, which is the major pollutant from tanneries. Its unique nonactivity on collagen enhances its industrial potential.
Material and methods
Fermentation of the marine isolate *Nocardiopsis dassonvillei* NRC2aza on whole-feather medium was performed for keratrinase enzyme production. Extraction of the enzyme was carried out by solid-state fermentation (SSF).
Results and conclusion
Nocardiopsis dassonvillei NRC2aza have excellent characteristics of crude keratinase, producing 1680 U/ml in a shaking submerged culture (SmF) and 19 760 U/g using SSF after 4 days at pH 7. The effect of inoculum concentration on SSF was studied, whereby higher concentrations (150–200%) lowered the activity. Fractional precipitation of the enzyme by ammonium sulphate produced four fractions, of which 70% was the most active and produced remarkable hide dehairing. A new locally isolated *Streptomyces* spp. from marine ecosystem produced a highly active keratinase enzyme that exhibits remarkable hide dehairing.

Keywords:
hide dehairing, keratin degradation, keratinase production, *Nocardiopsis dassonvillei* NRC2aza

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Introduction

Keratinases are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates. Increased attention has been devoted to these enzymes because of their several potential uses associated with the hydrolysis of keratinous substrates and other applications [1]. Keratin is an insoluble structural protein of feather and wool and is known for its high stability [2]. Keratin-rich wastes are difficult to degrade as the polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions. In addition, cross-linking of protein chains by several disulphide bonds confers high mechanical stability and resistance to proteolytic degradation of keratins by common proteases [3].

Despite their elevated resistance, keratins are recycled in nature and can be degraded by some microorganisms. Keratinolytic enzymes are produced by fungi, actinomycetes, and bacteria and have been frequently isolated from soils where keratinous materials are deposited [4–6]. A considerable amount of fibrous insoluble protein in the form of feathers, hair, nails, horns, and others are available as byproducts of agro-industrial processing [7]. Thus, keratinolytic enzymes may have important uses in the biotechnological conversion of keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. Insoluble feather keratins can be converted, after enzymatic hydrolysis, to feedstuffs, fertilizers, and films [7–9]. In addition, application of these enzymes for pharmaceutical and cosmetic purposes have also been described [1]. The leather industry converts hide (putrescible) into commercial leather (nonputrescible) using large amounts of chemicals, generating an environmental impact. Leather processing involves a series of units of operations, of which the process of unhairing is the first major step in leather making. The pelt has to be freed from the epidermis and hair follicles. During the unhairing process, large quantities of water and toxic chemicals such as sulphide are used, generating a huge amount of effluents that must be treated and solid wastes that could be reused or better treated to avoid soil and water contaminations.

The application of biotechnology in leather manufacturing, with the replacement of chemicals by enzymes, is an alternative for the reduction of the environmental impact [9–13]. Worldwide, it is estimated that 315 million bovine leathers are produced per year. Considering a waste treatment cost

of \$0.30/m^2 of leather produced, more than \$1 million is spent per day to treat the waste from tanneries around the world. We report here a novel keratinase from the locally marine *Streptomycete* spp. isolated from the water of the Mediterranean Sea achieving a good dehairing of the raw hide that has the potential to replace sodium sulphide in the chemical dehairing process.

Most keratinases are inducible, and different keratin-containing materials such as feathers, hair, and wool can be used as substrates for keratinase production [6]. Feather was the mostly utilized substrate as feathers are composed of over 90% protein and produce large amounts of waste during poultry processing worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage [7,14]. Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids but also consume large amounts of energy. Moreover, keratinase from *Bacillus licheniformis* PWD-1 can degrade the infectious form of prion, PrPsc, in the presence of detergents and heat treatment [15], which is very important for the utilization of animal meal as feed.

Materials and methods

Microorganism and culture conditions

Inoculum preparation

The marine isolate previously defined as *Nocardiopsis dassonvillei* NRC2aza on the basis of its morphological and phylogenetic characteristics [16] was maintained at 4°C on starch–peptone–yeast extract (SPY) medium and cultured on whole-feather medium as described by Letourneau *et al.* [17] with a slight modification. The medium contained the following (g/l): whole chicken feathers 20; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4$, 0.1; NaCl, 0.5. The pH was adjusted to 7.2. The feather (1 g) was weighed in a 250-ml Erlenmeyer flask, and then 50 ml mineral salt solution containing all the other medium components were added. The flasks were sterilized by autoclaving for 15 min at 121°C. The inoculum was prepared by suspending spores from a 1-week-old (SPY) slant of *Nocardiopsis dassonvillei* NRC2aza in 50 ml of sterile (SPY) liquid medium and incubated for 3 days at 37°C with shaking at 150 revolutions/min in a controlled-environment shaking incubator. All used media were prepared with marine water.

Keratinase production

Preparation of feather and skin wastes

Chicken feathers and raw animal skins were collected from local commercial poultry-processing market and slaughters. Crude feathers and raw animal skins were cut into small pieces, washed several times with sterile distilled water, and used as the sole C and N source for the production of keratinolytic enzyme.

Native chicken feather preparation: chicken feathers were washed extensively with water and detergent, dried under sunlight, and then further dried in a hot-air oven at 60 C for 48 h. The feathers were then milled in a mortar using glass beads and passed through a small-mesh gird to remove coarse particles and 2% was supplemented to the basal medium. Three millilitre of the prepared inoculum was transferred to each flask and incubated for 4 days on a shaking incubator at 180 rpm at 37°C. At the end of the incubation period, the culture filtrate was centrifuged at 10 000 rpm for 15 min. The supernatant was used for keratinolytic assay and protein content determination.

Preparation of keratin solution

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as the substrate. Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz *et al.* [18]. Native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were heated in a reflux condenser at 100°C for 2 h. Soluble keratin was then precipitated by the addition of cold acetone (1 l) at –70°C for 2 h, followed by centrifugation at 10 000*g* for 10 min. The resulting precipitate was washed twice with distilled water and dried at 40°C in a vacuum dryer. One gram of quantified precipitate was dissolved in 20 ml of 0.05 mol/l NaOH. The pH was adjusted to 8.0 with 0.1 mol/l Tris and 0.1 mol/l HCl and the solution was diluted to 200 ml with 0.05 mol/l Tris-HCl buffer (pH 8.0).

Keratinase activity determination

The keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in Tris-HCl buffer (0.05 mol/l, pH 8.0) was incubated with 1 ml keratin solution at 50°C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml of 0.4 mol/l trichloroacetic acid. After centrifugation at 1450*g* for 30 min, the absorbance of the supernatant was determined at 280 nm (UV-2102; UNICO Shanghai Corp., Shanghai, China) against a control. The control was prepared by incubating the enzyme solution with 2.0 ml trichloroacetic acid without the addition of keratin solution. One unit (U/ml) of keratinolytic activity was defined as an increase in corrected absorbance at 280 nm (A_{280}) of 0.01/min [19] with the control under the conditions described above and calculated by the following equation [20]:

$$U = 4 \times n \times A_{280}/(0.01 \times 10),$$

where *n* is the dilution rate, 4 is the final reaction volume (ml), and 10 is the incubation time (min).

Production of keratinase by solid-state fermentation

This was carried out by the fermentation of *Nocardiopsis dassonvillei* NRC2aza on solid waste medium composed of 10 g feather, 10 g wheat bran, 10 g feather+0.05 wheat bran and 10 g wheat bran+0.05 feather, which were prepared in 250-ml Erlenmeyer flasks and moistened with 10 ml of the basal medium at pH 7.2 (100% moisture content). The flasks were inoculated with freshly prepared (30%) inoculum and incubated for 4 days at 37°C (the time for optimum keratinase activity).

Enzyme extraction

The content of each flask was mixed with 100 ml of 0.1% Tween 80 distilled H_2O and shaken for 1 h at 180 rpm at room temperature. The solid mat was separated using a mesh cloth; then, the cell extracts were centrifuged in a cooling centrifuge at 10 000 rpm for 20 min. The supernatant was then used for the enzyme and protein assay.

Dehairing assay

To optimize the most suitable conditions for enzyme dehairing of animal skin, a piece of alcohol-sterilized animal skin (≈10 g) was incubated with 67.2 U/ml of crude enzyme in 50 ml of sterile Na carbonate buffer solution (pH 9) at 29°C under shaking conditions (50 rpm) with a contact time of 23 h; at the end of the process, the skin pieces were gently scraped with the fingers to loosen the hair. Complete skin depilation was observed after incubation of the skin with the partial pure enzyme 39.644 U/g bovine skin after 2 h.

Dehairing of skin by Na_2S/lime

Goat/bovine hides were soaked in 10% NaCl for 15 min, and then washed twice with a surfactant or a wetting agent (Egyptol PLM). The hides were subjected to the dehairing process as follows: the hides were limed by $Ca(OH)_2$ for 30 min, and then 3% Na_2S was added in two portions for 24 h. The dehaired hides were delimed by NH_4Cl and washed three times by water.

Scanning electron microscope

The scanning electron microscope (SEM) microscope offers high-quality imaging with high resolution. Experimental and control specimens were prepared as circular samples (10 mm) and then subjected to sputter coating of gold ions to prepare a conducting medium (sputter coater-Edwards-Model, S-150 A; UK). A scanning microscope (Jeol, Japan) JSM-T20 was used for the microscopic study.

Ammonium sulphate fractionation of Nocardiopsis dassonvillei NRC2aza keratinase

The culture filtrate of marine *Nocardiopsis dassonvillei* NRC2aza (500 ml) was fractionated with 40–100% ammonium sulphate, and each precipitate was dissolved in 0.05 mol/l Tris buffer pH 8.5. Each fraction was dialyzed against the same buffer solution and assayed for keratinase activity and protein content.

Results and discussion

The marine *Streptomyces* spp. isolate locally isolated from the water of the Mediterranean Sea was defined as *Nocardiopsis dassonvillei* NRC2aza and produced a highly active keratinase when whole feather was used as the sole C and N source in the fermentation broth with maximum enzyme activity (1680 U/ml) after 4 days of incubation at 37°C on shaking SmF. Moreover, it presented pronounced growth and complete hydrolysis of native chicken feathers. This could offer tremendous potential for the development of biotechnological methods for the hydrolysis of feather and its utilization as feedstuff. Therefore, the present work provides new data on chicken feather degradation by a marine isolate from sea water. This is consistent with earlier studies [5].

Solid-state fermentation

As shown in Figure 1, solid-state fermentation (SSF) was achieved using feather and the agro-industrial waste (wheat bran) in several combinations. Complete feather degradation was achieved when *Nocardiopsis dassonvillei* NRC2aza was grown on different combinations of feather and wheat bran as the substrate, producing maximum keratinase activity on the whole-feather substrate (19 760 U/g). However, the marine *Streptomyces* spp. produced a highly active enzyme on the whole wheat bran substrate (19 066.7 U/g). Wheat bran was one of the important agro-industrial wastes used for the production of microbial enzymes on SSF [21,22]. It was reported that *Nocardiopsis dassonvillei* NRC2aza produced keratinase enzyme both inducibly and constitutively; the same result was obtained by El-Gendy [23] who produced keratinase enzyme constitutively on solid rice straw. It was shown that SSF has several advantages over the SmF as it is a simple, cost-effective, and environmental friendly process of the solid waste management of feather and other keratinous wastes; it is also a potential method to improve the nutritional value of keratinous wastes as feed supplements. The effect of inoculum concentrations on enzyme production was

investigated, and as shown in Figure 2, the enzyme activities obtained at 50 and 100% were the same as the control (19 760 U/g). A higher inoculum concentration (150 and 200%) lowered the enzyme activity to 19 560 and 19 520 U/g, respectively.

Ammonium sulphate fractionation

Ammonium sulphate fractionation of *Nocardiopsis dassonvillei* NRC2aza keratinolytic enzyme is illustrated in Table 1. Six partially pure fractions were obtained, of which the 40% ammonium sulphate fraction showed the highest enzyme activity (7111.05 U/fraction) and the highest fold of purification (0.25). However, the 70% ammonium sulphate fraction (1982.2 U/fraction) exhibited a remarkably good dehairing of bovine animal hide within 2 h at 29°C.

Bovine skin dehairing enzyme/sulphide comparison

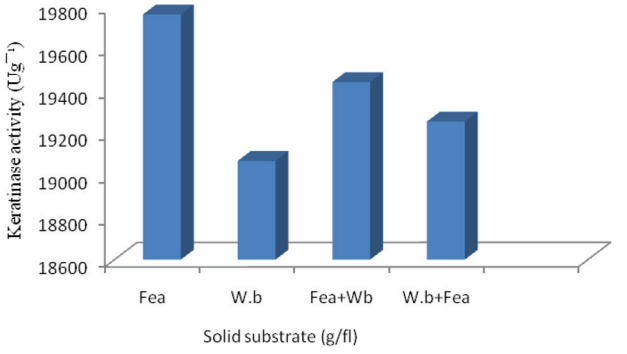
To compare the enzymatic process with the conventional method using lime and sulphides, soaked skins were cut into two halves; one half was treated with the crude enzyme and the other half was dehaired by the conventional chemical method. The two groups were then subjected to the same conventional operations. Applying the optimum enzyme-dehairing conditions obtained in the present study, the enzymatically dehaired skin was photographed by a SEM as shown in Figure 3, which shows that the fibres were not destroyed and the grain surface was normal. This confirms the possibility to use *Nocardiopsis dassonvillei* NRC2aza keratinase enzyme very safely in the dehairing process.

Scanning electron microscope

SEM is a useful technique for evaluating the effect of various treatments on the skin. Because SEM looks deeply into the hide fibre structure and shows the effect of different treatments on the fibre and the grain surface, a morphological study was carried out to assess the evaluation of the enzymatic dehairing process in comparison with the conventional dehairing method [24]. As shown in Figure 3, scanning micrographs of the grain surface (100 ×s) and the cross-section (200 ×s) of the skin were obtained to show the effect of the obtained enzymes on the grain surface and fibre bundles as dehairing agents [25].

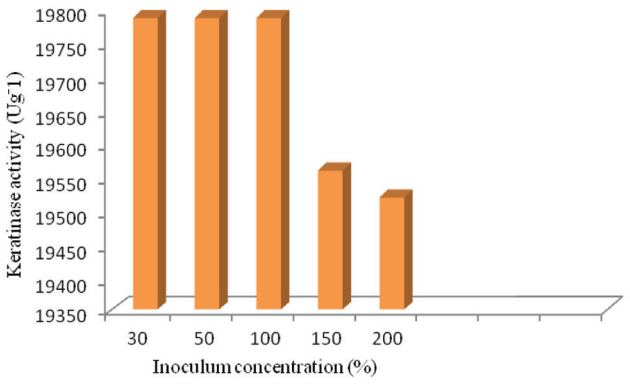
The cross-section micrographs showed that the fibre bundles of the enzymatically dehaired skin (Fig. 3a, c)

Figure 1



Production of keratinase enzyme by the local marine *Nocardiopsis dassonvillei* NRC2aza on solid-state fermentation using feather and wheat bran (WB) wastes after 4 days of incubation at 37°C, pH 7.2, with the following concentration: Feather (Fea), 10 g; WB, 10 g; Fea, 10 g + WB, 0.05 g; WB, 10 g + Fea, 0.05 g.

Figure 2



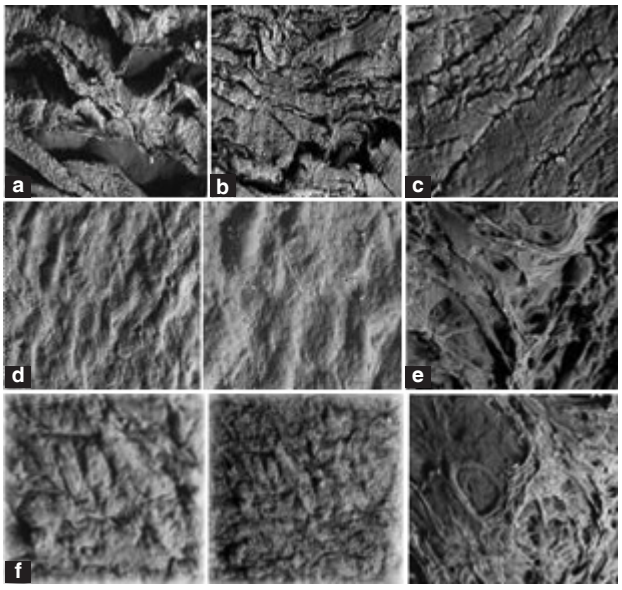
Effect of inoculum concentrations on keratinase production by marine *Nocardiopsis dassonvillei* NRC2aza on solid-state fermentation; control = 30%.

Table 1 Fractional precipitation of keratinase culture extract of local marine *Streptomyces* strain

Ammonium sulphate concentration (%)	Protein content of fraction (mg/f)	Recovered protein (%)	Total activity (U/f)	Recovered activity (%)	Specific activity (U/mg)	Fold purification
Culture filtrate	449.34	100	840 000	100	1869.41	1
40	15.00	3.34	7111.05	0.85	474.07	0.25
50	20.22	4.50	1733.32	0.21	85.72	0.05
60	19.47	4.33	1835.54	0.22	94.28	0.05
70	7.19	1.60	1982.20	0.24	275.69	0.15
85	14.04	3.13	1555.54	0.19	110.79	0.06
100	2.21	0.50	504.44	0.060	228.25	0.12

Total volume of cell extract = 500 ml.

Figure 3



(a) SE micrograph of the fibre bundles (×100), (×200) for enzymatically dehaired skin. (b) SE micrograph of the fibre bundles (×100) of chemically dehaired skin. (c) SE micrograph of the gain surface (×100), (×200) of enzymatically dehaired skin. (d) SE micrograph of the gain surface (×100), (×200) of chemically dehaired skin. (e) SE micrograph of the hair bulb (×100), (×200) for enzymatically dehaired skin. (f) SE micrograph of the hair bulb for chemically dehaired skin. Photographs (a–f) of bovine skin after 2 h of treatment with the 70% ammonium sulphate fraction of the marine Nocardiopsis *dassonvillei* NRC2aza keratinase enzyme (39.64 U/g bovine skin) compared with chemically dehaired skin.

were well-opened and the fibre network was cleaner in comparison with the chemically treated skin (Fig. 3b, d). In the same figure, (Fig. 3e), f indicated the degraded epidermis and hair bulb skin in both enzymatic and chemical dehairing. The dehairing process was accomplished by applying the 70% ammonium sulphate fraction of *Nocardiopsis dassonvillei* NRC2aza keratinase for 2 h at room temperature, which is a very good result.

Conclusion

The enzymatic process showed skin with good opening of the fibrous structure although it was not subjected to the bating step. This is the first time that a new keratinase was used for complete dehairing without sodium sulphate with the avoidance of collagen damage, which makes this new enzyme an exceptional candidate for dehairing, as it fulfills the industry tanning requirements. From this investigation, it was found that the keratinolytic activity of the marine *Nocardiopsis dassonvillei* NRC2aza isolated from the Egyptian shore had a high economical value and could be used for application in the trend of leather industry for the improvement of the Egyptian leather trade.

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Conflicts of interest

There are no conflicts of interest.

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