RESEARCH ARTICLE

Bacillus subtilis SLXX08 thermostable keratinase for decomposition and bioconversion of feather waste and dehairing

Rongxian Zhang[1, 2, 3, *], Jitong Sun[2, 4], Yubo Sun[2], Chunwei Hu[2], Renxiang Shen[2], Hezhen Wang[2], Yizhong Zhang[1, 3], Qunying Xiao[1, 3]

[1]School of Ecological Engineering, Guizhou University of Engineering Science, Bijie, Guizhou, China. [2]School of Life Sciences, Henan Institute of Science and Technology, Xinxiang, Henan, China. [3]Key Laboratory of Biological Resources Development and Ecological Restoration in Guizhou Province, Bijie, Guizhou, China. [4]Nanjing Biotogether Co., Ltd., Nanjing, Jiangsu, China.

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Chicken feathers, being discarded on a large scale worldwide, pose both environmental pollution risk and carriers of harmful microorganisms. Their highly recalcitrant nature, which renders them extremely hard to decompose, and their potential to inflict environmental harm, make the pursuit of effective disposal methods an urgent necessity. Employing microbial keratinase to decompose and utilize feathers offers a sustainable green pathway. This study identified an isolated *Bacillus subtilis* SLXX08 and optimized its production of keratinase. The optimal key components for the medium were 10 g/L of beef extract, 15 g/L of lactose, 2.4 g/L of $MgSO_4$, and 15 g/L of feather powder. The ideal conditions for keratinase production were an initial pH of 8.0, a temperature of 35°C, an inoculum volume of 6%, and a rotational speed of 220 rpm. The *B. subtilis* SLXX08 keratinase reached its peak activity at 50°C and pH 9.0, maintaining stable within the temperature range of 20 - 60°C and the pH range of 8.0 - 12.0. Notably, the enzyme showed relatively low activity towards type-I collagen. Furthermore, it demonstrated excellent capabilities in the biodegradation and bioconversion of feathers, producing over 1,200 mg/L of amino acids including eight essential amino acids. The enzyme displayed significant dehairing efficiency for goat skin within a time frame of 7 hours. This research supported the microbial degradation and valorization of feathers and highlighted the potential for clean dehairing processes.

Keywords: keratinase; *Bacillus subtilis*; feather decomposition; feather bioconversion; dehairing.

[*]**Corresponding author:** Rongxian Zhang, School of Ecological Engineering, Guizhou University of Engineering Science, Bijie 551700, Guizhou, China. Email: Rongxzhang@163.com.

Introduction

In the global meat market, poultry meat is a major part of the human diet. It is an excellent source of high-quality protein with a lower fat content than some red meats [1]. Its fat, mainly composed of unsaturated fatty acids like oleic acid, is healthier. Poultry also contains essential trace elements such as iron, zinc, and phosphorus for human metabolism. Therefore, the poultry industry has developed significantly worldwide. Feathers, which account for 5-7% of the total mass of poultry [2], are the major by-product of poultry processing with several million tons being generated annually [3, 4]. Nevertheless, feathers are composed of over 90% keratin [5]. Keratin, owing to the intensive cross-linking interactions by hydrogen bonds, disulfide bonds, and peptide

bonds within its molecular architecture, exhibits a highly compact and firm conformation with remarkable resistance to conventional proteases including pepsin and trypsin [5, 6].

The common methods for disposing of feathers are burial and incineration [4, 7]. However, burial unavoidably reduces soil sources, while incineration releases greenhouse gases and solid particles. Both methods can facilitate the proliferation of bacteria and viruses [8], endangering human welfare and the ecosystem. On the other hand, feather keratin, abundant in essential amino acids, represents a valuable nitrogen source [2]. Effectively harnessing feather protein resources can diversify feed protein sources, overcoming the current global shortage of digestible nitrogen in the feed industry and generating substantial economic value. Keratinases are highly effective in degrading the tightly structured keratin. Keratinases and their producers transform the highly structured feather keratin into nutrients, like soluble polypeptides and amino acids, that animals can readily assimilate and utilize. Moreover, due to their unique catalytic properties towards keratin substrates, keratinases and their producing microorganisms have wide-ranging potential applications such as the feed industry, the detergent industry, the leather industry, and the food industry [8-10]. Furthermore, in the bioprocessing of many industrial applications, crude enzymes possess several advantages over purified enzymes such as lower cost, higher productivity efficiency, better environmental tolerance, and enhanced storage stability. There characteristics render the crude more suitable for complex industrial working conditions. The main sources of keratinase are fungi [4, 11-13], actinomycetes [14], and bacteria [15, 16]. Certain bacteria capable of breaking down keratin, especially those from the *Bacillus* genus, have been reported [17, 18]. Bacterial keratinase resources have attracted significant attention because of their rapid growth, relatively high enzyme-producing capacity, and high safety level. However, because of the harsh working conditions in industrial applications, as well as the complexity of global patents, it is still essential to explore novel promising keratinases to meet the industrial demands. Orthogonal experimental design (OED) is a key method within fractional factorial design. By using OED, researchers can quickly and accurately identify the optimal reaction conditions [19]. This experimental approach not only determines the impact of each factor on the experimental outcomes but also elucidates the hierarchical importance of these factors. Owing to its remarkable capacity to extract highly valuable insights from a minimal set of experimental combinations, OED achieves high efficiency, streamlining the process and maximizing per-trial information, making it invaluable in scientific and industrial experimentation.

This study sought to isolate a keratinase-producing microbe and enhance its keratinase production to expand feed protein sources, ease the nitrogen shortage in the feed industry, and handle feather waste in an eco-friendly way. Subsequently, the potentials of crude *B. subtilis* SLXX08 keratinase (SLXX08 keratinase) in feather waste breakdown, utilization, and in dehairing were evaluated.

Materials and methods

Selection of feather-degrading strains

Soil samples were collected from the area in the market of Xinxiang, Henan, China, where feather keratin had accumulated after live-poultry slaughtering operations. One gram soil sample was mixed with 19 mL aseptic physiological saline. After being vigorously shaken for 20 minutes, the sample was left undisturbed for 30 minutes. The supernatant was carefully extracted and inoculated into the enrichment medium that was composed of 10 g/L feather powder, 0.5 g/L ammonium chloride, 0.5 g/L sodium chloride, 0.3 g/L dipotassium hydrogen phosphate, 0.4 g/L potassium dihydrogen phosphate, 0.1 g/L magnesium chloride, 1 g/L yeast extract with a pH of 7.0 and cultured at 37°C, 180 rpm, for 24 h

to obtain the enrichment culture.

To explore the potential strain, the cross-screening approach was employed. Preliminary screening was performed using the skim milk solid medium (10 g/L skim milk powder and 20 g/L agar powder, natural pH). After appropriate gradient dilution, the enrichment culture was spread onto the skim milk solid medium, cultured at 37°C for 24 h. Single colonies with clear hydrolysis zones were then selected. Secondary screening was carried out using the microbial strains acquired from the preliminary screening. The microbial strains were activated and subsequently inoculated into LB medium with an inoculum size of 1%. The cultures were incubated under 37°C, 220 rpm. The optical density value (OD) of the bacterial suspension at a wavelength of 600 nm was measured at intervals of 2 h. The growth curve of the strain was constructed based on the variation of OD_{600} value over time. The seed liquid in the logarithmic growth phase was inoculated into the initial fermentation medium (10 g/L feather powder, 1 g/L dipotassium hydrogen phosphate, 0.5 g/L potassium dihydrogen phosphate, and 0.5 g/L sodium chloride with an initial pH value of 7.0). The enzyme production status was monitored at intervals of 3 h.

Bacterial identification

Based on morphological identification, the strain was further characterized using physiological and biochemical methods. Additionally, the molecular identification was carried out by Personal Biotechnology Co., Ltd (Shanghai, China). Specifically, the target bacterial partial 16S rRNA gene was amplified using the 27F primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and the 1492R primer (5'-CTA CGG CTA CCT TGT TAC GA-3') in an ABI 2720 Thermal Cycler (Applied Biosystems, Westminster, CO, USA). The purified polymerase chain reaction (PCR) product was subjected to DNA sequence determination using an ABI 3730-XL sequencer (Applied Biosystems, Westminster, CO, USA). Furthermore, the National Center for Biotechnology Information (NCBI) Blast program was used to compare the assembled sequence with the data in the NCBI 16S database and obtain the species information with the highest similarity as the identification result.

Measurement of keratinase activity

The approach elucidated by Zhang *et al*. was utilized to ascertain keratinase activity [15]. The reaction system, which was composed of the properly diluted enzyme supernatant (0.5 mL) and 1% keratin substrate (1.5 mL) (J&K Scientific, Beijing, China), underwent the reaction at 40°C for 20 min before 2 mL of 0.4 M trichloroacetic acid (TCA) was added to terminate the reaction. After keeping the reaction system at room temperature for 10 min, the supernatants were obtained by centrifugation for 10 min. The Folin-phenol chromogenic reaction was carried out at 40°C for 20 min using the reaction system of 0.5 mL of the supernatant, 2.5 mL of 0.4 M Na_2CO_3 solution, and 0.5 mL of the Folin-phenol reagent. The activity of keratinase was quantified, whereby one unit of keratinase activity was designated as an increase of 0.01 in absorbance at a wavelength of 660 nm (OD_{660}) using an UV2400 spectrophotometer (Sunny Hengping, Shanghai, China).

Optimization of the enzyme-producing medium

The effect of the concentration of feather powder, which simultaneously served as the enzyme production inducer and the sole nitrogen and carbon source, on enzyme production was investigated. To analyze the influence of nitrogen sources on enzyme production, glucose, sucrose, cornmeal, starch, lactose, and maltose at 5 g/L were supplemented into the fermentation medium during fermentation. Additionally, the concentration of the optimal nitrogen source was also optimized. Concerning the effect of carbon sources on enzyme production, 5 g/L of sucrose, cornmeal, lactose, starch, and glucose were introduced to probe the repercussions of carbon sources on enzyme production during the fermentation procedure. Simultaneously, the concentration of the most favorable carbon source was optimized. Furthermore, during the process of optimizing the fermentation with

feather powder as the inducer and substrate, beef extract, lactose, and $MgSO_4$ exhibited relatively obvious effects on bacterial growth and enzyme production. Therefore, an orthogonal test was devised to determine the strain's optimal fermentation medium. The levels of the three factors were set as beef extract at 5, 10, and 15 g/L; lactose at 5, 10, and 15 g/L; and $MgSO_4$ at 1.2, 1.8, and 2.4 g/L.

Optimization of fermentation conditions

Different factors of fermentation conditions were adjusted to investigate the influence of single factor changes on enzyme production during fermentation. Specifically, the initial pH level of the culture medium, fermentation temperature, rotation speeds, and inoculum size were screened and evaluated.

Characterization of SLXX08 keratinase

To investigate the characteristics of SLXX08 keratinase, the effects of pH and temperature on its enzymatic activity were analyzed. The variation of SLXX08 keratinase activity within the pH range from 6.0 to 12.0 and the temperature range from 20 to 70°C was examined. The pH and thermal stabilities of SLXX08 keratinase were assessed following incubation under the corresponding conditions for 30 minutes. Additionally, the effects of various chemicals on SLXX08 keratinase activity were determined under the optimal reaction conditions, which included metal ions of Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Na^{+}, Al^{3+}, K^{+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, protease inhibitors of ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), surfactants of Tween 20, Tween 60, Tween 80, Triton X-100, sodium dodecyl sulfate (SDS), and the reducing agent β-Mercaptoethanol (β-ME). The enzyme activity in the absence of any chemical reagents was taken as 100%. Meanwhile, to explore its potential industrial application, the activities of SLXX08 keratinase towards insoluble substrates such as feather powder and wool powder, and soluble substrates like bovine serum albumin (BSA), type-I collagen, casein, and soluble keratin were also investigated. The substrates were prepared at 1% using pH 9.0 of Tris-HCl buffer solution at 50 mM and then explored the substrate specificity of keratinase. For the insoluble substrates, the reaction system contained the suspended 0.05 g of substrates in 0.5 mL of buffer solution, and 0.05 mL of the appropriately diluted keratinase. The reaction was carried out at 50°C for 1 h. Afterward, 1.0 mL of 10% TCA solution was incorporated into the reaction system to terminate the reaction, and then centrifugation at 15,000 rpm for 10 min. The absorbance value at 280 nm (OD_{280}) of the supernatant was determined. For each reaction, the reaction system in which TCA was added before the substrate was employed as a control. The enzyme activity was defined as an increase of 0.01 in OD_{280} as one unit. The substrate with the highest catalytic activity was designated as 100%.

Feather decomposition and bioconversion by SLXX08 keratinase

Two enzymatic methods were adopted for feather hydrolysis. After *B. subtilis* SLXX08 completed the fermentation for keratinase production, 10 g/L of intact chicken feathers were incorporated into the fermentation broth to initiate the feather degradation experiment. The degradation conditions were 50°C and 120 rpm. During this process, the degradation progress of the feathers was continuously monitored. The other method involved degrading feathers during the fermentation process of *B. subtilis* SLXX08. Specifically, the intact feathers were used to replace the feather powder in the fermentation medium. Then, the seed solution was inoculated, and fermentation was carried out for the enzyme production-related feather degradation. The free amino acids were determined by introducing 40 mL of 0.01 M hydrochloric acid (HCl) into the supernatant of feather hydrolysate. The mixture was vortex-mixed for 5 min, and then subjected to ultrasonication extraction for 5 min. After adjusting the volume to 50 mL and mixing well, the mixture was stored in the dark for 2 h. Subsequently, 5 mL of the solution was taken out and centrifuged at 4,000 rpm for 10 min. 1 mL of the supernatant was accurately combined with

an equal volume of 2-4% sulfosalicylic acid, then vortex-mixed for 1 min, stored in the dark for 60 min, and centrifuge at 15,000 rpm for 15 min. The supernatant was filtered through a 0.22 μm filter and then analyzed using a Hitachi LA-8080 ultra-high-speed amino acid analyzer (Hitachi, Tokyo, Japan).

Dehairing by SLXX08 keratinase

Three experimental treatments were designed with goatskins (about 6 cm × 6 cm) as follows. Treatment A was designed as negative control by treating the goatskin with tap water at room temperature and 180 rpm. Treatment B was designed as positive control by treating goatskin with a chemical method involving lime and sodium sulfide. Treatment C was the enzymatic treatment. The goatskin was immersed in SLXX08 keratinase with approximately 30,000 U and then incubated at 30°C and 180 rpm. The goatskins under the different treatments were collected at regular intervals to examine the depilation status during the depilation process. After the depilation process was completed, the goatskin was thoroughly rinsed with tap water. The efficiencies of both enzymatic and chemical depilation methods and the quality of the goatskin were analyzed using a stereomicroscope and other relevant techniques. Since there is no quantitative standard for evaluating the depilation effect, an integrated approach combining sensory evaluation and microscopic observation by a LEICA DM 2500 microscope (Leica Microsystems, Wetzlar, Germany) was employed to investigate the depilation effects of the enzyme.

Results and discussion

Isolation, screening, and identification of keratinase-producing bacterium

To investigate microbial resources for feather biodegradation, feather keratin was employed as the exclusive carbon and nitrogen source, and as an inducer for microbial keratinase production. Moreover, bacterial colonies that showed obvious zones on skim milk powder agar medium were processed. After isolation and screening, the keratinase-producing abilities of the strains were examined. Among the selected strains, strain SLXX08 showed the maximal keratinase activity reaching 312 U/mL under the initial fermentation medium with the specific culture conditions for 24 h. Meanwhile, strain J3 produced 220 U/mL after 24 h of fermentation. Consequently, strain SLXX08 was chosen for further investigation. The isolate SLXX08 was a Gram-positive rod and formed spore. Molecular identification of strain SLXX08 was conducted. The partial 16S rRNA sequence was submitted to the NCBI database, obtaining the accession number of OR793953, demonstrating a high similarity of over 99.9% with *B. subtilis* BCR10255 (Accession number 116017), *B. subtilis* DSM 10 (Accession number NR027552), and *B. subtilis* NBRC13719 (Accession number 112629). The phylogenetic tree constructed based on the 16S rRNA partial sequences comparison signified its position within the clade of *Bacillus subtilis* (Figure 1). Thus, the strain was designated as *Bacillus subtilis* SLXX08 and further deposited in China General Microbiological Culture Collection Center under the accession number of CGMCC No. 27266.

Improving keratinase production

(1) Growth, and keratinase production of strain SLXX08

B. subtilis SLXX08 got out of the lag phase and entered the logarithmic growth phase after 6 hour of cultivation in LB medium and accompanied by a rapid multiplication of the bacterial cells (Figure 2A). The appropriate seed cultivation time ranged from 12 to 14 h, corresponding to the middle-late period of the logarithmic growth phase. In addition, after *B. subtilis* SLXX08 was inoculated into the fermentation medium, the yield of *B. subtilis* SLXX08 keratinase was 418 U/mL when the cultivation lasted for 39 h (Figure 2B).

(2) Optimization of fermentation medium

Single-factor analysis was conducted to evaluate the influence of medium components on keratinase production. The impact of feather

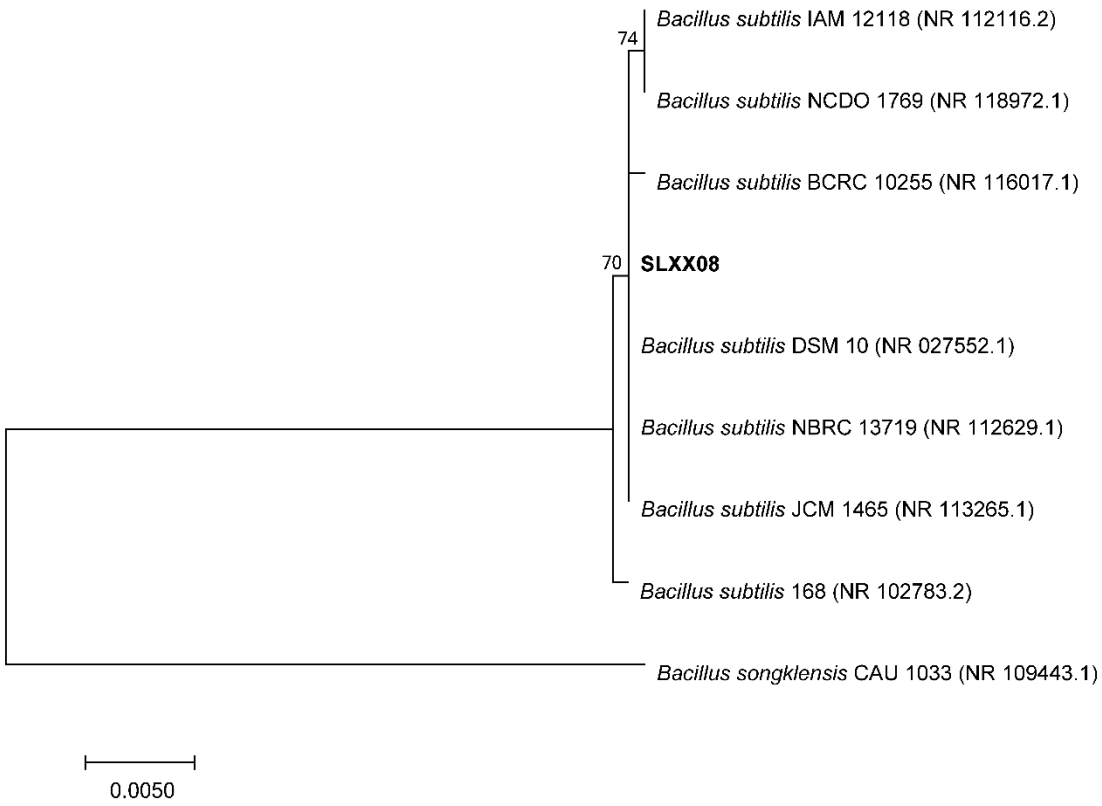

Figure 1. Phylogenetic tree of strain SLXX08 (NCBI accession number: OR793953).

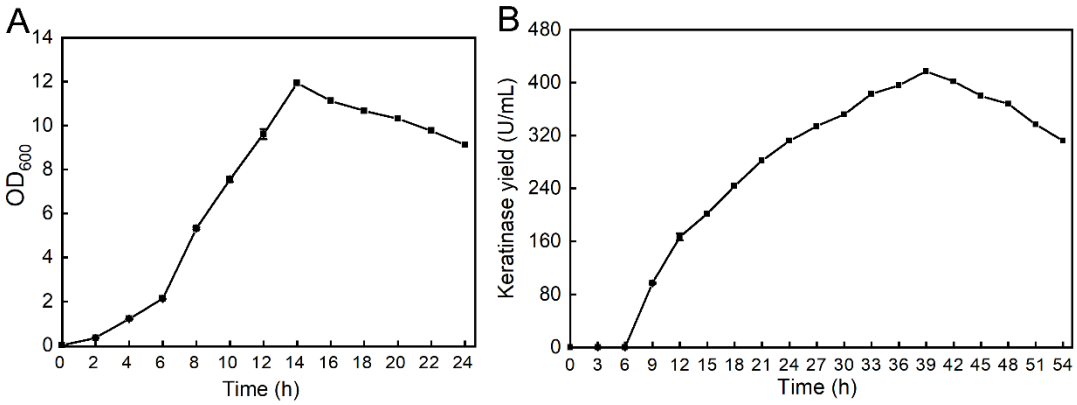

Figure 2. The growth curve and the enzyme production profile of Strain SLXX08.

powder, as an inducer for keratinase production [9], was investigated. The results demonstrated that enzyme production reached its peak when the concentration of feather powder in the culture medium was 15 g/L. Both excessively high and low contents of feather powder reduced the keratinase production of *B. subtilis* SLXX08 (Figure 3A). During the initial stage of fermentation, to obtain sufficient biomass for meeting the enzyme-production requirements of microorganisms, it was essential to introduce carbon substances, nitrogen substances, and mineral salts that could be easily accessible by microorganisms into the fermentation medium.

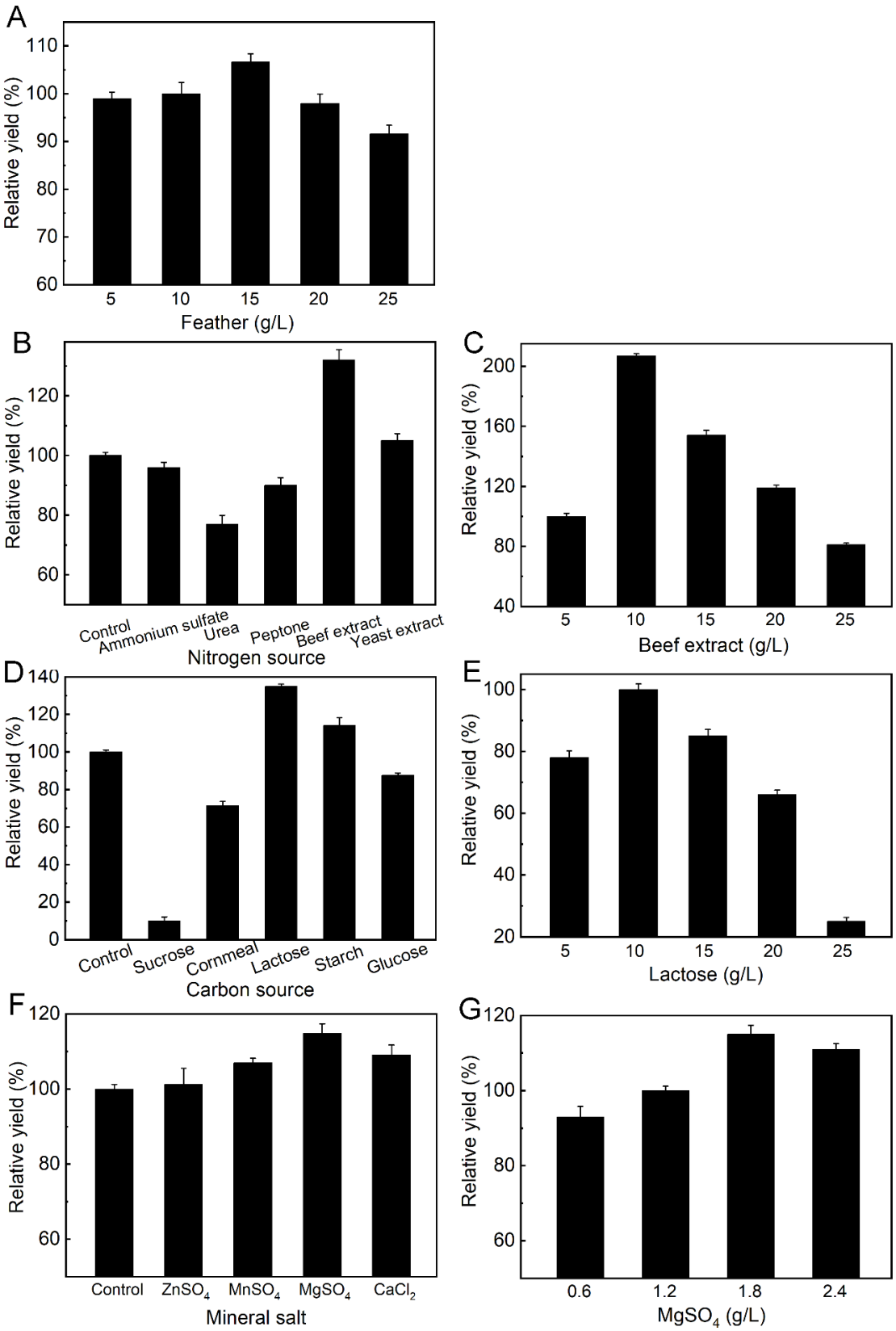

Figure 3. The impact of medium components on enzyme production by *B. subtilis* SLXX08. **A.** feather. **B**. nitrogen source. **C.** beef extract. **D**. carbon source. **E**. lactose. **F.** mineral salt. **G.** $MgSO_4$.

Nitrogen sources are crucial for bacterial growth. Appropriate nitrogen sources enable the bacteria to produce enough biomass in the early stage of fermentation. Research findings indicated that beef extract and yeast powder promoted enzyme production with beef extract showing a more prominent promoting effect (Figure 3B). In contrast, peptone, ammonium sulfate, and urea all inhibited the enzyme production by the bacteria to a certain degree. Consequently, the next-step experiment is to explore the optimal addition amount of beef extract. The enzyme production by *B. subtilis* SLXX08 reached its maximum when the additional amount of beef extract was 10 g/L (Figure 3C). It has been reported that beef extract enhanced protease production by *B. pumilus* NJM4 and *Bacillus aerius* NSMk2 [20, 21], and yeast extract improved keratinase production by *Amycolatopsis* sp. MBRL 40 [22]. Among the tested carbon sources, lactose followed by starch promoted enzyme production. However, glucose, cornmeal, and sucrose inhibited enzyme production to different extents (Figure 3D). Further research found that the enzyme production reached its peak when the lactose addition amount was 10 g/L, and a repressive effect on the enzyme production was observed when the lactose addition amount was 20 - 25 g/L (Figure 3E), which was similar to the improvement of lactose on the keratinase yield of *B. velezensis* ZBE1 [23] and the stimulatory effect of starch on the keratinases yields of *Bacillus pseudofirmus* FA30-01, *B. cereus* YQ15, and *B. cereus* [15, 24, 25]. In the research for optimal inorganic salts, it was found that magnesium sulfate, zinc sulfate, calcium chloride, and manganese sulfate all had a stimulatory effect on enzyme production (Figure 3F). Among them, magnesium sulfate exhibited the most prominent promoting effect, which was in line with that magnesium ions enhanced enzyme production by *B. pumilus* YQ15 and *B. pumilus* A1 [15, 26]. Moreover, the optimal addition amount of magnesium sulfate was 1.8 g/L, at which the enzyme production was the highest (Figure 3G). Based on the preceding optimization, it was determined that beef extract, lactose, and magnesium sulfate affected the keratinase production by *B. subtilis* SLXX08. These three factors were further investigated using an orthogonal design strategy to optimize the enzyme production. The outcomes of the orthogonal experiments indicated that the significance ranking of the three factors in descending order was beef extract > lactose > $MgSO_4$. The optimal combination was found to be 10 g/L beef extract, 15 g/L lactose, and 2.4 g/L $MgSO_4$, which was not included in the orthogonal experiment combinations. Subsequently, the optimal combination of the fermentation medium was validated. A three-batch experimental verification process was conducted, and the strain demonstrated a stable enzyme-producing capacity under the conditions of this medium. The average value reached 1,560 U/mL, presenting a 2.73-fold elevation compared to the pre-optimization level. Therefore, the optimal medium for *B. subtilis* SLXX08 consisted of 10 g/L beef extract, 15 g/L lactose, 2.4 g/L magnesium sulfate, 15 g/L feather powder, 0.5 g/L sodium chloride, 0.5 g/L potassium dihydrogen phosphate, and 1 g/L dipotassium hydrogen phosphate.

(3) Optimization of the *B. subtilis* SLXX08 culture conditions

The optimal cultural temperature for *B. subtilis* SLXX08 was 37°C (Figure 4A), which was the same as that for *Bacillus* sp. FK 46 [27]. Moreover, the enzyme production reached its maximum when the initial pH of the fermentation medium was 8.0. Therefore, a pH value of 8.0 was selected as the optimal culture pH (Figure 4B), which was in line with the optimal pH range for keratinase producers that thrived under neutral to alkaline conditions [28]. The rotation speed affects the dissolved oxygen and mass transfer in the medium. The requirement of *B. subtilis* SLXX08 for the rotation speed can indirectly reflect the strain's demand for dissolved oxygen. When the rotation speed was adjusted from the initial 180 rpm to 220 rpm, the enzyme production level increased significantly and then decreased (Figure 4C). Therefore, the optimal culture rotation speed was determined to be 220 rpm,

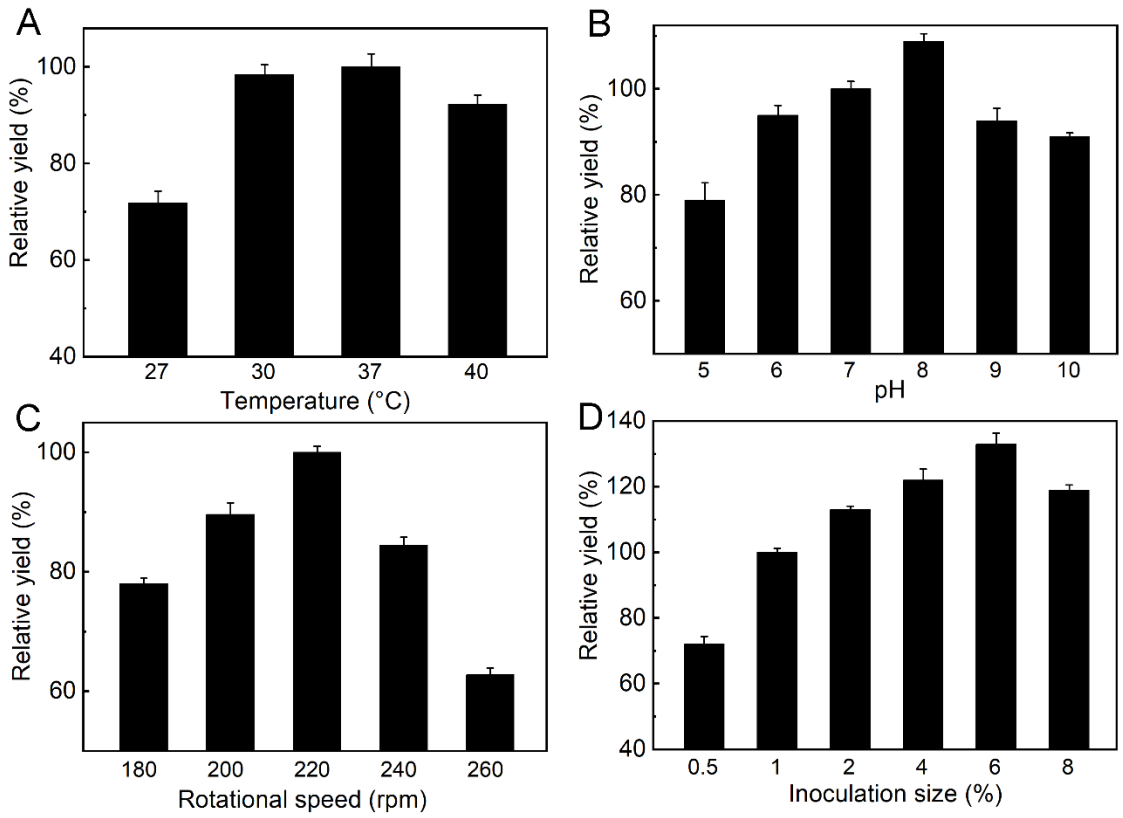

Figure 4. The impact of culture conditions on enzyme production by strain SLXX08. **A.** temperature. **B.** pH. **C.** rotational speed. **D.** inoculation size.

which guaranteed oxygen transfer efficiency during the fermentation. Furthermore, the experiments revealed that, when the inoculum size was 6%, the enzyme production level of *B. subtilis* SLXX08 reached an inflection point (Figure 4D). Thus, an inoculum size of 6% was selected as the optimal culture inoculum size. The relatively high inoculum size was correlated with that of *B. velezenis* HFS_F2 [17]. Combining the above-optimized fermentation conditions, the keratinase production stability by *B. subtilis* SLXX08 was further measured. After verification *via* three batches, the average enzyme production of the strain was 2,280 U/mL under the conditions of 37°C, a rotation speed of 220 rpm, an inoculum volume fraction of 6%, and an initial pH of 8.0.

Characterization of crude SLXX08 keratinase

In comparison with the purified enzyme, the crude enzyme usually boasts advantages such as lower cost, higher productivity efficiency, better environmental tolerance, and storage stability, rendering it more suitable for complex industrial working conditions. Consequently, the characteristics of crude SLXX08 keratinase were investigated to explore its industrial application potential.

(1) The impact of pH and temperature on SLXX08 keratinase activity

SLXX08 keratinase showed maximal enzyme activity at pH 9.0, which was consistent with that of bacterial keratinases [15]. The optimal reaction temperature was determined to be 50°C, consistent with that of *Stenotrophomonas Rhizophila* MT1 [16]. Moreover, the residual activity of SLXX08 keratinase exceeded 86% at 20 - 60°C, indicating its excellent thermal stability. The broad thermostability is of crucial significance for its suitability in industrial applications. It was also noted that the enzyme's residual activity was over 60% within the pH range of 8.0 - 12.0. Thus, SLXX08 keratinase

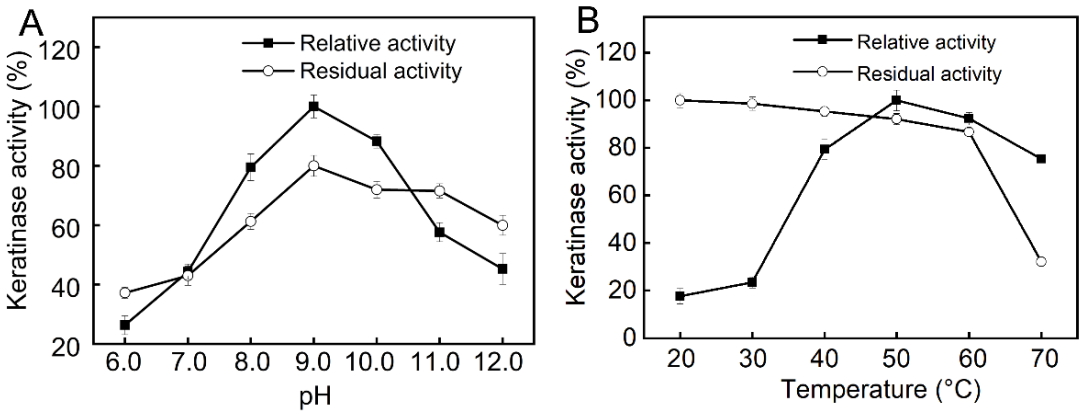

Figure 5. Influence of pH and temperature on the activity and stability of SLXX08 keratinase.

exhibited thermal and alkaline stability at 20 - 60°C and a pH range of 8.0 - 12.0 (Figure 5). The remarkable thermal stability and alkaline stability exhibited by SLXX08 keratinase implied its high enzymatic efficiency and low risk of microbial contamination during industrial processes [29].

(2) The impact of a variety of chemicals on SLXX08 keratinase activity

The results showed that Ca^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} demonstrated promoting effects on the activities of SLXX08 keratinase with the enzyme activities being increased by 0.2 to 0.9 times correspondingly (Table 1). The stimulation of Ca^{2+}, Mn^{2+}, and Mg^{2+} on SLXX08 keratinase was in accordance with those on the keratinases of *B. cereus* YQ15 and *Bacillus mojavensis* SA [15, 30]. A significant inhibition of the enzyme activities was observed for Cu^{2+}, Fe^{2+}, Al^{3+} with 50 - 65% of activity and Fe^{3+} with 25.56% of activity. Moreover, the protease inhibitors, EDTA and PMSF, exhibited substantial inhibition on keratinase activities with inhibition rates of 75% and 90%, respectively. The findings implied that SLXX08 keratinase probably required metal cofactors, and serine residues might be present in the catalytic center. This was similar to *B. subtilis* S1-4, and *B. pumilus* NRC21 keratinases, which were also inhibited by EDTA and PMSF [31, 32]. Furthermore, the reducing agent β-ME had a certain promotional effect on SLXX08 keratinase. In addition, SLXX08 keratinase exhibited stable activity in the presence of the surfactant Tween-60. However, its activity was inhibited by Tween 20, 80, Triton X100, and SDS.

Table 1. Effects of multiple chemical reagents on SLXX08 keratinase.

Chemicals	Concentration	Relative activity (%)
Zn^{2+}	5 mM	118.19±2.43
Cu^{2+}	5 mM	60.87±1.50
Ca^{2+}	5 mM	188.35±2.63
Mn^{2+}	5 mM	165.00±1.44
Na^{+}	5 mM	100.05±1.48
Al^{3+}	5 mM	62.76±0.27
K^{+}	5 mM	99.03±0.57
Mg^{2+}	5 mM	131.34±0.38
Fe^{2+}	5 mM	52.21±0.57
Fe^{3+}	5 mM	25.56±0.13
β-ME	1 mM	112.45±1.90
EDTA	5 mM	25.69±0.23
PMSF	5 mM	10.01±0.25
Tween 20	1%	30.77±1.93
Tween 60	1%	112.69±5.93
Tween 80	1%	29.56±0.53
Triton X-100	1%	93.07±1.19
SDS	1 mM	68.30±1.26

(3) The catalytic activity of SLXX08 keratinase towards various substrates

It is well known that substrate specificity is a crucial property of an enzyme for understanding its enzymatic properties and exploring its potential industrial application value. Thus, the enzymatic activity of SLXX08 keratinase towards a variety of substrates was studied. The results

showed that the catalytic activities of SLXX08 keratinase in descending order were casein > soluble keratin > keratin in wool powder > BSA, and it also had relatively high catalytic activity towards keratin in feather powder, while its catalytic activity towards type I collagen was the lowest one (Figure 6). Due to its low collagenolytic activity, SLXX08 keratinase did not cause excessive collagen depletion in leather. In combination with the specific catalytic properties towards keratinous substrates, as well as its robustness under alkaline pH conditions and thermostability, SLXX08 keratinase held significant potential for application in the leather industries. Additionally, its high activity toward feather powder substrate endowed it with the capacity for feather degradation. Consequently, SLXX08 keratinase exhibited favorable application value in both feather-degradation application and depilation of the leather industry.

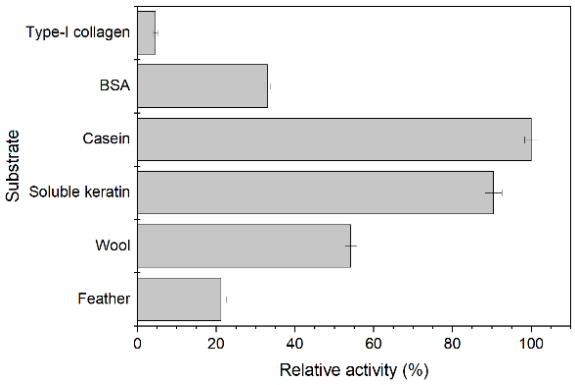

Figure 6. The catalytic activity of SLXX08 keratinase towards various substrates.

Application of crude SLXX08 keratinase

(1) Decomposition and bioconversion of feather

Given the significant quantity of feathers generated annually worldwide, the foremost step in the management and recycling of feathers is the biological decomposition of feathers, which is considered a prospective and sustainable approach for feather management [7]. Additionally, this method features high efficiency and environmental friendliness, in line with the principles of ecological civilization. Due to the pronounced keratinase activity of SLXX08 keratinase, further investigations were carried out on the decomposition and bioconversion of feathers. The results of feather hydrolysis showed that the degradation rate of intact feathers was over 85% after 60 h of treatment with crude SLXX08 keratinase (Figure 7A - 7C). Moreover, during the fermentation, the degradation rate of feathers could reach 83.5% after 72 h. As the hydrolysis time was prolonged to 96 - 100 h, feathers were entirely degraded (Figure 7D - 7F). Considering that the intact feathers were incorporated into the system at the beginning of the fermentation, the decomposition time encompassed both the biomass accumulation and enzymes production phases. Therefore, the decomposition efficiency of the combination of *B. subtilis* SLXX08 cells and the resulting enzyme systems was similar to that of crude SLXX08 keratinase. The reason could be that *B. subtilis* cells contributed to the process of feather decomposition. This is consistent with the previous reports that complete feather degradation occurred in the presence of living cells during the fermentation process [33]. The results demonstrated that both crude SLXX08 keratinase solution and the combination of the bacterial cells and complex enzymes during the fermentation process were capable of degrading intact feathers with high efficiency.

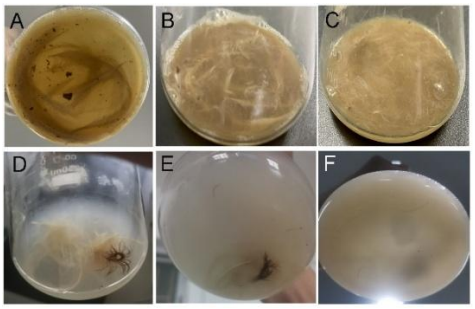

Figure 7. Decomposition of feathers by SLXX08 enzyme. **A.** enzymatic hydrolysis for 12 h by *B. subtilis* SLXX08 crude keratinase. **B.** enzymatic hydrolysis for 36 h by *B. subtilis* SLXX08 crude keratinase. **C.** enzymatic hydrolysis for 60 h by *B. subtilis* SLXX08 crude keratinase. **D.** feather degradation after 12 h during the fermentation process. **E.** feather degradation after 36 h during the fermentation process. **F.** feather degradation after 72 h during the fermentation process.

Feathers are a well-known source of valuable amino acids. Considering the shortage of feed nitrogen sources worldwide, releasing valuable amino acids from feathers is an alternative solution. When microbial keratinases hydrolyze feather waste efficiently under mild conditions, amino acids can be released and utilized as nutrition in the feed industry [9, 34]. Given that SLXX08 keratinase could decompose feathers, the amino acids in the enzymatic hydrolysate of feathers were assayed. The results manifested that SLXX08 keratinase could liberate amino acids from feathers [7]. The feather hydrolysate produced by crude SLXX08 keratinase was abundant in isoleucine, leucine, tryptophan, threonine, phenylalanine, lysine, methionine, and valine (Figure 8). These amino acids are essential for the growth of animals. Moreover, the total amino acid content surpassed 1,200 mg/L. Among them, cysteine (233 mg/L) and leucine (177 mg/L) had relatively high contents and were the principal amino acids in the hydrolysate followed by alanine, valine, phenylalanine, and glutamic acid with their contents varying from 90 to 120 mg/L. The contents of threonine, histidine, and serine were the lowest with all less than 10 mg/L. The contents of the other amino acids were in the range of 15 - 71 mg/L. Therefore, *B. subtilis* SLXX08 keratinase could efficiently recycle the waste of feather keratin and acquire its amino acids for further industrial applications or research.

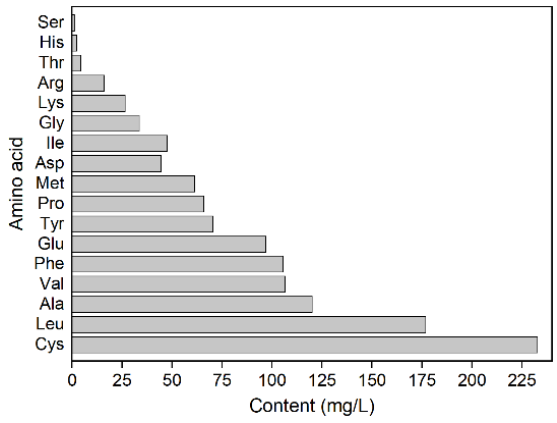

Figure 8. Amino acids types and contents in feather hydrolysate.

(2) Hair removal for leather processing

Dehairing is an essential step in the leather manufacturing industry. Conventional dehairing techniques that rely on chemicals like sodium sulfide lead to pollution-related issues [35]. In contrast, the protease-based dehairing process is eco-friendly and yields favorable products [36]. Alkaline keratinase with relatively low enzymatic activity toward collagen manifests high dehairing efficiency and effectively avoids damage to the leather. Therefore, considering its relatively low collagen activity, the hair removal efficacy of the alkaline SLXX08 keratinase was assayed. The dehairing results demonstrated that SLXX08 keratinase could complete the dehairing of the goatskin within 7 h. The results showed that the wool in the negative control was attached to the goatskin (Figure 9A). In the chemical treatment, the wool had been completely removed (Figure 9B). However, the goatskin appeared darker in color and the texture of the cortex was relatively stiff. In the enzymatic treatment, as could be discerned by the naked eye, the wool had also been completely removed without any residue (Figure 9C). Additionally, the goatskin exhibited a lighter color and the cortex was soft. Microscope examination revealed that the chemical depilation was thorough (Figure 9D). The image of the goatskin depilated by SLXX08 keratinase under stereomicroscope observation showed that the enzymatic depilation was comprehensive. There was no residue remaining on the goatskin, and the surface of the goatskin was smooth, indicating that enzymatic dehairing could eliminate the epithelial cell layer and hair follicles (Figure 9E). The results coincided with the relevant reports. In this process, the effectiveness of alkaline keratinase could be attributed to the fact that alkaline conditions caused the loosening of the attachment of the hair roots and follicles. Subsequently, the keratinase action in alkaline conditions exhibited optimal activity towards the structural constituents including the hair follicle protein and hair root keratin, which facilitated the gentle and efficient removal of hair [11]. Therefore, SLXX08 keratinase had the potential to serve as a substitute for lime-sulfide in dehairing process.

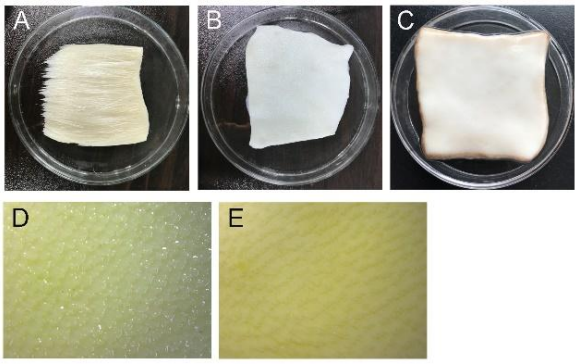

Figure 9. Hair removal by SLXX08 keratinase. **A.** goatskin subjected to water treatment. **B.** chemical depilation of goatskin. **C.** enzymatic depilation of goatskin. **D.** microscopic observation of the goatskin following chemical depilation. **E.** microscopic observation of the goatskin following enzymatic depilation.

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