RESEARCH ARTICLE

Biosynthesis of keratinase from *Stenotrophomonas maltophilia* Wu2 from poultry and its effect on poultry feather waste bioutilization, washing and dehairing

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Chicken feathers, globally discarded in large amounts, are considered an environmental pollutant and a source of pathogenic microorganisms. Given their recalcitrant nature and potential environmental implications, their disposal has become critical. Exploring keratinase-producing microorganisms for degrading feathers is a sustainable and environmentally friendly approach. In this study, an isolated feather-degrading *Stenotrophomonas maltophilia* Wu2 was identified. Moreover, the culture components and cultural conditions for its keratinase production were optimized using the one-variable-at-a-time (OVAT) method. The key optimal medium compositions and cultural conditions were lactose, beef extract, magnesium sulfate, an initial fermentation pH of 7.0, incubation at 25°C and 160 rpm, and a 5% inoculum. Furthermore, the keratinase (SmKWu) displayed excellent stability within the wide pH range from 7.0 to 12.0 and the temperature range from 30 to 50°C. Interestingly, non-ionic surfactants enhanced the activity of SmKWu. Additionally, SmKWu showed high tolerance to detergents and low activity toward collagen. Application experiments revealed that *S. maltophilia* keratinase could degrade more than 80% chicken feathers within 5 days, resulting in a high content of amino acids over 1,100 mg/L in feather hydrolysate. Interestingly, SmKWu could wash blood stains from cotton pieces in 18 min and dehair wool in 6 h. This work suggested that SmKWu exhibited significant potential for the efficient degradation and bioutilization of feather waste, functioning as an additive in the detergent industry and contributing to hair removal within the leather industry.

Keywords: chicken feather waste; keratinase; *Stenotrophomonas maltophilia*; feather degradation; bioutilization.

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Introduction

With the rise of people's living standards and growing concern for health, poultry meat and egg production plays a crucial role in the global economy and provides healthy protein sources and other nutrients for human consumption [1]. Along with the high consumption of poultry products, the poultry industry is highly developed around the world for the reasons of its economic

significance, globalization, and trade. As the primary byproduct of poultry processing industries, millions of tons of feather waste abundant in crude protein are produced annually in the world, which presents not only environmental concerns but also economic impacts. Feathers mainly consist of keratin, accounting for approximately 90% by weight [2, 3]. Keratin is replete with hydrogen bonds and disulfide bonds [1]. These rich contents of disulfide bonds and hydrogen bonds contribute to the unique molecular arrangement within the keratin matrix of feathers, conferring upon them remarkable hardness and durability. So, feather keratin exhibits a strong recalcitrance to digestion and degradation by common enzymatic processes.

To date, the majority of poultry feathers are either directly incinerated or disposed of in landfills, while only a small fraction is utilized for decorative purposes or composting due to the lack of effective and safe degradation methods. During the incineration process of feathers, the release of solid particles, pollutant gases such as a major anthropogenic greenhouse gas, and other substances gives rise to environmental pollution. Given that feathers in slaughterhouses are typically blood-stained and wet, their accumulation will facilitate pathogenic microorganisms' growth and spread into the environment, thereby leading to a risk of water pollution, which is a major threat to human life and health [4]. Hence, there is an urgent need for efficient methods of feather degradation. These methods should be not only low-cost but also environmentally friendly for feather waste management. On the other hand, a notable shortage of feed protein resources worldwide poses a significant challenge to the agricultural and livestock industries. Feather keratin contains abundant amino acids, and it has been reported that the hydrolysis products of feathers by microbial enzymes contain high contents of amino acids, especially essential amino acids for animals [4]. Keratinase (EC 3.4.21/24/99.11) is capable of specifically decomposing the coiling keratin substrates and converting them into nutrient composition including soluble protein, polypeptides, or amino acids. Owing to its distinct advantages, especially the unique catalytic properties towards keratinous substrates and its robustness, keratinase has attracted extensive attention. The feather waste broken down by microbial keratinases undergoes hydrolysis and is transformed into soluble molecules with beneficial properties including easy digestibility and bioavailability. Effective utilization of feather waste can not only reduce the amount of discarded and incinerated feathers to alleviate environmental pollution but also make up for the shortage of feed protein resources, thereby achieving the goal of transforming waste into valuable resources. Therefore, finding ways to utilize feather keratin has profound implications for both environmental protection and the optimization of feed resources. The main sources of keratinases are diverse microorganisms including bacteria such as widely reported *Bacillus species* [5-7], fungi such as *Aspergillus* species and *Trichophyton* species [8, 9], and actinomycetes such as *Streptomyces genus* [10, 11]. Among bacteria, in addition to widely reported Gram-positive strains, a few Gram-negative strains are capable of producing feather-degrading enzymes, like *Xanthomonas* sp. [12], *Arthrobacter* sp. [13], *Pseudomonas* sp. [4], and *Stenotrophomonas* sp. [14]. However, only a few of those enzymes have been reported for commercial application [15]. New keratinase discovery in nature is necessary because of the better performance required in industrial applications and the patent situation's complexity worldwide. The bacterial fermentation of the process for feather treatment has distinct advantages including high efficiency, low energy consumption, and eco-friendliness. High efficiency can protect substances and the produced nutrients in the system from being damaged. Furthermore, no harmful byproducts in the process are generated, making it environmentally friendly. Interestingly, the enzyme-producing systems have been reported to have potential applications in multiple industries including degradation and

bioconversion of feathers, the detergent industry, and the dehairing process within the leather industry [7, 16]. Additionally, the robustness and low cost of crude enzymes promote their industrial applications.

This study focused on exploring microbial keratinase that could convert feather waste into value-added nutrients. A newly isolated strain *Stenotrophomonas maltophilia* Wu2 was identified, and the keratinase production was improved. Furthermore, the characteristics of the produced keratinase were examined, which extended the possibility of its potential applications such as converting feather waste into nutrients, as an additive in detergent, and for dehairing. Further application experiments were conducted to evaluate the enzyme's capabilities of degrading feathers, removing hair, and cleaning.

Materials and methods

Isolation and screening of bacteria capable of degrading feather

The soil samples were obtained from Xianlu Village egg and chicken farm (Huixian, Xinxiang, Henan, China). One gram of each sample was dispersed in 30 mL of 0.9% NaCl solution for 0.5 h. The feather-decomposing microorganisms in the supernatant were enriched according to the method of Zhang *et al*. [7]. One milliliter of supernatant was inoculated in 30 mL of the enrichment medium containing 10 g/L feather powder, 0.1 g/L $MgCl_2$, 1 g/L yeast powder, 0.5 g/L NH_4Cl, 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, and 0.4 g/L KH_2PO_4 with neutral pH and subsequently cultured in a ZWY-2102C shaker (Shanghai Zhicheng Analysis Instrument Manufacturing Co., Ltd., Shanghai, China) at 37°C and 200 rpm for 24 h. Serial dilution was then carried out to screen the keratinase-producing bacteria. The target bacteria were further screened by cross-fostering on the preliminary screening medium and the secondary screening medium after enrichment cultivation.

Keratinase assay

Microbial keratinase activity was measured according to the method reported by Zhang *et al*. [7]. The reaction system was composed of 0.5 mL of properly diluted enzyme supernatant and 1.5 mL of 1% keratin substrate (J&K Scientific Ltd., Beijing, China). The reaction was performed in a DZKW-S-6 water bath (Shanghai Yuzhuo Instrument Co., Ltd., Shanghai, China) at 40°C for 20 min before 2 mL of 0.4 mol/L trichloroacetic acid (TCA) was added to terminate the reaction. After being maintained at room temperature for 10 min, the supernatants were obtained by centrifugation for 10 min. The Folin-phenol chromogenic reaction was then performed using 0.5 mL of the supernatant, 2.5 mL of 0.4 mol/L Na_2CO_3, and 0.5 mL of the Folin-phenol reagent (Hefei Bomei Biotechnology Co., Ltd., Hefei, Anhui, China) at 40°C for 20 minutes. The activity of keratinase was quantified using UV2400 spectrophotometer (Shanghai Sunny Hengping Scientific Instrument Co., Ltd., Shanghai, China) at a wavelength of 660 nm (OD_{660}). One unit (1 U) of keratinase activity was defined as an increment of 0.01 in absorbance.

Preparation of microbial seeds

The bacterial strains stored in frozen glycerol stock were activated using LB-agar medium at 37°C for 18 - 24 h with an LRH-250 incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China). The fresh seed was obtained by inoculating the activated culture into 30 mL of LB medium followed by cultivation at 37°C and 220 rpm for 18 - 24 hours. The cells were collected into a container filled with LB liquid medium to prepare a bacterial suspension. By adjusting its OD_{600} to 0.1, this bacterial suspension served as the fresh inoculum for the fermentation process.

Microbial identification

The target strain cultured with the LB medium in the logarithmic growth phase was used for morphological identification. The bacterial strain was observed using a Quanta 200 scanning electron microscope (SEM) (FEI Company, Hillsboro, Oregon, USA) [17]. Furthermore, the strain's 16S rRNA was sequenced by Personal

Biotechnology Co., Ltd (Shanghai, China) and was uploaded to the National Center for Biotechnology Information (NCBI) database. Meanwhile, its phylogenetic tree was constructed using MEGA7 software (https://www.megasoftware.net/).

Enhancement of keratinase production

To enhance the target strain keratinase production, the effects of key fermentation medium components and conditions were explored using the one-variable-at-a-time (OVAT) method. Owing to the insoluble feathers in the medium, the microbial biomass was unable to be accurately determined. Hence, keratinase production during the fermentation process was regarded as the primary assessment criterion.

Preparation of crude keratinase

After the completion of fermentation, the crude keratinase, SmKWu, was collected by centrifugation of fermentation broth at 4,746 *g*, 4°C for 15 min.

Effects of pH and temperature on SmKWu

The effects of pH on SmKWu activity were examined at the pH range of 5.0 - 12.0 in the buffer systems of 50 mmol/L acetic acid - sodium acetate buffer for pH 5.0, 50 mmol/L K_2HPO_4-NaOH buffer for pH 6.0 - 7.0, 50 mmol/L Tris-HCl buffer for pH 8.0 - 9.0, 50 mmol/L glycine-NaOH buffer for pH 10.0 - 11.0, and 50 mmol/L Na_2HPO_4-NaOH buffer for pH 12.0. The effects of temperature were detected at the temperature range of 20 - 80°C. After being incubated at the corresponding pH environments and temperatures for 60 min, respectively, the pH stability and thermal stability of SmKWu were determined.

Effects of various chemicals on SmKWu

After incubating the mixture of SmKWu and metal ions of sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), zinc sulfate ($ZnSO_4$), and manganese chloride ($MnCl_2$), inhibitors including ethylene diamine tetraacetic acid (EDTA) and phenylmethanesulfonyl fluoride (PMSF), and surfactants of Tween 20, Tween 60, Tween 80, Triton X-100, and sodium dodecyl sulfate (SDS) for 60 min, the residual activities were detected at the optimal pH value and the optimal temperature. The effects of 10 mM metal ions, 5 mmol/L PMSF and EDTA, 5% surfactants, and 1% SDS on the activity of SmKWu were investigated with the keratinase activity without adding any chemicals under the same conditions as 100%.

Substrate specificity of SmKWu

Various protein substrates including insoluble substrates and soluble substrates were utilized to detect SmKWu's substrate specificity. The enzyme reaction towards insoluble substrates consisted of 0.5 g of substrate in 1.5 mL of 50 mmol/L buffer (pH 10.0) and 0.5 mL of properly diluted enzyme solution. The reaction was kept at 60°C for 60 minutes and was halted by adding 1 mL of 0.4 mol/L TCA. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was collected, and the absorbance at 280 nm (OD_{280}) was measured for human hair, chicken feather powder, and wool powder. The methods for assessing enzyme activity on soluble substrates like type-I collagen, bovine serum albumin (BSA), and casein were similar to those described in the keratinase assay above under the conditions of the enzyme's optimal pH and temperature.

Compatibility of SmKWu with commercial detergents

The compatibility of SmKWu with various brands of commercial detergents at concentrations of 0.7 and 1.0 mg/mL was evaluated. The compared commercial detergents included WhiteCat (Shanghai White Cat (Group) Co., Ltd., Shanghai, China), Super and Diao (Nice Group Co., Ltd., Zhengding, Hebei, China), Bluemoon (Bluemoon (China) Co., Ltd., Guangzhou, Guangdong, China), OMO (Unilever (China) Investment Co., Ltd., Shanghai, China), Tide (Procter & Gamble (China) Ltd., Guangzhou, Guangdong, China), and Liby (Guangzhou Liby Enterprise Group Co., Ltd., Guangzhou, Guangdong, China). The endogenous enzymes in the detergents were deactivated by boiling for 30 min. Subsequently, the residual activities were measured following a 30-minute

incubation of SmKWu and the deactivated detergents at 40°C to analyze the compatibility of SmKWu with these detergents.

Feather degradation and analysis of hydrolysis products

The degree of feather degradation was observed under the following treatments, which included the negative treatment with an equal amount of intact feather being added to 30 mL of tap water, an equal amount of intact feather being used instead of feather meal in 30 mL of the medium, an equal amount of intact feather being added to 30 mL of fermentation supernatant (SmKWu), and an equal amount of intact feather being added to 30 mL of culture after inoculum 48 h of *S. maltophilia* Wu2-enzyme system. After cleaning the remaining feathers, drying them at 50°C for 12 h, and weighing them, the feather's degradation rate was calculated as follows.

$$\text{Degradation of feather (\%)} = \frac{\text{the loss weight of feather}}{\text{the initial weight of feather}} \times 100\%$$

After the supernatant of feather hydrolysis was mixed with 40 mL of 0.01 mol/L HCl and subjected to vortex for 5 min followed by ultrasonic extraction for 5 min, the mixture was fixed to 50 mL and kept in the dark for 2 h before centrifugation at 4,000 rpm for 10 min. One milliliter supernatant was blended with 1 mL of 2 - 4% sulfosalicylic acid by vortex mixing for 1 min. After being kept in darkness for 1 h, the supernatant was obtained by centrifugation at 15,000 rpm for 15 min, then filtered with 0.22 µm cutoff. The contents of free amino acids were assayed by using Hitachi LA-8080 automatic amino acid analyzer (Hitachi, Ltd., Tokyo, Japan).

Cleaning performance

The cleaning performance of SmKWu was evaluated by staining cotton cloths with 150 µL of blood and then keeping at 60°C for 30 min to fix the blood stain [18]. The stained cotton cloths were then treated with 100 mL of tap water as negative treatment group, 100 mL of 0.7% detergent in tap water as positive treatment group, 100 mL of SmKWu (10,000 U) as enzyme treatment group, and 100 mL of SmKWu blended with 0.7% detergent that had its endogenous enzyme inactivated by boiling for 30 min in advance as mixture treatment group, respectively. All four groups were incubated at 25°C for 10 - 20 min. Meanwhile, the washing effects of the stained cloths were observed every 1 min after 10 min incubation.

Dehairing performance

Because of its low activity toward type-I collagen, the potential application in hair removal of the keratinase was subsequently studied. Three treatments to sheep skin (6 cm × 6 cm) were set up with tap water treatment as negative control, a chemical method involving lime and sodium sulfide as traditional treatment [19], and keratinase supernatant (about 20,000 U) co-incubation at 37°C and 200 rpm as enzymatic treatment. The dehairing effects were observed at regular intervals. The dehairing situation and skin quality were evaluated by the naked eyes and a LEICA DM 2500 metallographic microscope (Leica Microsystems, Wetzlar, Germany). Because there is no specific standard for the effect of hair removal, the effect of enzymatic hair removal was assayed by combining with their senses and stereomicroscope observation.

Results and discussion

Screening, isolation, and identification of feather-degrading bacteria

Feathers are rich in keratin with about 90% of crude protein in feathers is keratin [2]. However, the keratin components in feathers are resistant to multiple enzymes and chemical reagents. The combination of the difficulties in feather decomposition and low utilization ratio leads to their accumulation, which causes serious environmental pollution globally. Exploring microbial resources for degrading feather waste has become a viable alternative approach, which can not only address the environmental issues related to feather waste but also mitigate the shortage of protein resources for animal feed and promote the efficient utilization of feather

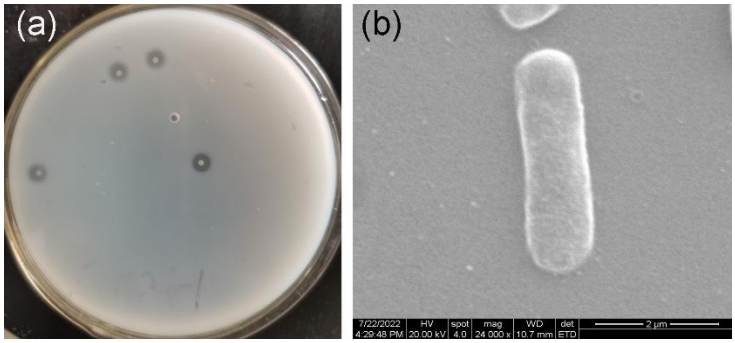

Figure 1. Morphological image of *S. maltophilia* Wu2. (a) colony formation. (b) the bacterium image observed by SEM.

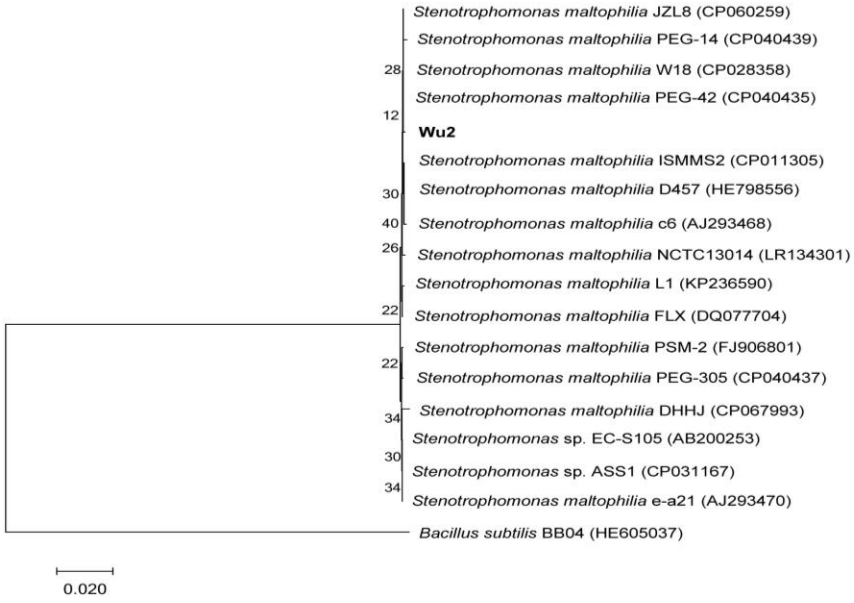

Figure 2. Phylogenetic tree of strain Wu2 using the neighbor-joining method. *B. subtilis* BB04 was used as the outgroup.

keratin resources. It has been reported that microbial strains capable of degrading and utilizing feathers often generate keratinase [20, 21]. Given that chicken feather keratin can serve as the substrate for microbial keratinase production, chicken feather powder was selected as the sole nitrogen and carbon source for target strains' growth, and as a keratinous inducer for screening keratinase-producing bacteria in this study. Based on the ratios of the diameters of protein-degrading halos and those of colonies on skim milk agar, 12 bacterial strains were screened out and isolated. Among these strains, five isolates exhibited high keratinase activity. Notably, Strain Wu2 with a high keratinase activity of 286 U/mL was used in this study. The colony of Wu2 strain was light yellow with a smooth surface and edge. The morphology of the strain cell observed under a microscope revealed that the strain was Gram-negative, had a straight rod-shaped structure, and did not form a spore (Figure 1). To identify the strain, the sequence of its partial 16S rDNA was uploaded to the GenBank database and designated as MW282849. Based on BLAST analysis and aligning with the relevant sequences, a phylogenetic tree was generated (Figure 2). As shown in the phylogenetic tree, strain Wu2 exhibited 99% similarity to the sequences of *S. maltophilia* strains (CP04035, CP028358,

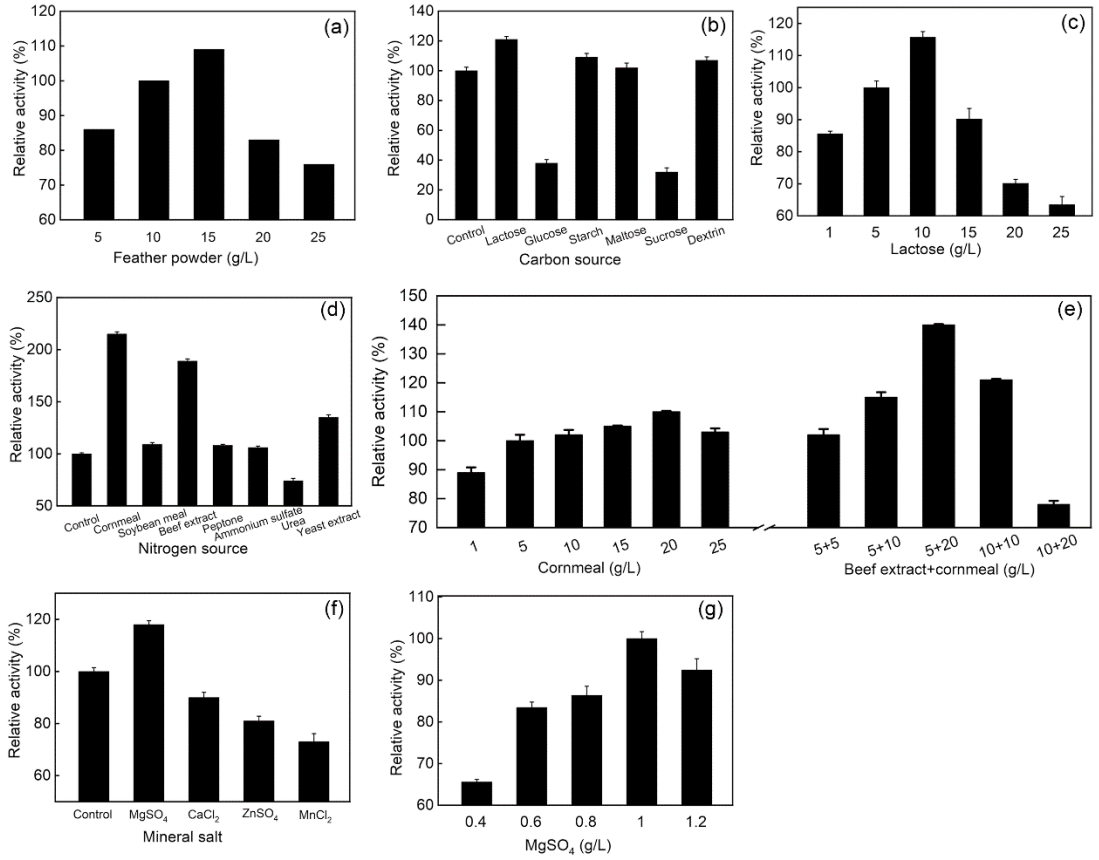

Figure 3. Effects of components of fermentation medium on keratinase production by *S. maltophilia* Wu2.

CP0404039, and CP060259), indicating that it was in the clade of *Stenotrophomonas maltophilia*. Accordingly, the strain was named as *Stenotrophomonas maltophilia* Wu2 and was preserved in the China Center for Type Culture Collection (No. M2021606. Wuhan, Hubei, China).

Improving keratinase production

It is well known that the components of the culture medium have an important impact on enzyme production by microorganisms. In this study, the OVAT method was employed to promote keratinase production by *S. maltophilia* Wu2. Feather acting as the keratin inducer and a carbon and nitrogen source in the fermentation medium usually affects microbial keratinase production [3]. Thus, the effects of feather keratin in the fermentation medium on the keratinase production by *S. maltophilia* Wu2 were studied. The results showed that the contents of feather powder in the medium affected the keratinase production by *S. maltophilia* Wu2 (Figure 3a). The maximal keratinase production by *S. maltophilia* Wu2 was observed when the concentration of the feather powder was 15 g/L. The results were consistent with the previous report on chicken feather keratin improving the keratinase yield of *B. cereus* YQ15 [7]. In addition, other components of the medium are also important for microbial growth and keratinase production. Among the tested co-carbon sources, lactose, starch, and dextrin showed promoting effects on keratinase production with activity of the enzyme increased by 0.21-, 0.09- and 0.07-fold, respectively, yet fructose and glucose exhibited inhibitory effects on keratinase production (Figure 3b). When the concentration of lactose was 10 g/L, the keratinase production by *S. maltophilia* Wu2

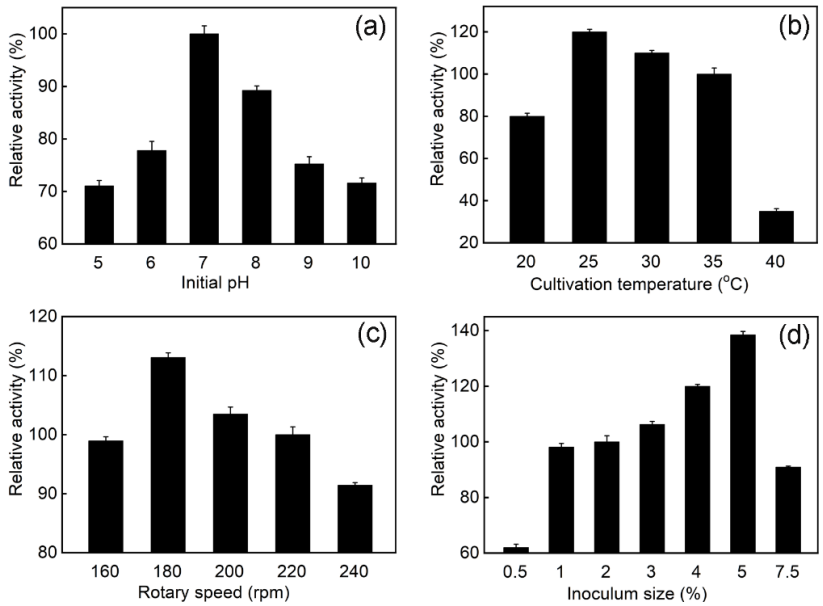

Figure 4. The effects of cultural conditions on keratinase production by *S. maltophilia* Wu2.

reached its maximum of 404 U/mL. When it was in the concentration range of 15 - 25 g/L, the keratinase production decreased significantly (Figure 3c). The results were similar to the enhancement of lactose on *B. licheniformis* PWD-1 keratinase production [22], and starch and dextrin stimulated the keratinase yields of *B. pumilus* NJM4 and *B. pseudofirmus* FA30-01 [23, 24]. Moreover, various co-nitrogen sources including organic nitrogen and inorganic nitrogen sources were selected to investigate their effects on keratinase production by *S. maltophilia* Wu2. Interestingly, it was found that compared with the control without additional nitrogen source, cornmeal and beef extract exhibited distinctly promoting effects on enzyme production with activity increased by 1.15- and 0.89-fold, respectively, while urea and ammonium sulfate showed inhibitory effects on enzyme production (Figure 3d). It has been reported that beef extract enhanced the keratinase yield of *B. pumilus* NJM4 [23]. Thereby, the combination effects of beef extract and cornmeal were also studied. The maximal keratinase production of 1,316 U/mL was obtained when beef extract and cornmeal were at 5 g/L and 20 g/L, respectively (Figure 3e). Of mineral salts, magnesium sulfate showed a promoting effect on enzyme production, while other ions did not facilitate keratinase production (Figure 3f). The supplemental magnesium sulfate at 1 g/L led to an increase in keratinase production of 0.18-fold (Figure 3g). It has been observed that magnesium sulfate enhanced keratinase synthesis by *B. cereus* YQ15 and *S. maltophilia* Kb2 [7, 25]. Suitable fermentation conditions can not only affect the absorption and utilization of nutrients by microorganisms but also interfere with the activity of enzymes in microbial metabolic pathways for promoting bacterial growth and production of enzymes afterward. The cultural conditions for *S. maltophilia* Wu2 to produce keratinases were further studied. The results indicated that the maximal production was observed when the initial medium pH was neutral (Figure 4a), which was in line with the optimal fermentation pH for *S. maltophilia* R13 to produce keratinase [26]. Moreover, keratinase production increased with temperature below 25°C followed by a slight decrease (Figure 4b). Consequently, the optimal cultivation temperature was 25°C (Figure 4c), which was lower than that of production keratinase by *S. maltophilia* DHHJ (40°C) [27]. The results also illustrated the high productive efficiency of *S. maltophilia* Wu2 with less energy cost. The

enzyme production by *S. maltophilia* Wu2 increased when the rotary speed was adjusted from 160 rpm to 180 rpm and then decreased (Figure 4d). Hence, the rotary speed was chosen as 180 rpm, which was the same as that of keratinase production by *S. maltophilia* MT1 [28]. At an inoculum size of 5%, keratinase production by strain Wu2 was the highest. The optimal inoculum size was correlated with the *B. velezensis* HFS_F2 [5]. Thus, the optimal cultural condition for *S. maltophilia* Wu2 keratinase production was pH 7.0, 25°C, 160 rpm, and an inoculum size of 5%. By using the optimal fermentation medium and optimal cultural conditions for *S. maltophilia* Wu2 to produce keratinase obtained from the above study, the maximal keratinase yield of 1,866 U/mL was obtained after 48 hours of cultivation with a 5.52-fold increase (Figure 5). Subsequently, the yield showed a slow decline as cultivation time was prolonged. It has been reported that a relatively extended fermentation time was required as *S. rhizophila* MT1 and *S. maltophilia* FF11 reached their maximum enzyme activity after 60 h cultivation [28, 29].

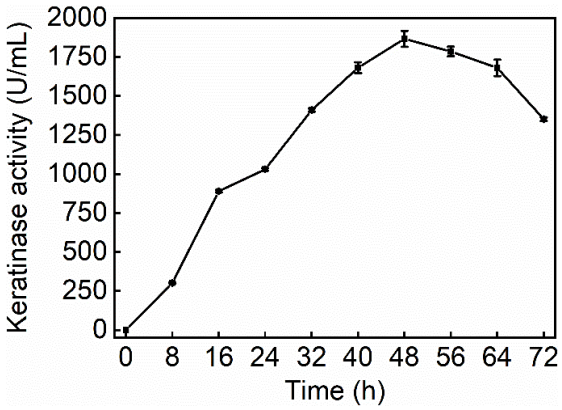

Figure 5. The keratinase production by *S. maltophilia* Wu2.

Effects of pH and temperature on SmKWu

The conformation of the enzyme is influenced by pH and temperature, thereby resulting in the change of activity and stability of the enzyme molecule. SmKWu showed an optimal pH of 10.0, which was in line with those of the keratinases from *B. cereus* YQ15, *B. licheniformis* MP1, and *B. pumilus* GRK [7, 30, 31]. It remained more than 80% residual activity within the broad pH range of 7.0 to 12.0 (Figure 6a). The optimal temperature of SmKWu was 60°C, which was in line with that of keratinases from *B. cereus* YQ15, and *B. pumilus* FH9 [7, 32], and higher than those of *S. rhizophila* MT1 (50°C) [28], *S. maltophilia* L1 and *Brevibacillus brevis* US575 (40°C) [27, 33], and *Pseudomonas geniculate* H10 keratinase (30°C) [4]. The thermal stability of SmKWu was also tested. The residual activity of SmKWu was more than 75% within the temperature range of 30 to 50°C and decreased obviously when the temperature was higher than 50°C (Figure 6b). The thermostability of SmKWu agreed with other microbial keratinases [4]. SmKWu's optimal pH and excellent stability in a wide alkaline pH range and wide temperature range suggested its potential applications in leather processing procedures and as detergent additives in the formulation [6, 15, 30, 34].

Effects of chemicals on SmKWu

The influence of inhibitors, surfactants, and metal ions on SmKWu's activity showed that SmKWu's activities were severely inhibited by protease inhibitors (PMSF and EDTA), which indicated that metal ions were crucial for maintaining the enzyme's active conformation and there might be a serine residue within the active center of the enzyme. Thus, SmKWu was likely to be a serine metalloprotease. Additionally, the results showed that Ca^{2+}, Mn^{2+}, Mg^{2+}, K^{+}, and Na^{+} activated the activity of SmKWu in different degrees. While Zn^{2+}, Fe^{2+}, and Cu^{2+} severely inhibited the activity of keratinase, Fe^{3+} inhibited the activity completely. The significant activation of Ca^{2+} was similar to the multiple proteases [27, 28], and the increasing effects of Mn^{2+}, Mg^{2+}, K^{+}, and Na^{+} on SmKWu were following *B. thuringiensis* AD-12 keratinolytic proteinase and *B. subtilis* HQS-3 protease [35, 36]. The severely inhibitory effects of heavy metals (Cu^{2+} and Zn^{2+}) on SmKWu were similar to *S. rhizophila* MT1 and *S. maltophilia* L1 keratinase [27, 28]. Also, Zn^{2+}, Fe^{2+}, and Fe^{3+} inhibited remarkably the enzymes' activities of *S.*

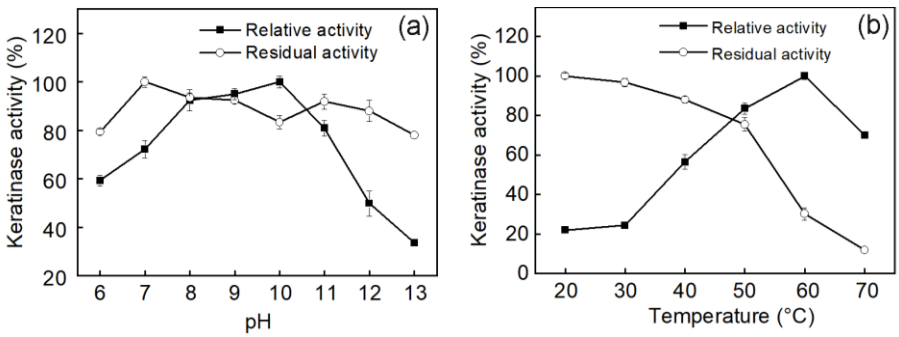

Figure 6. The effects of pH and temperature on the activity of SmKWu.

Table 1. Effects of chemicals on SmKWu activity.

Chemicals	Concentration	Relative activity (%)
Ca^{2+}	10 mmoL/L	150.96 ± 2.51
Zn^{2+}	10 mmoL/L	82.09 ± 0.59
Mn^{2+}	10 mmoL/L	118.73 ± 2.26
Mg^{2+}	10 mmoL/L	180.44 ± 4.83
Fe^{2+}	10 mmoL/L	33.06 ± 0.57
Fe^{3+}	10 mmoL/L	Not detected
K^{+}	10 mmoL/L	172.45 ± 3.94
Na^{+}	10 mmoL/L	118.18 ± 4.17
Cu^{2+}	10 mmoL/L	45.73 ± 1.23
PMSF	5 mmoL/L	39.96 ± 2.28
EDTA	5 mmoL/L	55.86 ± 2.67
Tween 20	5%	160.92 ± 2.35
Tween 60	5%	182.12 ± 2.92
Tween 80	5%	152.62 ± 3.88
Triton X-100	5%	103.07 ± 1.90
SDS	1%	67.31 ± 0.38

maltophilia FF11 keratinase [29]. Furthermore, the study on the effects of various surfactants on keratinase activity indicated that, although the keratinase activity was inhibited in the presence of 1% SDS, multiple surfactants at a concentration of 5% enhanced the enzyme's activity to different degrees (Table 1). Specifically, Tween 20 increased the activity by 60.92%, Tween 60 by 82.12%, Tween 80 by 52.62%, and Triton X-100 by 3.07%. It was generally known that enzymes used in detergent formulas are active and stable with multiple surfactants [7, 37]. Consequently, the activation of those surfactants on SmKWu suggested the potential of SmKWu in the detergent industry.

Substrate specificity of SmKWu

Enzyme substrate specificity is a crucial characteristic in comprehending its enzymatic properties and is demonstrated in potential applications. The results demonstrated that SmKWu varied activities towards diverse substrates with the exhibition of high activities against the casein substrate, the soluble keratin substrate, and BSA (Figure 7). SmKWu was also capable of catalyzing wool keratin substrate and feather keratin substrate and manifested the lowest catalytic activity against type-I collagen substrate. Given its low collagen activity, the enzyme could neither cause damage to collagen in skin during detergent application nor result in

excessive loss of collagen in leather. Keratinases with better substrate specificity could degrade a variety of substrates and remove various stains such as accumulated keratinous stains on collars, sleeve cuffs, and blood stains on cloth [15, 37]. Coupled with the specific catalytic properties toward keratinous substrates and robustness in the alkaline pH, SmKWu could thus be utilized in the detergent and leather industries. Additionally, the high activity of the enzyme toward feather powder substrate indicated that SmKWu had the potential for feather degradation.

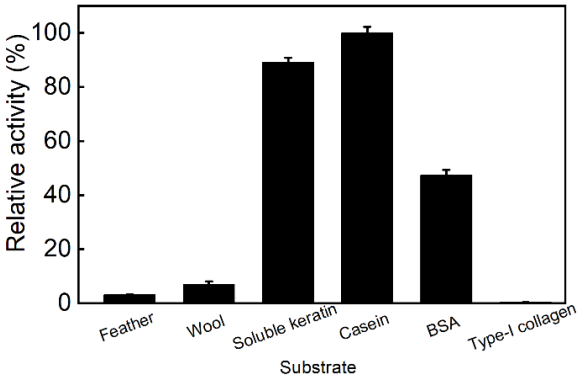

Figure 7. Substrate specificity of SmKWu.

Compatibility with commercial detergents

Based on its relatively low collagenase activity, high activity toward protein substrates, particularly the attractive stability within the alkaline pH range, and its stability and even activation with various surfactants that were commonly employed in detergents, SmKWu might be suitable as a detergent additive. However, the complex components in the detergents could affect the enzyme's activities and catalytic efficiency [37]. Therefore, to explore its potential applicability in detergent industry, the compatibility of SmKWu with various commercial detergents was evaluated. The results showed that SmKWu remained at 80-90% of residual activity when the systems contained detergents at a concentration of 0.7%. Interestingly, the commercial brand detergents including Bluemoon and Diao brands demonstrated high residual enzyme activities at the high detergent concentrations (Figure 8). The high compatibility and stability of enzymes with commercial detergents were considered an important property for their industrial detergent application [7, 15]. Therefore, SmKWu had the potential to be a candidate for the detergent additive.

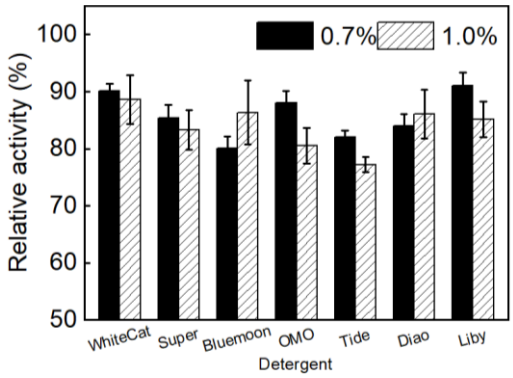

Figure 8. Compatibility of SmKWu with commercial detergent.

Feather degradation and analysis of amino acid in hydrolysate

With the worldwide rising consumption of poultry products, a large number of feathers are generated annually. The degradation of feathers by microbial enzymes attracts the attention of scientists [38]. Thus, the performance of *S. maltophilia* Wu2 in feather degradation was explored in this study. The results showed that the feather treated with tap water was still intact (Figure 9a), while part of the feathers was degraded in the treatment for 5 d using the intact feather to replace the feather meal inducer in the fermentation medium (Figure 9b). The feather was degraded distinctly in the treatment for 5 d by SmKWu (Figure 9c). The feather was completely degraded in the *S. maltophilia* Wu2-enzyme system where intact feathers were introduced after 48 hours of fermentation (Figure 9d). Compared with centrifugation to obtain the supernatant, the *S. maltophilia* Wu2-enzyme system had a lower cost. Thus, the feather degradation rates in the *S. maltophilia* Wu2-enzyme system were further analyzed. The

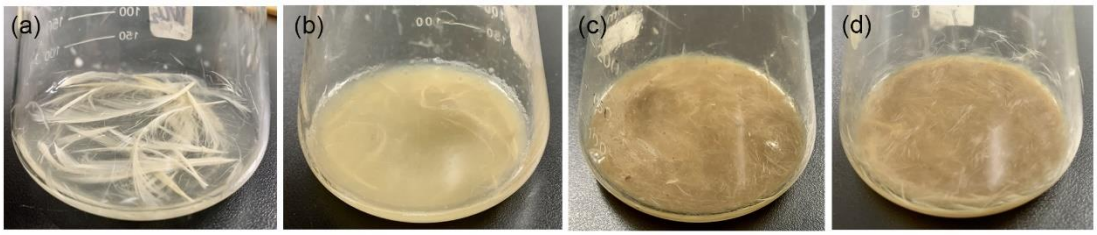

Figure 9. Feather degradation results. (a) feather treated with tap water. (b) the intact feather was used to replace the feather powder inducer in the medium. (c) feather treated with SmKWu. (d) feather treated with *S. maltophilia* Wu2-enzyme system.

results showed that the degradation rate of feathers exceeded 80% and more than 83% for 5 days and 7 days, respectively. As the enzymatic time extended, the feather's degradation rate was further enhanced and could exceed 88%.

Protein sources are scarce in the feed industry worldwide. Therefore, scientists are also attempting to find appropriate alternative protein resources. Recycling keratin from feathers into nutrients that animals can absorb and utilize through the biological enzymatic method is essential in easing the shortage of protein and in managing feather waste in an environmentally friendly way [34]. Given *S. maltophilia* Wu2's capability of degrading feathers, the analysis of amino acids in feather hydrolysate produced by the *S. maltophilia* Wu2-enzyme system was conducted. The results showed that the total content of amino acids in the hydrolysate after feather degradation for 5 days was over 1,100 mg/L, and it was rich in essential amino acids like Lys, Met, Trp, Leu, Ile, Phe, Val, and Arg with Leu and Val showing the highest concentrations of 247.59 mg/L and 145.51 mg/L, respectively, while Thr and His were not detected (Table 2). The high content of free amino acid nitrogen, especially essential amino acids in the hydrolysate, indicated its potential use as a feed additive that is usually expensive in the diet. Thus, it might reduce the feed cost and benefit the breeding industry [6, 39]. Therefore, the conversion of recalcitrant low-value and inexpensive keratin-rich feathers by the *S. maltophilia* Wu2 system into valuable nutrients is beneficial for sustainable development for environment protection and resource utilization.

Table 2. Analysis of amino acids in feather hydrolysate for 5 days.

Amino acid	Content (mg/L)
Asp	34.79
Thr	Not detected
Ser	0.77
Glu	97.44
Gly	38.27
Ala	162.54
Cys	20.78
Val	145.51
Met	72.91
Ile	65.56
Leu	247.59
Tyr	12.71
Phe	79.19
Lys	28.75
His	Not detected
Arg	15.57
Pro	99.53

Washing performance

With people's increasing concern for the environment and the pursuit of high efficiency and high quality of life, there is an urgent need to search for an alternative to the chemical organic compounds and surfactants used in detergents. Detergent-stabilizing enzymes attract researchers' attention because of their potential use as additives to detergent formulation [38]. Based on the attractive stability of SmKWu with various surfactants and good compatibility with commercial detergents, as well as high catalytic activity toward various protein substrates including recalcitrant keratin substrates, the washing performance of Wu2 keratinase was conducted to further explore the potential of SmKWu in detergents. Removing blood stains

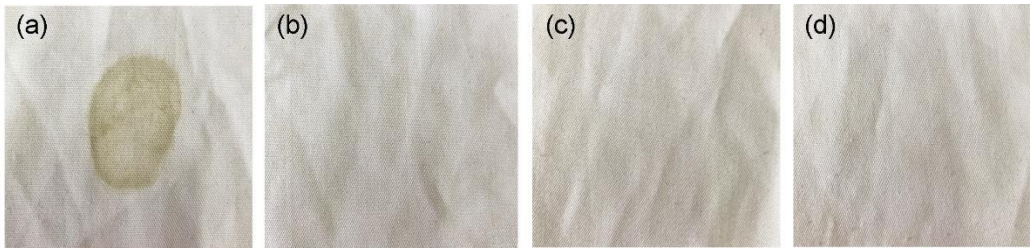

Figure 10. Washing performance of SmKWu. (a) treated with tap water for 18 min. (b) treated with 0.7% Bluemoon detergent in tap water for 18 min. (c) treated with SmKWu for 18 min. (d) treated with combining 0.7% of Bluemoon and SmKWu for 13 min.

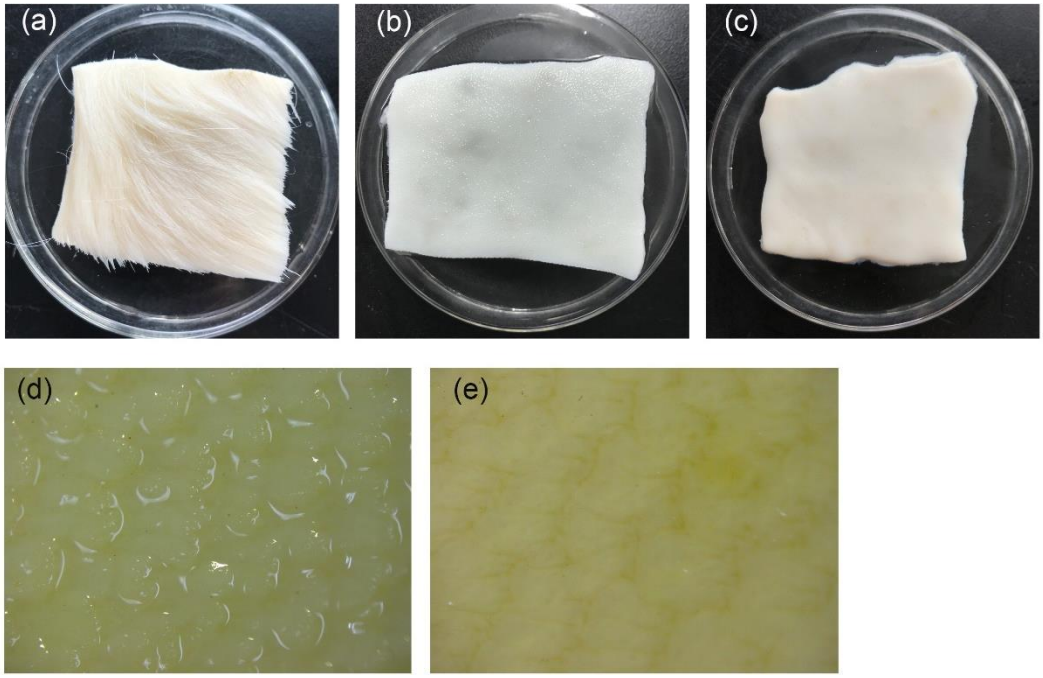

Figure 11. Dehairing performance of SmKWu. (a) treated with tap water. (b) treated with chemical method. (c) treated with SmKWu. (d) microscopic image of chemical treatment. (e) microscopic image of SmKWu treatment.

from cotton cloth, which are considered to be stubborn stains, represents the decontaminating ability. The results demonstrated that an obvious blood stain on the cotton cloth could be removed by the commercial detergent and SmKWu, respectively. Combining inactivated detergent with SmKWu could also wash the blood stain out (Figure 10). The results indicated that SmKWu could not only remove blood stains with satisfying washing effects but also promote the washing efficiency of the detergent, which suggested its prospects in the laundry industry. The compatibility with detergent ingredients, attractive stability with various surfactants, and high washing efficiency endowed SmKWu with the potential for use as a bio-additive in detergent.

Dehairing performance

In view of sustainable development, the leather industry demands an alternative to traditional chemical-based dehairing process that causes ecological impact. The objective of the dehairing process is to remove hair with high functional efficiency and to reserve collagen. The sustainability of leather requires a reduction in the disposal of pollutants into environments, without the use of detrimental reagents such as

sodium sulfite and lime [38, 40]. Environmental pH in the dehairing process is commonly in the range of 8.0 to 10 because the skin is swollen, and it is easy to erase hair roots from the skin pores in the alkaline pH [9]. Considering the low activity toward collagen substrate and excellent alkaline stability of SmKWu, its dehairing efficiency from sheepskin was further evaluated to explore its potential in leather processing. The results showed that the wool on the sheepskin treated by tap water was intact, while the sheepskin treated with the chemical method was dark and the cortex was hard compared to the sheepskin treated with the enzyme method, which was clean, light, soft cortex, and no wool visible by the naked eye (Figures 11a-11c). The depilation effects were further observed under a microscope. The results showed that the enzymatic method presented the same dehairing efficiency as the traditional chemical method. For both methods, the depilation was complete and there was no residual wool on the surface of the sheepskin (Figures 11d and 11e). The efficient hair removal performance of SmKWu and no damage to the skin suggested its potential application as a dehairing agent in the leather industry.

Conclusion

A novel feather degrading *Stenotrophomonas maltophilia* Wu2 strain was isolated in this study. The keratinase yield of strain Wu2 was promoted by the OVAT method. The results showed that SmKWu exhibited excellent stability in the alkaline environment and the temperature range of 30 - 50°C. The keratinase was stable and even activated toward several surfactants at 5%. Additionally, SmKWu was highly compatible with various commercial detergents and exhibited satisfying washing efficiency. Furthermore, SmKWu could convert feather waste into valuable nutrients and be used for dehairing sheep skin. Therefore, SmKWu might be suitable for applications in keratin waste management, feed additives, detergent additives, as well as dehairing process.

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