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 (Dudyn´ski 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 (Thani-kavilan 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...
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 (Molynieux, 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; Kornilowicz-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; Fakh-Zouari et al., 2010; Cedriola 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$_2$HPO$_4$ 1, KH$_2$PO$_4$ 1, NaCl 1 and 100 µl Tween-201. 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 (BioFlo110, 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$_2$CO$_3$ 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 1000g 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 (Leudeking 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)).
where $C_s$ is dry cell weight (g/l); $C_p$ is keratinase production (U/ml); $C_r$ is residual wool (%); $a$, $b$, $c$, and $d$ are dependent parameters; $t$ is the corresponding fermentation time (h).

From the simulation curves in Fig. 1, it showed that increase of keratinase was significant before the exponential phase of biomass. At the end of exponential phase, however, total yield of keratinase had never increased. We inferred that cell growth significantly influenced the yield of keratinase during fermentation process. Fermentation kinetics also showed that 23.3 h was the mid-exponential phase of cell growth, and 12.85 h was the maximum point of keratinase production rate. It was contrast to earlier reports (Brandelli et al., 2010; Tiwary and Gupta, 2010) that production rate of keratinase was lower during exponential phase. Nonlinear curve fitting indicated that modeling value of keratinase production reached a stable phase after 24 h. On the other hand, the biomass still largely increased after 24 h. It could deduce that S. maltophilia BBE11-1 secreted extracellular keratinase before exponential phase of cell growth. Besides, there was no increase of keratinase yield and specific growth rate of dry cell weight before 6 h. Through nonlinear curve fitting (Eqs. (2) and (4)), we also found that fitting functions of keratinase production and substrate consumption were similar, and the biodegradation of wool keratin was closely related to cell growth. We thus deduced that increase of biomass probably led to high yield of keratinase and subsequent wool degradation. Consequently, S. maltophilia BBE11-1 has the potential of efficient keratinase production under suitable fermentation strategies.

### 3.2. Three fermentation strategies in 3-L fermenter

As we inferred that high biomass was benefit for keratinase production, three strategies that aimed to seek the relationship between biomass and keratinase production were studied. These strategies were according to fermentation kinetics in 3-L fermenter.

From the previous discussion of Leudeking-Piret model in 3-L fermenter, we could know that 12 h was the maximum point of keratinase increasing rate and 24 h was the stable phase of enzyme production. In order to determine the optimal temperature in different stages for efficient temperature-shift process, keratinase activities at 12, 24, and 48 h were surveyed. As shown in Fig. 2a for 12 h fermentation, 30 °C had the maximum increase of keratinase activity while 37 °C was the minimum. After 24 h fermentation, 30 °C still maintained the highest yield (806 U/ml) despite of good yield at 23 °C. However, the total yield at 30 °C decreased and 23 °C showed the maximum activity (853.8 U/ml) after 48 h. The decrease of activity at 37 °C fermentation was the largest. It could infer that 23 °C was beneficial for latter stage fermentation, while 30 °C was suitable for earlier stage. The temperature-shift process enhanced the growth rate of biomass (0.033 g/l/h) within 6 h and obtained the maximum keratinase activity (863.4 U/ml) after 16 h which nearly shortened 24 h at 23 °C fermentation. It seemed that high temperature for initial fermentation improved biomass, and could effectively increase keratinase production that reduced fermentation time. High temperature at 37 °C stimulated cell growth but decreased the yield of keratinase. Therefore, 30 °C used for first stage fermentation might favor the cell growth and affected some on gene expression of keratinase. The expression of keratinase gene was not always enhanced at elevated temperature which resulted in poor stability of the messenger RNA of keratinase (Wang and Shih, 1999). Thus, 23 °C probably did the positive impact on gene expression and was used for second stage fermentation.

Dissolved oxygen (DO) control had been always used in fermentation process (Hwang et al., 1991; Wang and Shih, 1999). Sufficient dissolved oxygen might improve activity of living cell and keratinase. In Fig. 2b, 45% DO value was suitable for cell growth, however, its keratinase yield was lower than 25% DO. High DO value of 65% inhibited keratinase production. As DO value was decreased in the early stage of exponential phase, we controlled agitator speed to maintain DO value when it reached 25%. This process had improved cell growth rate in exponential phase, and resulted in 0.024 g/l/h growth rate and maximum keratinase production (834.3 U/ml) within 24 h.

The third strategy used the constant glucose feeding to change cell growth rate. Feeding strategy was always used to change metabolic pathway fluxes and improve enzyme production.
During the fermentation period, glucose consumption was reduced, which restrained biomass yield. As shown in Fig. 2c, high feeding rate could greatly enhance growth rate but 1.8 g/l of glucose feeding resulted in the maximum enzyme yield, which we used for further studies. The fed-batch process clearly showed improved growth rate to 0.042 g/l/h. The maximum keratinase yield (1282.7 U/ml) with a 62.2% improvement was achieved at 18 h. However, keratinase activity dropped down after 18 h.

During the improvement of biomass or cell growth rate before onset of keratinase production, fermentation time for keratinase production was all shortened though these strategies. Temperature-shift and fed-batch process had shortened nearly 18 h to reach the maximum keratinase yield and two-stage DO control shortened 12 h. During exponential phase of growth, the culture required oxygen to metabolize glucose. However, high DO value or feeding speed had a negative impact on keratinase production. It indicated that excessive oxygen and glucose feeding probably led to cell growth only or acetate accumulation which might seriously reduce enzyme productivity (Åkesson et al., 2001). Thus, a new fermentation strategy avoiding inhibition should be developed for the improved keratinase yield.

### 3.3. Developing glucose fed-batch control in 30-L fermenter

According to our previous fermentation strategies in 3-L fermenter, glucose fed-batch process was the most productive one for keratinase production. However, stability of keratinase activity
dropped after 20 h, that might be related to continuous feeding of glucose. In order to maintain enzyme activity and enhance productivity during fermentation, this strategy was improved for scale-up fermentation in 30-L batch fermenter (Fig. 3).

Though the first procedure of two-stage DO control combined with temperature-shift process resulted the lowest keratinase yield (827 U/ml), the decrease of activity was not obvious. It corresponded to two-stage DO control in 3-L fermenter which increased the stability of keratinase to some extent. On the other hand, keratinase activity in third strategy was clearly decreased from maximum activity 1408–1021 U/ml at the later stage. It further proved that continuous feeding of glucose decreased keratinase activity that resulted in a negative impact on productivity and wool degradation.

Constant feeding was thus replaced with intermittent feeding in the second and fourth strategies. Temperature-shift process and two-stage DO control were also combined with feeding-time course. As DO value was related to the growth status of *S. maltophilia* BBE11-1, we tried to control the addition of glucose to adjust DO value during the fermentation period. Glucose stimulated cell growth but decreased DO value to 25% which was verified as the optimal DO for keratinase production (Fig. 2b). As shown in Fig. 3, the maximum enzyme activity achieved within 18 h by fourth strategy was 1728 U/ml that was 117.7% improvement compared with the initial data in 3-L fermenter. Without temperature-shift process, the second strategy achieved the maximum yield after 24 h which was slower than 18 h in fourth strategy. It further proved that continuous feeding of glucose decreased keratinase activity that resulted in a negative impact on productivity and wool degradation.

In shake-flask fermentation, production of keratinase from *Bacillus pumilus* KS12 was enhanced up to seven fold by statistical methods (Rajput and Gupta, 2013). However, a 30-L fermenter was close to pilot scale level and its fermentation conditions could be easily developed for scale-up fermentation (Zaghoul et al., 2011). Wang and Shih (1999) used feather and soy flour to improve keratinase yield with scale-up procedure of 100-L fermenters by *B. licheniformis* PWD-1. Feeding strategies had been widely used in scale-up fermentation to enhance enzymes or other secondary metabolites (Jiang et al., 2010). In this study, DO value and glucose concentration were the important factors related to cell growth rate as well as keratinase production. We optimized the scale-up fermentation by coordinating each key factor, which shows the important of rigorous procedure in scale-up fermentation to achieve maximum enzyme activity and stability.

### 3.4. Characterization of biodegrading wool waste

The newly developed strategy using glucose fed-batch combined with two-stage DO control and temperature-shift process was used for wool degradation by *S. maltophilia* BBE11-1. The wool medium for keratinase production by *S. maltophilia* BBE11-1 also showed remarkable biodegradation of wool waste. After 2 days, 66.7% degradation rate of native wool was observed in 30-L fermenter. So structure changes of wool and free amino acids in fermentation broth after biodegrading process were studied. As shown in Supplementary Fig. 3A, native wool had intact and unbroken cuticle layers, whereas they were remarkably separated after 2 days biodegradation. It indicated that cortex fibers were more accessible for *S. maltophilia* BBE11-1, which also verified by Fourier transform infrared spectroscopy (FTIR). Wool keratins mainly consisted of α-helical protein chain and it was proved by the existence of Amide I 1650 cm⁻¹ (−CO−NH−). The absorption around 1519 cm⁻¹ suggested the bending vibration band of N–H of Amide II. The increased of transmittances of those two absorption peaks indicated chain scission of peptides which probably resulted from keratinase hydrolytic action. The separation of absorption at 1116.38 cm⁻¹ after biodegradation suggested that −C(=O)− was generated from ester groups of cortex lipids and converted into acids or alky groups and carbon dioxide. However, C–S bonds in both control sample and degraded wool were not observed at 880 cm⁻¹ according to former reports (Kumar et al., 2008). We deduced that C–S bonds were not produced during wool degradation. However, they were dissolved in fermentation broth as soluble cysteine, which was proved by the increase of cysteine concentration (Table 1).

After four days fermentation, wool fibers were easily broken down and gradually degraded. When 17 free amino acids were assayed from cell-free fermentation broth (Table 1), there was a large increase of total amino acid concentration from 2 days to 4 days. Essential amino acid phenylalanine was the largest value, reaching 92.67 mg/l after 4 days. On the contrary to feather degradation by *S. maltophilia* R13 (Jeong et al., 2010) which produced rare aspartic acid and glutamic acid, those amino acids from wool degradation by BBE11-1 were abundant and ranked after phenylalanine. Amino acids such as serine and histidine were rare and not affected, while cysteine and proline increased by about 10-fold during 4 days. Since histidine and lysine are low in wool keratin and important for cell growth (Kornillowicz-Kowalska and Bohacz, 2011), total quantities was low. The different elements in feather and wool as well as the growth mechanism of keratin-degrading strains could distinguish products of amino acids (Kornillowicz-Kowalska and Bohacz, 2011; Gupta et al., 2012).

Crude keratinase produced from 30-L fermenter was characterized in Supplementary Fig. A4, showing that pH 8.0–10.0 and temperature 40–50 °C were suitable for keratinase activity. The applications of keratinase had been widely discussed in other reports (Gupta and Ramnani, 2006; Brandelli et al., 2010; Gupta et al., 2012). It was found that keratinase had immense potential.

![Fig. 3. Development of glucose fed-batch control for keratinase production in 30-L batch fermenter. First: two-stage DO control combined with temperature-shift process (○), agitator controlled 25% DO; Second: glucose fed-batch combined with two-stage DO control (■), intermittent glucose feeding controlled 25% DO; Third: glucose fed-batch combined with temperature-shift process (▲), intermittent glucose feeding controlled 25% DO; All glucose feeding was begun after 6 h.](image-url)
in various environmental and biotechnological sectors where conventional proteases showed poorer ability (Gupta et al., 2012). In this study, S. maltophilia BBE11-1 secreted keratinases that could well degrade wool waste, and thus, applications for treatment of waste water and rough wool in fertilizer industry and textile industry could be developed.

4. Conclusion

Production and productivity of keratinase was significantly improved using different fermentation strategies in both 3-L and 30-L fermenters with the main medium of wool waste. Especially the glucose fed-batch procedure in 30-L fermenter enhanced keratinase yield remarkably as well as wool degradation. Other applications of fermentation broth also showed great potential for fertilizer and textile industry.

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