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Unhairing wastewater treatment by *Bacillus pumilus* and *Bacillus cereus*

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ABSTRACT

Two proteolytic bacteria *Bacillus pumilus* and *Bacillus cereus* were isolated from an aerobic tannery effluent treatment tank. Both strains were characterized and used for the treatment of unhairing wastewater during batch experiments. Chemical oxygen demand (COD), biomass production and protease activity were determined during the incubation period. The maximum COD removals were of 41 and 44% after 10 days incubation in *B. cereus* and *B. pumilus*, respectively. Optimum pH and temperature for protease and keratinase production by both strains were also investigated. Results showed that the optimum temperature and pH of protease production in the cultures of *B. pumilus* and *B. cereus* were of 30°C and 9.0, respectively. Under these optimum conditions, proteolytic activities were 283.8 and 515.8 U/mL in the cultures of *B. cereus* and *B. pumilus*, respectively. Optimum keratinase production by cultures of both bacteria occurred at the same temperature and pH of the protease production.

Keywords: Tannery wastewater; Biological treatment; *Bacillus pumilus*; *Bacillus cereus*; Protease; Keratinase

1. Introduction

Tanning is one of the oldest industries in the world. With human population growth, there is an increasing requirement for leather and leather-based products, leading to the establishment of large commercial tanneries [1]. Tanneries generate wastewaters that contain high levels of salinity, organic loading, inorganic matter, dissolved and suspended solids, ammonia, organic nitrogen and specific pollutants such as sulphides, chromium and other toxic metal salt residues [2].

The tanning process is based on four main steps: beamhouse, tanning, post tanning and finishing [3]. Chrome tanning and unhairing, which occurs in the

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beamhouse process, are the most polluting steps during leather manufacture [4]. In order to overcome the pollution generated by the tanning industry, several treatment processes has been explored. In general, biological treatments of tannery wastewater, such as activated sludge system, have been studied [5]. Thus, activated sludge processes comprise complex biological communities including bacteria, protozoa, fungi and metazoa that contribute to the degradation of organic components in wastewater [6].

The unhairing step removes substances that are not important for the leather production process, such as natural oils and proteins [3], through the addition of lime and sodium sulphide. Unhairing wastewater, therefore, is characterized by an abundance of protein, particularly keratin, which is a component of epidermal and skeletal tissues [7].

Keratinous wastes represent a source of valuable proteins and amino acids, and could find application as fodder additives for animals or sources of nitrogen for cultivation of plants [8]. For this purpose, degradation of the rigid keratin structure and proteins is necessary [9]. Biodegradation of proteins and keratin by micro-organisms possessing proteolytic and keratinolytic activity represents an alternative method for bioconversion of keratin wastes from poultry and leather industries [10]. Proteases produced by microbial sources are important hydrolytic enzymes that have gained importance in industrial sectors [11]. For example, keratinolytic enzymes, which may have important uses in biotechnological processes [10], are generally produced by a wide range of micro-organisms including bacteria such as *Bacillus* species [12,13], actinomycetes [14,15] and fungi [16,17].

The aim of the work paper was to study the biodegradation of unhairing wastewater by *B. pumilus* and *B. cereus,* which were isolated from aerobic bioreactor treating this effluent. As the unhairing wastewater contains a large quantity of proteins, an assessment of proteolytic activity was made. The optimum physico-chemical conditions for production of proteases and keratinases by *B. pumilus* and *B. cereus* were also determined.

2. Materials and methods

2.1. Raw wastewater

Effluent samples were obtained from an unhairing-liming bath at tannery in southern Tunisia. Effluents were filtered through 140 μm mesh sieves to remove hair and pieces of skin. After collection, samples were stored in the dark at 4 ± 1 ℃ until use. The characterization of unhairing wastewater is presented in Table 1.

Table 1
Raw unhairing effluents characteristics

Parameters	Values range
pH	11−12.5
Chemical oxygen demand (COD) (mg/L)	9,000–20,000
Biological oxygen demand (BOD) (mg/L)	3,200–7,600
Suspended solid (SS) (mg/L)	4,500–8,100
Total solids (TS) (mg/L)	16,100–8,800
Fat (mg/L)	40–155
Total Kjeldahl nitrogen (mg/L)	1,100–3,250

2.2. Inoculum

2.2.1. Micro-organisms

The bacteria used in this work were isolated from an activated sludge inoculated reactor used to treat unhairing wastewater and from the endogenous biomass. The organisms were strain BA6-3 (16 S rRNA gene accession number JQ712508) and strain BE4-3 (16 S rRNA gene accession number JQ712503).

2.2.2. Genomic DNA isolation

Genomic DNA from cultivable bacteria was isolated by a simple boiling protocol. Small quantity (1/2 loop) of bacteria growing in LB medium was homogenized in 50 μl sterile MQ water, boiled at 96 ℃ for 10 min, centrifuged at 17,000 rpm for 5 min and the pellet used as a PCR template.

2.2.3. PCR amplification and phylogenetic analysis

Two sets of oligonucleotide primers were used to amplify the bacterial 16S rRNA genes. To identify culturable bacteria, PCR was performed using primers fD1 and rP2 [18]. The reaction mixture of 25 l consisted of 0.2 mM dNTPs, 0.5 μM of each primer, 4 ng template DNA, 2 units of recombinant *Taq* DNA polymerase (MBI Fermentas, Vilnius, Latvia) and 1 × PCR buffer amended with 2 mM $MgCl_2$. The presence and yield of PCR products were determined in 1% agarose gel run at 80 V for 2 h in 0.5 × TBE buffer, and stained with ethidium bromide.

Phylogenetic analysis was done using SEQMATCH of Ribosomal database project II (RDP).

2.3. Media and culture conditions

B. pumilus and *B. cereus* were routinely cultivated in 25-mL Luria–Bertani broth medium (comprising g/L: peptone10; yeast extract, 5; NaCl, 5) in 250 mL Erlenmeyer flasks. Flasks were incubated on rotary shaker

(180 rpm) at 37˚C for 24 h. The substrate used in the experiments was unhairing wastewater, with the initial pH was adjusted to 7.0 prior to sterilization [19]. Batch cultures were prepared in 500 mL Erlenmeyer flasks containing 150-mL substrate. The inoculum size was 10% (v/v). Cultures were incubated on a rotary shaker (180 rpm) for 12 days at 32˚C. The culture medium was centrifuged at 10,000 rpm for 5 min at 4˚C, and the cell-free supernatant was used to estimate protease and keratinase activities.

2.4. Enzyme assays

2.4.1. Protease activity

Protease activity was determined according to Kembhavi et al. [20], using casein as the substrate. A 0.5 ml aliquot of the culture supernatant, suitably diluted, was mixed with 0.5 mL of 100 mM Tris- HCl (pH 7.0) containing 1% (w/v) casein and incubated for 15 min at 50˚C. The reaction was stopped by the addition of 0.5 mL of trichloroacetic acid (TCA; 20%, w/v). The mixture was allowed to stand at room temperature for 15 min before centrifuging at 13,000 rpm for 15 min to remove the precipitate. Absorbance of the acid-soluble material was measured spectrophotometrically at 280 nm. A control assay, without the enzyme, was performed and used as a blank in all spectrophotometric measurements. A standard curve was generated using solutions containing 0–50 mg/L tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 g of tyrosine per minute under the experimental conditions given above. Protease activities reported represent the means of at least two determinations.

2.4.2. Keratinolytic activity

Keratinolytic activity was assayed with soluble keratin as a substrate, according to the method of Takiuchi et al. [21]. An appropriately diluted enzyme solution (0.5 mL) was mixed with 0.5 mL of 100 mM glycine-NaOH buffer (pH 9.0) containing 0.8% (w/v) hoof and horn keratin (Sigma-Aldrich). After 1 h of incubation at 55˚C, the enzyme reaction was stopped by adding 0.4 mL of 10% (w/v) TCA before centrifuging at 10,000 rpm for 15 min at 4˚C. The absorbance of the supernatant was measured at 280 nm against a control. One unit (U) of keratinolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance at 280 nm of 0.1 under the above conditions. The data presented are mean values of two parallel determinations.

2.5. Effect of initial medium conditions for enzyme production

2.5.1. Effect of incubation temperature

To investigate the effect of temperature on keratinolytic and proteolytic activities using unhairing wastewater medium, *B. cereus* and *B. pumilus* were cultivated at a range of temperatures between 20 and 45˚C. For shaken cultures, 120 mL of the unhairing wastewater at an initial pH of 7.0 and initial COD of 9 g/L was dispensed into 500 mL Erlenmeyer flasks 12 mL inoculum added. Cultures were incubated on a rotary shaker at 170 rpm for 10 days before centrifuging at 13,000 rpm for 15 min. Supernatants were used as crude enzyme preparations.

2.5.2. Effect of initial pH

The effect of pH on protease and keratinase production was investigated by varying the unhairing wastewater pH between 5.0 and 12.0. Batch cultures were prepared in 500 mL Erlenmeyer flasks containing 120 mL of unhairing wastewater, which have initial COD 9 g/L, and 12 mL of inoculum, with incubation and processing as described above. During the experiments, the initial pH of unhairing effluents was adjusted to 5.0, 7.0, 9.0 and 12.0 by adding HCl solution (10 N) for pH 5.0, 7.0 and 9.0 or NaOH solution (10 N) for pH 12.0, after that adequate buffer was added. The following buffer systems were used to adjust pH: 100 mM sodium acetate buffer, pH 5.0–6.0; 100 mM Tris-HCl buffer, pH 7.0–8.0; 100 mM glycine–NaOH buffer, pH 9.0–10.0; 100 mM KCl-NaOH, pH 12.0–13.0. Cultures were incubated at 30 ˚C on a rotary shaker at 170 rpm for 10 days before centrifuging at 13,000 rpm for 15 min. Supernatants were used as crude enzyme preparations.

2.6. Analytical methods

The measurement of COD, BOD, TS, SS, fat, total kjeldahl nitrogen (TKN) and volatils suspended solids (VSS) reflecting the biomass concentration were performed using standard methods [22]. Each analysis was performed in triplicate and mean values presented as results.

3. Results and discussions

3.1. 16S rRNA gene sequence analysis of the bacterial isolates

All PCR products were sequenced and results processed by a Blast search of the GenBank database (www.ncbi.nlm.nih.gov.cn/BLAST). Homology

searches revealed that most strains were related to members of the genus *Bacillus* in both reactors. Phylogenetic analysis showed that strain BA6-3 was closely related to *Bacillus pumilus* (T); ATCC 7061; with a 16 S rRNA gene sequence similarity value of 99%, while strain BE4-3 was closely related to *Bacillus cereus* ATCC 14579 with a 16 S rRNA gene sequence similarity value of 99.

3.2. Batch unhairing wastewater treatment

3.2.1. COD removal efficiency

Fig. 1 depicts the COD removal during batch treatment of unhairing wastewater. After two days of inoculation, the initial COD of 9.45 g/L was decreased to 7.14 and 6.05 g/L for *B. pumilus* and *B. cereus* cultures, respectively (Fig. 1(a)), corresponding to COD removals of 24.44 and 35.98%, respectively. COD removal efficiency increased from the 2nd day to the 10th day of incubation, reaching 41% after 10 days of treatment with *B. cereus*. In *B. pumilus* cultures an increase in the degradation of organic matter occurred until 10 days after inoculation, when COD removal was 44% (Fig. 1(b)). From day 10 to the end of the incubation period, COD removal was stable in *B. pumilus* culture, but decreased slightly to 36.5% at the end of treatment with *B. cereus*. This decrease was probably due to an accumulation of substances inhibitory to bacterial growth.

3.2.2. Biomass growth

Two days after the inoculation of the wastewater, VSS concentration increased from 1 g/L to 2 and 2.5 g/L in *B. cereus* and *B. pumilus* cultures, respectively (Fig. 2). During the stationary phase, from the 8th to the 10th day, the biomass growth was stable and the average values of VSS were of 3.59 and 3.75 g/L for *B. cereus* and *B. pumilus* cultures, respectively. These results suggested that *B. pumilus* gave the best growth under these conditions and had, as a consequence, the most potential to reduce the COD during the 10-day treatment period (Fig. 1). The decrease in VSS concentration may result from a decline in viable cell numbers [23] and explains the decrease in COD removal later in the treatment processes in the two bacterial cultures. These results are in agreement with those of Mlaik et al. [24] who indicated that the decrease of biomass concentration leads to the decrease of COD during batch treatment of unhairing effluent.

3.2.3. Protease activity

Protease activity increased progressively over the first 10 days of incubation in both the *B. cereus* and *B. pumilus* cultures (Fig. 3). The increase of protease activities from the second day of incubation can be due to the production of different proteases by *B. pumilus* and *B. cereus* in the both cultures.

At day 8 of incubation, activity was 315 U/mL for both bacteria. Highest activities occurred at day 10, when 445 and 571 U/mL were measured for *B. cereus* and *B. pumilus*, respectively. Subsequently, protease activity decreased rapidly in cultures of the two bacteria. During treatment, the kinetics of enzyme activities in cultures of the two bacteria were similar, but overall, there was greater activity in *B. pumilus* cultures.

3.3. Effect of initial temperature and pH on production of protease and keratinase

3.3.1. Effect of cultivation temperature

Proteases from both *B. pumilus* and *B. cereus* were active at temperatures from 25 to 45°C with an optimum temperature at approximately 30°C (Fig. 4(a)). After 10 days of incubation, the optimum protease activities were 415 and 255.63 U/mL in the *B. pumilus* and *B. cereus* cultures, respectively. Thus, proteolytic activity of *B. pumilus* was higher than that of *B. cereus* during the degradation of unhairing wastewaters, whereas the two bacterial cultures had similar kinetics of enzyme production. Under different conditions for inducing proteases in *B. cereus*, protease activity was 127 U/mL in starch, soybean meal and calcium carbonate medium [25].

Keratinases produced in cultures of both bacteria were active from 25 to 45°C, with optimum activity after 10 days incubation at approximately 30°C (Fig. 4(b)). At optimum temperature, keratinase activities were approximately 4.28 and 8.85 U/mL in *B. pumilus* and *B. cereus* cultures, respectively. These results were similar to those reported by Fakhfakh-Zouari et al. [26], who showed that highest keratinolytic activity levels of *B. pumilus,* incubated in a rotary shaker (200 rpm) for 24 h at 37°C, were obtained with chicken feather meal (25.92 U/mL) and a hair-derived keratin source resulted in a relatively weak enzyme production of 4.3 U/mL.

It is noted that protease and keratinase activities occurred at the same optimum temperatures, but keratinolytic activity was significantly lower than protease activity.

3.3.2. Effect of medium's initial pH on protease and keratinase production

B. pumilus and *B. cereus* were grown in the medium containing unhairing wastewater, the pH

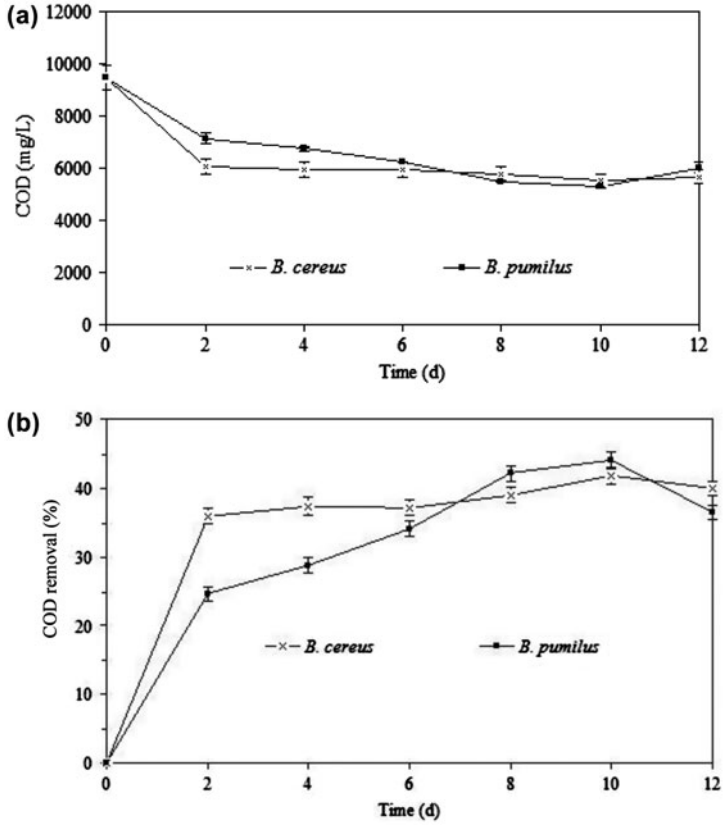

Fig. 1. Effects of treatment of unhairing wastewaters with *B. cereus and B. pumilus* on (a) COD of treated effluent and (b) COD removal efficiency. (Error bars represented the standard errors of the mean).

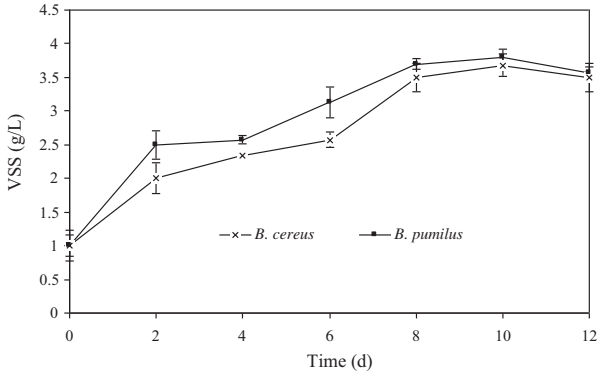

Fig. 2. Changes in VSS during treatment of unhairing wastewater by *B. cereus and B. pumilus.*

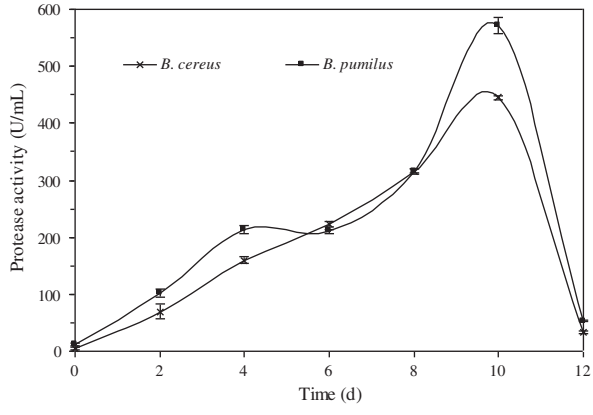

Fig. 3. Changes in protease activity during batch treatment of unhairing wastewater by *Bacillus cereus* and *B. pumilus.* (Error bars represented the standard errors of the mean).

values of which was adjusted to 5.0–12.0 with appropriate buffer solutions prior to sterilization. After 10 days of incubation, both protease and keratinase activities were determined. Higher protease activities were observed at pH 7.0 and 9.0, but in both cultures the optimum activity was at pH 9.0 (Fig. 5(a)). In contrast, El-Refai et al. [27] showed that an isolate of *B. pumilus* designated strain FH9 produced protease with an

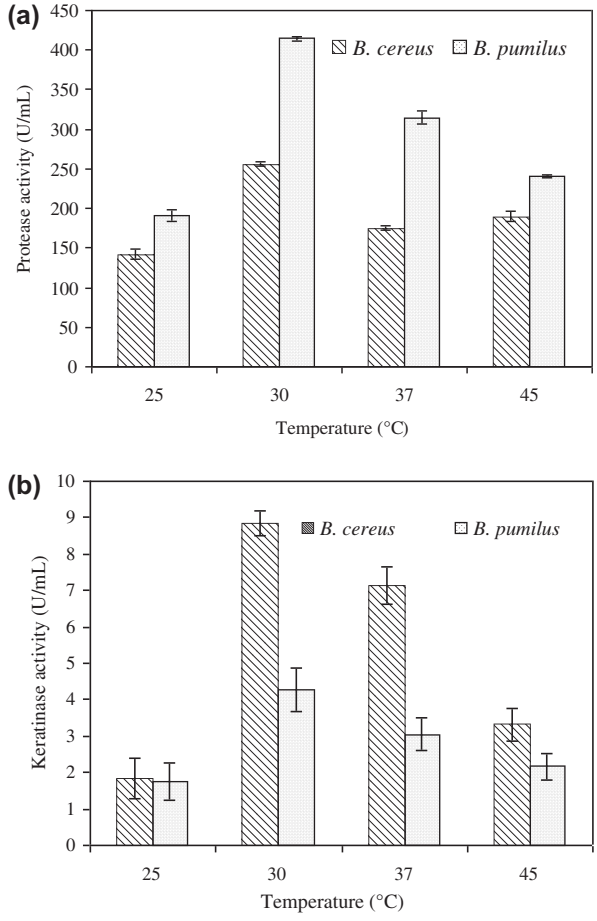

Fig. 4. Effect of temperature during batch treatment (operating condition pH 7; COD = 9 g/L; 170 rpm) on: (a) protease activity; (b) keratinase production by *B. cereus* and *B. pumilus*. (Error bars represented the standard errors of the mean).

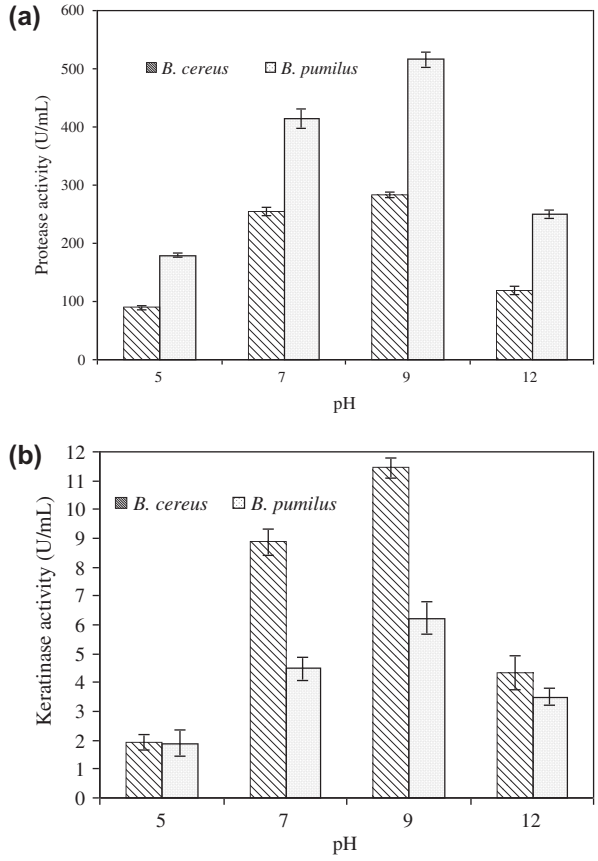

Fig. 5. Effect of pH on (a) protease and (b) keratinase production by *B. pumilus* and *B. cereus* during unhairing wastewaters treatment (Operating condition: Temperature = 30°C; COD = 9 g/L; 170 rpm). (Error bars represented the standard errors of the mean).

optimum pH of 8.0 when grown in a basal medium containing 1% chicken feather meal.

Under optimum pH conditions, the protease activities were 283.8 and 515.8 U/mL for *B. cereus* and *B. pumilus*, respectively. Although *B. pumilus* produced higher protease value after 10 days of incubation compared to *B. cereus*, the behaviour of *B. cereus* and *B. pumilus* proteases over the pH range tested were similar.

The optimum initial medium pH value for keratinase production by both bacteria was also 9.0 (Fig. 5(b)). Under these conditions, keratinase activities were 11.44 and 6.23 U/mL in *B. cereus* and *B. pumilus* cultures, respectively. Thus, *B. cereus* produced more keratinase than *B. pumilus*. The production of keratinase by both bacteria was considerably suppressed in the lower pH range.

4. Conclusion

In this study, unhairing wastewater was treated during batch assay by *B. pumilus* and *B. cereus* cultures, which were isolated from the reactors treating this effluent. The maximal performance of wastewater treatment was obtained after 10 day of incubation and can be explained to the maximum growth of *B. pumilus* and *B. cereus*.

At 10th day of incubation, COD removal was 41 and 44% and protease activities were 445 and 571 U/mL in *B. cereus* and *B. pumilus* cultures, respectively.

Optimal culture conditions for protease and keratinase production by *B. pumilus* and *B. cereus* had an initial pH value of 9 and an initial temperature of 30°C. Under these conditions, keratinase activities were 11.44 and 6.23 U/mL in *B. cereus* and *B. pumilus* cultures, respectively. Keratinase production by both bacterial cultures was very weak in the presence of unhairing wastewater as the keratin source.

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