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Production, characterization and application of a keratinase from *Chryseobacterium* L99 sp. nov.

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ABSTRACT

Keratins are important bioresources for apparels and feedstuffs, but recalcitrant to common enzymes. Now, it is popular and essential to develop keratinolytic enzymes for environmental prevention and improvement of keratin product quality. In the study, the medium optimization, purification, characterization and application of the keratinase from a newly isolated *Chryseobacterium* L99 sp. nov. were conducted. Exogenous sucrose, malt sugar, glucose, starch, tryptone, Mg^{2+}, Zn^{2+}, Ca^{2+} and Cu^{2+} could promote the keratinase production, while exogenous urea, NH_4Cl and yeast extract exhibited strong inhibition effects. Response surface methodology predicted a maximum keratinase yield of 213.8 U mL^{-1}, at (g L^{-1}) sucrose 16.8, $MgCl_2{\cdot}6H_2O$ 1.9, feather keratin 40.0, $NaH_2PO_4{\cdot}2H_2O$ 6.0 and $K_2HPO_4{\cdot}6H_2O$ 1.0, where dry cell weight nearly had a minimum 8.58 g L^{-1}. Then, a serine keratinase about 33 kDa was purified, and its optimal activity was acquired at 40 °C and pH 8.0 with K^+, Zn^{2+} or Co^{2+}. Compared with Savinase 16 L and transglutaminase, the L99 keratinase could efficient prevent shrinkage and eliminate directional frictional effect of wool, indicating it as a promising prospect in the biotreatment of wool fibres.

1. Introduction

Environmental concerns and market competition are inducing a great revolution in wool finishing technologies. Many enzymes such as lipases [1], protein disulphide isomerases [2], transglutaminases [3], cellulases, hemicellulases, and pectinases [4] have played important roles during this process. In particular, proteinases are intensively studied because of their efficient ability to impart shrink-resistance and to improve handling properties [5–7]. One disadvantage of the proteolytic process is difficult to control, thus often leading to results unpredictable and irreproducible, such as a high level of weight loss and strength reduction [8]. This greatly limits the application of proteinase.

Keratinases are good at degrading insoluble and stable proteins including feather [9], hair, wool [10] and nails [11]. In biotreatment of wool textiles, substitution of normal proteinases with specific keratinases might restrict the enzymatic action towards the wool scale surface, thus reducing/eliminating the observed internal fiber degradation and subsequent tensile strength loss [12]. Gushterova et al. had investigated *Streptomyces flavis* 2BG and *Microbispora aerata* IMBAS-11A producing keratinases during growth on sheep wool wastes [13]. Ignatova et al. isolated an extracellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus* [14]. Riessen and Antranikian reported a novel thermophilic *Thermoanaerobacter keratinophilus* and the potential application of its keratinase on modification of wool fibers [15]. Recently, we isolated a wool biodegradation bacterium and identified as *Chryseobacterium* L99 sp. nov. producing a serine keratinase.

In this work, we optimized the culture medium of *Chryseobacterium* L99 sp. nov. using response surface method, purified the keratinase, investigated its characterization and explored its application on the modification of wool textiles.

2. Materials and methods

2.1. Materials

Keratin azure (Sigma–Aldrich, St. Louis, MO, USA) and azocasein (Sigma–Aldrich) were purchased from Sigma–Aldrich Trading Co. Ltd. (Shanghai, China). The protease preparation of Savinase 16 L (20 kU mL^{-1}) was supplied by Novo Nordisk Industri (Bagsvaerd, Denmark). The microbial transglutaminase (60 U mL^{-1}) isolated from *Streptoverticilium mobaraense* was bought from Ajinomoto Inc. (Tokyo, Japan). The plain weave wool fabrics from yarns spun around 22.3 μm wools were presented by Zhejiang Queping Textile Chemical Technology Holding Co., Ltd. (Zhejiang, China). All other chemicals were of analytical grade purity and commercially available. Chicken feathers obtained from a poultry processing plant were cleansed of impurities, washed extensively with water and detergent, dried thoroughly at 65 °C, milled to powder, passed through a 0.2-mm screen to remove coarse particles, and then used as feather keratin.

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2.2. Microorganism

Chryseobacterium L99 sp. nov. was isolated and kept in the China General Microbiological Culture Collection Center (CGMCC) with an accession number of CGMCC 2295. In our lab, the strain was maintained on nutrient agar (pH 7) slants or in 20% (v/v) glycerol stocks. Sub culturing was done periodically.

2.3. Inoculum preparation and flask culture

A loopful of cells from a slant was transferred into an Erlenmeyer flask (250 mL) containing 50 mL seed medium composed of ($g\,L^{-1}$): tryptone 10.0; NaCl 10.0; yeast extract 5.0, and incubated at 30 °C and 200 rpm for 16 h. Inoculum (6%, v/v, pH 7.5) containing approximately 3×10^7 cells mL^{-1} was transferred into an Erlenmeyer flask (250 mL) containing 50 mL of fermentation medium whose composition was varied based on the experimental designs. The basal medium was composed of ($g\,L^{-1}$) feather keratin 40.0, $NaH_2PO_4{\cdot}2H_2O$ 2.0 and $K_2HPO_4{\cdot}6H_2O$ 5.0. All media were autoclaved for 20 min at 121 °C. The flasks were incubated for 30 h on a rotary shaker at 30 °C and 200 rpm. Triplicate experiments were carried out and the mean value was calculated.

2.4. Measurement of cell mass

The cell mass was determined by measuring the dry cell weight (DCW). After cultivation, keratin powders were filtered out using eight layers of gauze. Cells were collected by centrifugation 10 mL of the filtrate at 3000 × *g* for 10 min, washed twice with distilled water, and dried at 105 °C to a constant weight.

2.5. Determination of keratinase activity

The keratinase activity was generally determined by the modified method of Letourneau et al. [16]. 1.5-mL eppendorf tubes with 400 μL of enzyme solution, 100 μL of 150 mM Tris–HCl buffer (pH 8.0) and 10 mg of finely chopped keratin azure were incubated in a water bath shaker at 50 °C, 200 rpm for 30 min. The enzyme reaction was stopped by adding 500 μL of 10% trichloroacetic acid (TCA), and the samples were further incubated for 15 min. Then, they were centrifuged for 10 min at 12,000 rpm in a high speed centrifuge (Sigma 3K18, Germany). The absorbance of the supernatant was measured spectrophotometrically at 595 nm (UV-2401PC, Shimadzu, Japan, Photometric repeatability ±0.001 Abs) against a control. The same method was applied to detect the keratinase activity on hair, sheep wool, and feather keratin at 280 nm. One unit (U) of keratinase activity was defined as an increase of corrected A_{595} (or A_{280}) for 0.001 within 1 h under the above conditions. Sheep wool was used as substrate for the study of keratinase purification and characterization. Keratin azure was used as substrate for the study of fermentation to avoid the interference of hybrid protein at A_{280}. All assays were run in triplicate, and the data presented are mean + SD of the triplicate assays.

2.6. Experiment design

2.6.1. Fractional factorial design (FFD)

The two-level fractional factorial design from SAS 8.0 (SAS Institute, Cary, NC, USA) is based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i x_i \tag{1}$$

where Y is the predicted response, β_0 is the intercept, β_i is the linear coefficient and x_i is the coded independent factor. The factors significant at 95% of confidence level ($p < 0.05$) were considered to have greater effects on keratinase production and were further optimized by a central composite design.

2.6.2. Response surface methodology and central composite rotary design (RSM–CCRD)

Response surface methodology and central composite rotary design were widely applied as an integral part of data analysis in optimization and data interpretation. In this study, a central composite rotary design from SAS 8.0 was employed to optimize the most significant factors for enhancing keratinase production. Variables were coded according to the following equation:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i} \quad i = 1, 2, 3, \ldots, k \tag{2}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_{cp} is the real value of an independent variable at the center point and ΔX_i is the step change of real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i.

Experimental data were respectively analyzed by the response surface regression (RSREG) procedure of SAS 8.0 to fit the second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j \tag{3}$$

where Y is the dependent variable or response variable (keratinase activity), β_0, β_i, β_{ii} and β_{ij} are constant regression coefficients, x_i and x_j are the uncoded independent variables, and k is the amount of single factors. All figures were plotted with MATLAB 7.0 (Mathworks Inc., Natick, Massachusetts, USA).

2.7. Keratinase purification

Broths were centrifuged at 6000 × *g* for 15 min, discarded cells, added 226 $g\,L^{-1}$ of ammonium sulfate slowly with ice bath cooling, stirred for 60 min, centrifuged again at 12,000 × *g* for 20 min, and discarded the pellet. The supernatant solution was added additional 120 $g\,L^{-1}$ of ammonium sulfate in an ice bath, stirred for 60 min, centrifuged, and discarded the supernatant. The pellet was dissolved in minimum volume of 10 mM Tris–HCl buffer (pH 8.5), removed salts with a column (5.0 cm × 50 cm) of Sephadex G-25, applied to a column (5.0 cm × 25 cm) of Q-Sepharose fast flow pre-equilibrated with a solution containing 10 mM Tris–HCl (pH 8.5). The column was equilibrated with the same buffer containing 0.2, 0.5 and 08 M NaCl separately. The fractions positive for keratinase activity was pooled, lyophilized, solved and added to the top of a column (2.6 cm × 80 cm) of Sephadex G-75 pre-equilibrated and equilibrated with a solution containing 10 mM Tris–HCl pH 8.0. The purified enzyme was freeze-dried and stored at −20 °C.

2.8. Methods to detect the keratinase characterization

The effect of pH on keratinase activity was studied with citrate–phosphate (50 mM, pH 3.0–7.0), Tris–HCl (50 mM, pH 7.0–9.0), and glycine–NaOH (50 mM, pH 9.0–10.0). The L99 keratinase was separately preincubated in these buffers at 80 °C for 2 h and then assayed activity at 40 °C. The optimum temperature was explored from 40 °C to 80 °C at pH 8.0. Temperature stability was tested from 30 °C to 80 °C in 50 mM Tris–HCl buffer at pH 8.0. Chemicals were separately solved with 50 mM Tris–HCl buffer at pH 8.0. Then, these buffers were used to study the effects of chemicals on keratinase activity. The time course curves of the absorbance of products were acquired with 10 mg mL^{-1} of substrates at 40 °C, Tris–HCl pH 8.0.

2.9. Enzymatic treatments and efficiency evaluations of wool textiles

2.9.1. Wool pre-treatments and enzymatic treatments

The commercially scoured fabrics were bleached in 0.1 M Na_2CO_3/$NaHCO_3$ buffer containing 1% H_2O_2 (30%) on the mass of wool fabric at pH 9.0 and 55 °C for 60 min. Then, the wool fabrics were thoroughly washed with distilled water and allowed to air dry. Enzymatic treatment of scoured and bleached wool fabrics was performed in 30 mM Tris–HCl buffer of pH 8.0 at the liquor to fibre ratio of 20 mL g^{-1} at 40 °C for 40 min. The amount used was 0.5%, 1%, 2% or 5% o.w.f. (of weight of fabrics) for Savinase 16 L or transglutaminase; 20, 40, 200 or 400 U mL^{-1} for the L99 keratinase.

2.9.2. Weight loss

Wool fabrics were conditioned at 105 ± 3 °C for 2 h and desiccated until their weight kept constant (considered as differences between successive weights inferior to 1 mg). Since long-time exposure under high temperature might result in denaturation, the initial weight of fabrics for testing was calculated from the weight ratio of the same fabrics desiccated and not desiccated. The measurements were carried out in triplicate.

2.9.3. Tensile strength resistance and dimensional stability

The tensile properties of wool samples were determined using a SDL tensile tester equipped with a load cell having a maximum capacity of 2 kgf. Wool fabric (250 mm × 250 mm) shrinkage was determined after washed with 1.5 $g\,L^{-1}$ detergent at the liquor to fibre ratio of 20 mL g^{-1} for 180 min in a full automatic fabric shrinkage tester YG701D (Changzhou No. 2, Textile Machinery Co. Ltd., Changzhou, China).

2.9.4. Directional frictional effect (DFE)

Thirty wool fibers from the fabric were incubated at a condition of 65% relative humidity and 20 °C for 48 h. Frictional coefficients (μ) were tested with JN-B precise torsion balance and calculated by:

$$\mu = \frac{\ln f - \ln(f - m)}{\pi} \tag{4}$$

where m is 200 mg and f is the data on precise torsion balance. Directional frictional effects (DFE) were calculated by:

$$\text{DFE}\ (\%) = \frac{\mu_a - \mu_s}{\mu_a + \mu_s} \times 100 \tag{5}$$

where μ_a is the coefficient of friction against the scales and μ_s is the coefficient of friction along the scales.