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Biodegradation of wool waste and keratinase production in scale-up fermenter with different strategies by *Stenotrophomonas maltophilia* BBE11-1 ☆

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HIGHLIGHTS

- Wool medium was first optimized for keratinase production.
- Cell growth rate did a major impact on keratinase production.
- A new strategy of glucose fed-batch process was developed.
- High production of keratinase with wool medium was achieved in 30-L fermenter.
- *Stenotrophomonas maltophilia* showed great potential for waste management.

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ABSTRACT

A keratin-degrading strain *Stenotrophomonas maltophilia* BBE11-1 was grown in a 3-L batch fermenter containing wool waste as the main medium and cell growth rate was determined as the key factor to affect keratinase yield. Three strategies of temperature-shift procedure, two-stage DO control and fed-batch process were used to change growth rate. And a 62.2% improvement of keratinase yield was achieved. With the glucose fed-batch procedure in 30-L fermenter, keratinase production was significantly improved up to 117.7% (1728 U/ml) as compared with initial data (793.8 U/ml) in a 3-L fermenter and with much shortened fermentation time within 18 h. Significant structure changes and high levels of free amino acids from wool decomposition indicated the possible applications for wool waste management and fertilizer industry. The remarkable digestion of wool cuticle also suggested its potential utilization in textile industry.

1. Introduction

There are million tons keratin wastes discard without sufficient reuse every year (Kornillowicz-Kowalska and Bohacz, 2011). Feather, wool, hair and horn are the common keratin waste (Dudyński et al., 2012). Keratin waste is mainly composed of keratinous protein and classified into α, β and γ- keratin since the diverse percentage of disulfide bonds (Hill et al., 2010). The α-keratin of wool waste is one of the important protein resources from leather industry and poultry farm. However, wool is not easily degraded in nature and causes serious pollution in leather processing (Thanikaivelan et al., 2004). Alpha keratin, which is also called hard-keratin, has higher cysteine content (up to 14%) to form S–S bonds between cross-linking protein chains, contributing ability to resist common proteolytic enzymes such as pepsin, trypsin or papain (Onifade et al., 1998; Brandelli et al., 2010). In addition, the hydrophobic groups of spiral coil in wool make it more difficult for biodegradation than feather (Kornillowicz-Kowalska and Bohacz, 2011).

Nowadays, chemical and high thermal methods have been extensively explored for keratin decomposition and reuse (Brandelli et al., 2010). Environmentally friendly and economical methods of microbial degradation are not universally used for α-keratin reuse especially wool waste. However, microbial decomposition still seems to be an attractive approach to manage those

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wastes without energy wastage and amino acids loss (Gupta and Ramnani, 2006; Brandelli, 2008).

Stahl et al. (1950) was the first one to discuss microbiological degradation of wool, then fungus, bacillus and thermophilic actinomycetes (Molyneux, 1959; Weary et al., 1965; Gousterova et al., 2005) were found to produce keratinolytic enzyme during wool degradation. However, Gram-negative bacteria were rarely used to digest wool waste.

Because of the high protein content of wool, hydrolysates including peptides and amino acids could be potential source for animal feed and fertilizer (Brandelli et al., 2010; Kornillowicz-Kowalska and Bohacz, 2011). In our previous studies (Fang et al., 2013), wool was remarkably degraded by a keratinolytic strain *Stenotrophomonas maltophilia* BBE11-1 and its derived keratinases showed a great utilizability for wool treatment in textile industry. Many reports had discussed keratinase production with feather medium in details (Lin et al., 1999; Wang and Shih, 1999; Fakhfakh-Zouari et al., 2010; Cedrola et al., 2012). However, keratinase production with wool medium has rarely been discussed, particularly on those relationships between fermentation variables and strategies.

This study aims to reuse wool waste by keratinolytic strain *S. maltophilia* BBE11-1. After optimizing fermentation in 3-L and 30-L fermenters, degradation rate and keratinase production were significantly enhanced. Further fermentation strategies were investigated in 30-L batch fermenter for keratinase production. Wool degradation, structure and hydrolysate analyses were also studied.

2. Methods

2.1. Keratinolytic strain and culture medium

A keratinolytic strain *S. maltophilia* BBE11-1 (GenBank JQ619623) was cultivated in wool medium for 2–4 days at initial pH 9. Wool medium consisted of (g/l): native wool 10, Asp 1.5, soy peptone 1.45, glucose 4.25, K_2HPO_4 1, KH_2PO_4 1, NaCl 1 and 100 μl Tween-20 l^{-1}. The medium composition was initially optimized with response surface method in shake flask (shown in Supplementary data).

2.2. Laboratory-scale batch fermentation

Laboratory-scale batch fermentation in 3-L fermenter (Bio-Flo110, New Brunswick Scientific Co.) was kept at 23 °C, initial pH 9.0, 400 rpm agitation and 1.5 l/min air flow rate. Three fermentation strategies including temperature-shift process, two-stage DO control and glucose fed-batch process were conducted. Different initial temperature for temperature-shift process was investigated. This was according to that of Wang and Shih (1999) by which 6 h cell growth at high temperature followed by a shift to low value for keratinase production. We also confirmed suitable DO value and glucose feeding speed for other two strategies. Two-stage DO control was conducted by changing agitation speed after 6 h to maintain constant DO value. The last strategy of glucose feeding was also begun after 6 h.

In a 30-L batch fermenter (Bioengineering AG, Switzerland), a new strategy of glucose fed-batch combined with two-stage DO control and temperature-shift process was studied. In this strategy, intermittent feeding of glucose was used to control DO value. DO measurement was coupled to computer control to adjust feeding speed when DO value exceeded 25%. Temperature controller was used to change temperature. DO control and temperature shift were all begun after 6 h. The maximum agitation speed did not exceed 1000 rpm. The air flow rate was stabilized at 15 l/min. pH was controlled at above 8.0 but below 9.0 by adding 1 M NaOH or HCl.

2.3. Analytical methods

Keratinolytic activity assay was according to Yamamura et al. (2002) with a slight modification. Sample containing 1 ml of 50 mM Gly/NaOH buffer (pH 9.0) including 50 mg wool powder was mixed with 1 ml enzyme solution and incubated at 50 °C for 1 h. The reaction was terminated with 2 ml 4% trichloroacetic acid (TCA). Based on Folin–Ciocalteu method, 200 μl supernatant was mixed with 1 ml 4% Na_2CO_3 and 200 μl Folin–Ciocalteu reagent at 20 °C for 1 h. Absorbance at 660 nm was measured. According to tyrosine standard curve, one unit of keratinolytic activity was defined as 1 μmol tyrosine liberated per hour. For the control, TCA solution was added before enzyme reaction. All experiments were repeated in three times and the mean value and standard deviation were recorded.

Biomass was determined using dry cell weight after filtration through glass fabric, and wool weight loss also depended on dry weight collected from that filter. Wool was washed with 0.1% (v/v) Triton X-100 three times to separate biomass. Dry weight was obtained after drying at 60 °C. All experiments were repeated in three times and the mean value and standard deviation were recorded.

The concentration of different amino acids in fermentation broth was determined by an amino acid analyzer (L-8900, Hitachi, Japan). The supernatant was obtained from fermentation broth centrifugation at 1000*g* for 10 min.

Nature and degraded wool samples were fixed in 4% glutaraldehyde. After washed in phosphate buffer (pH 7.2), fixed samples were dehydrated and coated with platinum alloy. Scanning electron microscope XL-30 ESEM (Netherlands) was used to observe microstructure of wool after degradation.

Fourier transform infrared spectroscopy (Perkin Elmer, Germany) was used to investigate the changes of functional groups in wool degradation. Dry samples were grinded with KBr and made into transparent pellets at 1 MPa pressure. The measurements were carried out in the mid-infrared range from 4000 to 400 cm^{-1}.

3. Results and discussion

3.1. Fermentation curves in 3-L fermenter

In the course of investigation of wool degradation, the relationship of three variables (dry cell weight, keratinase activity, and residual wool) and corresponding datum were recorded in 3-L fermenter. As shown in Fig. 1, keratinase production was related to cell growth. Along with cell growth, remarkable increase rate of keratinase activity was noted from 8 h to 20 h and increase of activity was terminated at 40 h. The maximum value (793.8 U/ml) was obtained while the biomass tended to increase until 48 h. This was contrast to earlier reports (Tiwary and Gupta, 2010), in which the reduction of keratinase activity was not obvious in the stationary phase of cell growth. *Bacillus subtilis* KD-N2 (Cai and Zheng, 2009) for hair degradation achieved the maximum keratinase activity after 36 h and *Bacillus licheniformis* ER-15 (Tiwary and Gupta, 2010) achieved maximum enzyme production after 60 h. Dry weight of wool was obviously decreased which resulted in 60% weight loss at the end of time course.

Fermentation kinetics model was obtained through nonlinear curve fitting using Leudeking–Piret model (Luedeking and Piret, 1959) to explain the relationship between keratinase production and cell growth (Eq. (1)). Substrate consumption course was also fitted through Log-modified model as follow (Eq. (3)).