Isolation and Molecular Identification of Keratinase-Producing Bacteria from the Sludge of Qeshm Island

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ABSTRACT

Background and Objectives: Keratinase is an enzyme commonly used in the production of detergents, cosmetics, drugs, leather, and other industries. Considering the high cost of traditional methods for decomposition of feather, hair, hooves, nails, and wool that contain high levels of keratin, their biodegradation with keratinase-producing bacteria can be a valuable solution. The present study aimed for isolation and molecular identification of keratinase-producing bacteria in Qeshm Island and Peyposht village in Iran.

Methods: Water and sludge samples from the Qeshm Island and Peyposht village were collected. The bacteria isolates were screened for keratinase production using the Lowry method. Effect of pH and temperature was assessed on the production of keratinase and on the growth of the isolates. Colony-polymerase chain reaction was used for molecular identification of the isolates.

Results: *Bacillus berevis* and *Enterobacter cloacae* were isolated in this study. Keratinase production in *B. berevis* was highest at pH 7.5 and 35 °C. In addition, the highest level of enzyme production by *E. cloacae* was observed at pH 7 and 37 °C.

Conclusion: It seems that the bacterial strains isolated from sludge in the study area have relatively favorable keratinase production capacity.

Keywords: Bacteria, Colony PCR, Identification, Keratinolytic protein, Sewage.

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INTRODUCTION

Keratinase is an extracellular protease that decomposes scleroprotein, keratin. Microbial keratinases are alkaline or neutral proteases that functions best in pH of 7.5-9 and at 40-85 ℃ (1). The most important producers of keratinase include fungi (*Microsporum, Trichophyton, Aspergillus flavus* and *Aspergillus fumigatus*), bacteria (*Bacillus pumilus, Bacillus cereus, Bacillus licheniformis* and *Bacillus subtilis*), and *Actinomycetes* (*Streptomyces albidoflavus* and *Streptomyces pactum*) (2-5) that are capable of hydrolyzing insoluble keratin. Most keratinolytic strains are able to decompose feather keratin within 48h of keratinase production, indicating the potential applicability of the enzyme for bioconversion of keratin wastes into valuable products (6). Keratinous waste is widely generated by various industries. Feathers are byproducts of poultry production that can provide suitable conditions for the growth of anaerobic or aerobic bacteria if not removed from the environment (7, 8). Thus, it is necessary to investigate the production of keratinase with particular emephisis on enzyme extraction technology at industrial scale. We aimed to study the isolation and molecular identification of keratinase-producing bacteria in Qeshm Island and Peyposht village in Iran.

MATERIAL AND METHODS

Water and sludge samples collected from separate beaches of Peyposht and Qeshm were transferred to the laboratory of Islamic Azad University of Falavarjan, Iran. Biologocal oxygen demand (BOD) and chemical oxygen demand (COD) were measured as indicators of water quality.

In order to prepare feather powder, raw chicken feathers were cut into small pieces (1-3 cm) and then rinsed with water several times. Next, 500g of the pieces were placed in 1L of 1:1 chloroform-methanol mixture (v/v) for two days. The pieces were then placed in a 4:3:1 chloroform-acetone-methanol mixture (v/v) for two days for degreasing. In order to remove residual solvents, the small feather pieces were washed with distilled water several times, rinsed and dried for three days at 60 °C. Finally, the pieces were powdered using a home mill and then kept in a capped sterile bottle until use (2).

In order to isolate keratinase-producing bacteria, 10 ml of the samples along with 2 to 3 chicken feathers were added to 90ml of distilled water and then stored at 30 ℃ in a shaking incubator (at 180 rpm) for 24 hours. Serial dilutions (10^{-1}-10^{-6}) were prepared from the samples. Then, 100 ml of each dilution was added to FMB medium containing 0.1 g/L yeast extract, 0.5 g/L NaCl, 0.5 g/L NH_4Cl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.1 g/L $MgSO_4$, 10 g/L feather powder, and 0.0001 g/L cyclohexamide. Bacterial growth was assessed after transferring 1ml of sample-FMB mixture was to FMA (solid feather culture medium with 2% agar). In order to obtain colonies, the bacteria were first cultured on nutrient agar by streaking and then incubated at 37 ℃ for 24h (9, 10). After characterization with Gram staining and morphology, the colonies were transferred and incubated in skim milk agar to separate hemolytic bacteria (11). Keratinase-producing isolates were cultured in liquid FMB medium containing 100 g of powdered feathers for 72h. After filtration through a Whatman paper, the medium containing the enzyme was centrifuged at 3500 rpm for 30min. Next, 1 ml of supernatant was mixed with 1 ml of 50 mM Tris-HCl buffer (Merck, Germany) at pH 8 and 1 ml of 0.5% soluble keratin (Sigma, USA). After heating at 50 °C for 10 min and then adding 2 ml of 0.4M CH_3Cl_3 (Sigma, USA), the mixture was centrifuged at 3500 rpm and for 10 min at 4°C. Optical density (OD) of the solution was read at 280nm against a control prepared by mixing 2 ml of 0.4M TCA with 1 ml of enzyme extract (0.01U) (12). Lowry protein assessment kit was used to measure total protein, and standard curve was plotted using different concentrations of BSA. To determine the optimal pH for the growth of bacteria, FMB medium was prepared at different pH values (6.4, 6.8, 7, 7.2 and 7.5). A colony of bacteria was added to shake flasks (at 180 rpm), which were later incubated at 30 ^{c}o. Serial dilutions (10^{-1}-10^{-6}) were prepared in six tubes containing 9 ml of sterile distilled water. After 24h of incubation at 30 ˚C, the number of colonies was counted. To determine the optimal growth temperature, a bacterial colony was inoculated in FMB medium (pH 7.5). The flasks were placed in a shaking incubator at 180 rpm for three days at various temperatures

(28, 30, 32, 34 and 36 °C) for 12, 24, 48, 72 and 96 hours. In order to isolate keratinase-producing bacteria, 10 ml of the samples along with 2 to 3 chicken feathers were added to 90ml of distilled water and then stored at 30 ℃ in a shaking incubator (at 180 rpm) for 24 hours. Serial dilutions (10^{-1}-10^{-6}) were prepared from the samples. Then, 100 ml of each dilution was added to FMB medium containing 0.1 g/L yeast extract, 0.5 g/L NaCl, 0.5 g/L NH_4Cl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.1 g/L $MgSO_4$, 10 g/L feather powder, and 0.0001 g/L cyclohexamide. Bacterial growth was assessed after transferring 1ml of sample-FMB mixture was to FMA (solid feather culture medium with 2% agar).

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To determine the optimal growth temperature, a bacterial colony was inoculated in FMB medium (pH 7.5). The flasks were placed in a shaking incubator at 180 rpm for three days at various temperatures (28, 30, 32, 34 and 36 °C) for 12, 24, 48, 72 and 96 hours. FMB liquid medium with a pH range of 5-9 was prepared by addition of HCl or NaOH. After inoculation of bacteria into the culture medium with different pH values, each tube was placed in a shaking incubator for 72h at 37 ℃. A sample was taken from each tube for analysis of keratinase production at different temperatures (20, 25, 30, 37, 40 and 45℃) (12).

For molecular identification of keratinase-producing bacteria, polymerase chain reaction was performed universal primers with the following sequences: (forward) 5’AGGAGGTGATCCAACGCA3’ and (reverse) 5’AACTGGAGGAAGGTGGGGAT3’ for gram positive bacteria and (forward) 5’CCATTGTAGCACGTGTGT3’ and (reverse) 5’CCATTGTAGCACGTGTGT3’ for gram negatives bacteria (13). Cycling conditions were as follows: initial denaturation at 95 ℃ for 5 min, denaturation at 94 ℃ for 30 sec, annealing at 50 ℃ for 30 sec, extension at 72 ℃ for 70 sec, and final extension at 72 ℃ for 5 min. After performing colony PCR and gel electrophoresis, the results were interpreted and evaluated on the NCBI database.

RESULTS

As shown in table 1, pH of both samples was in the acidic range with a minor difference. BOD of the sample collected from Peyposht was higher than that of the Qeshm Island.

Table 1-Biological properties of the sludge samples collected from beaches in Qeshm

Sampling site	Temperature (℃)	pH	BOD (mg/L)	COD (mg/L)
Qeshm	33	5	760	1510
Peyposht	31	6	930	1434

PCR products were identified using a 50 bp DNA ladder and then were sent for sequencing. The samples from Qeshm had 99% similarity with 16S rRNA of *B. berevis*, while the sequence of samples from Peyposht had 96.5% similarity with *E. cloacae*. The optimal pH for the growth of *B. berevis* and *E.cloacae* was 7.2 and 7.5, respectively. These bacteria were also able to grow at 28, 30, 32, 34 and 36 ℃. Nevertheless, the optimal temperature for the growth of *B. berevis* and *E. cloacae* in FMB was 32 ℃ and 34 ℃, respectively.

Keratinase production by the two bacteria was measured in the FMB medium with different pH values. Maximum keratinase production (71.24 U/mL) by *B. berevis* was observed in pH 7.5 (Figure 5). In addition, maximum keratinase production (52.29 U/mL) by *E. cloacae* was observed in pH 7 (Figure 6).

Figure 1- Growth of *B. berevis* in different pH ranges

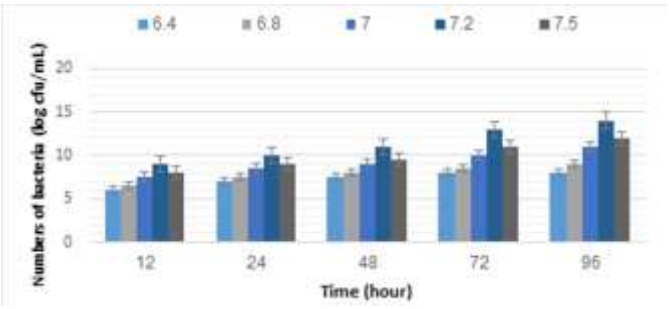


Figure 2- Growth of *E. cloacae* in different pH ranges

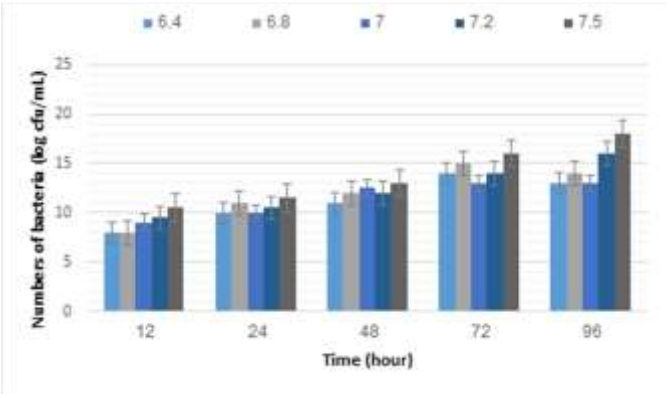


Figure 3- Growth of *B. berevis* at different temperatures

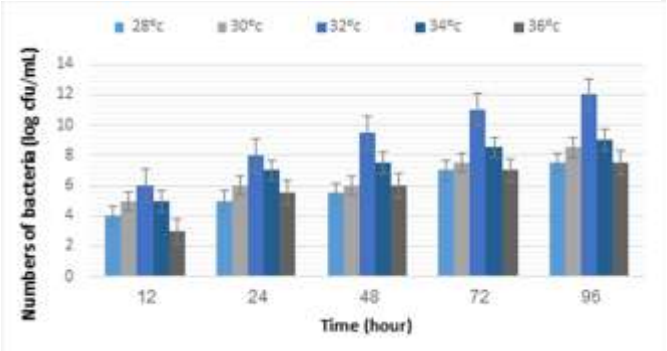
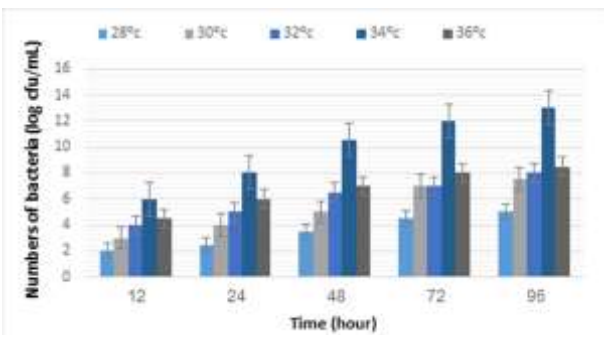


Figure 4- Growth of *E. cloacae* at different temperatures



Keratinase production by the two bacteria was measured in the FMB medium with different pH values. Maximum keratinase production (71.24 U/mL) by *B. berevis* was observed in pH 7.5 (Figure 5).

In addition, maximum keratinase production (52.29 U/mL) by *E. cloacae* was observed in pH 7 (Figure 6). We also measured keratinase production by the two bacteria in different temperatures. Maximum keratinase production (59.4 U/mL) by *B. berevis* was noted at 35 ℃, while minimum enzyme production (14.07 U/mL) was observed at 20 ℃ (Figure 7). Moreover, maximum enzyme production (68.88 U/mL) by *E. cloacae* was recorded at 37 ℃, while minimum enzyme production (25.29 U/mL) was observed at 20 ℃ (Figure 8).

Figure 5- Keratinase production by *B. berevis* after 72 hours of incubation at 37 ℃ in different pH values

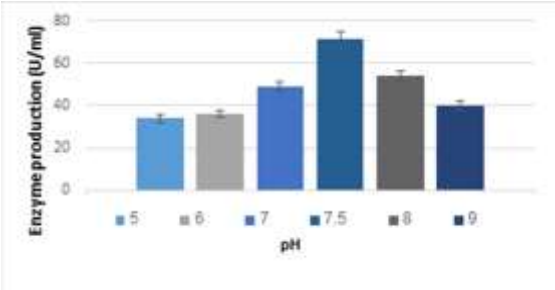


Figure 6- Keratinase production by *E. cloacae* after 72 hours of incubation at 37 ℃ in different pH values

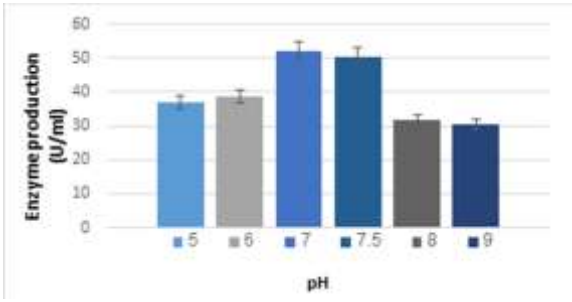


Figure 7- Keratinase production by *B. berevis* at different temperatures (after 72 hours)

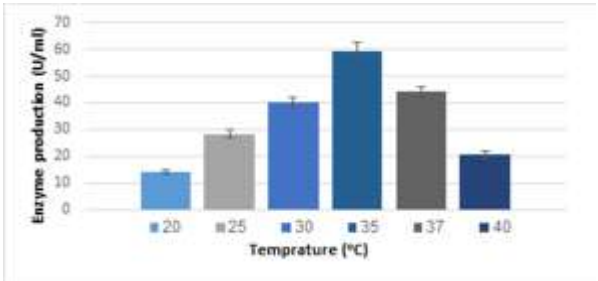
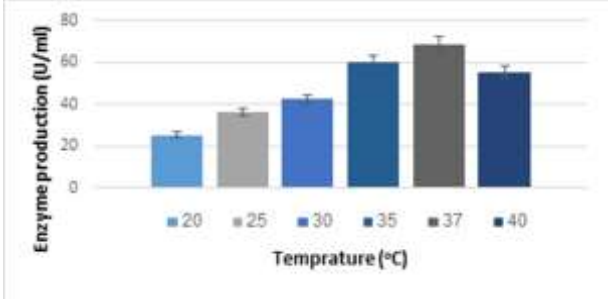


Figure 8- Keratinase production by *E. cloacae* at different temperatures (after 72 hour)



The protein content of *B. berevis* and *E. cloacae* at 650 nm was 0.649 mg/mL and 0.669 mg/mL, respectively.

DISCUSSION

Today, environmental water pollution and the increasing amount of industrial wastewater poured into waters require proper management. Due to its insoluble nature, keratin is usually resistant to degradation by proteolytic enzymes such as pepsin, trypsin and papain. Keratin-degrading microorganisms can grow in various environments such as soil, air, forage, and waste (14). In this study, we aimed at isolation and molecular identification of bacterial strains from sludge samples collected from Peyposht and Qeshm, which have high quantities of keratin-rich waste.

In a study by Tork et al., 7 of 23 bacterial isolates with bright halos on skim milk agar were identified as keratinase producer (15). A study by Sangali and Brandli reported the keratinolytic activity of *Vibrio sp.* strain *KR2* in a medium containing raw feather (16). In 2007, Joshi et al. isolated 3 keratinolytic *Bacillus sp.* from the Amazon River, which had 90% similarity with the 16S rRNA sequence of *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis* (17). On the contrary, some studies relied on the biochemical detection and isolation of keratinolytic bacteria. For instance, Pandian et al. found colonies with high keratinase production by using the Bergey's Manual (18). However, we identified the isolates by microscopic and macroscopic examinations and based on the 16S rRNA sequence of the isolates. The results showed that the isolates from the Qeshm beach are most likely (96.5%) *B. berevis*, while the isolates from the Peyposht village are likely to be (99%) *E. cloacae*. We also determined the optimal pH and temperature for the growth of these isolates. In study of Sangali and Brandeli, the optimal growth of keratinolytic *Vibrio sp.* strain *KR2* was observed at pH=7 and 30 ˚C (16).

The isolated bacteria could grow at 28, 30, 32, 34 and 36℃ but the optimal growth temperature after 96 hours for *Bacillus* and *Enterobacter* was 32 ℃ and 34℃, respectively.

In this study, the amount of BOD and COD of sludge of water taken from the Qeshm beach and Peyposht village was notably higher than the standard value, indicating the high and hazardous level of contamination in these areas. Higher COD values compared to BOD_5 is observed when toxic compounds are present in the wastewater, which inhibits the activity of organisms that degrade organic materials.

In this study, we examined the effect of pH on keratinase production by *B. berevis* and *E. cloacae*. The results showed that maximum keratinases production by *B. berevis* (71.24 U/ml) and *E. cloacae* (52.29 U/ml) is at pH 7.5 and 7, respectively. Nadia et al. reported that the optimal pH for keratinase production in *B. berevis* is 7.5, 8 and 9 (19). Kim et al. also claimed that keratinase production in *B. cereus, B. subtilis* and in *B. pumilus* is highest at pH 7, 5-9, and 5-6, respectively (20).

The highest and lowest level of keratinase production in *B. berevis* was observed at 35 ˚C and 20 ˚C, respectively. However, Nadia et al. reported 40 ˚C as the optimal temperature for keratinase production (19). This difference can be related to physicochemical conditions of the two studies and the genus of bacteria examined.

In 2009, Xu et al. measured the amount of soluble proteins produced by *B. licheniformis K-19* using folin phenol and bovine serum albumin. After 36 hours of incubation at 37 ˚C, the concentration of soluble protein was almost 3 mg/ml (21).

Matikeviciene et al. studied the level of keratinase production in some *Bacillus* species at 565 nm and using the Lowry method. According to their findings, *B. subtilis*, *B. licheniformis*, *B. subtilis*, and *B. pumilus* produced 1.23, 1.17, 1.16, and 1.05 mg/ml keratinase, respectively (22). However, in the present study, the amount of enzyme produced by *B. berevis* and *E. cloacae* was 0.649 mg/ml at 650 nm, respectively. This inconsistency could be attributed to the difference in bacterial species and the wavelength used for measurement of keratinase production.

CONCLUSION

Keratin waste is a favorable environment for the growth of keratinase-producing bacteria. Most of these bacteria are of *Bacillus* genus since they can produce spore and survive adverse environmental conditions. The optimal pH for the growth of keratinase-producing bacteria is in the neutral to acidic range, while the optimal pH range for keratinase production

is in the neutral to alkaline range. In addition, the optimal temperature for the growth of bacteria and keratinase production is 32-34 ℃ and 35-37 ℃, respectively.

The bacteria isolates in our study were gram-negative spore-forming *Bacillus sp.* with high enzyme production.

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CONFLICT OF INTEREST

None declared.

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