Isolation and molecular identification of keratinase-producing bacteria from the sludge of Qeshm Island

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ABSTRACT:
Keratinase enzyme in the production of detergents, cosmetics, pharmaceuticals, leather industry, and dietary supplements production is used by livestock, and also in newer fields such as the analysis of prions for Bovine Spongiform Encephalopathy (BSE) treatment is used. Since the use of traditional methods for decomposition of feathers, hair, hooves, nails, and wool that contain high levels of keratin, is costly and leads to elimination of amino acids, biodegrading of the mentioned cases by bacterial keratinase can be a valuable solution. The aim of this study was, isolation and molecular identification of bacteria in Qeshm Island and Peyposht village that were able to break down keratin. For this purpose, water and sludge samples from Qeshm Island and Peyposht village were collected. After enrichment of bacteria, screening enzyme-producing species keratinases was performed for isolates by Lowry method, in the next phase, the effect of pH elements and temperature on production of keratinase enzyme as well as the growth of microorganisms was examined, and then for molecular identification of the isolates, colony-PCR method, were used. Bacillus berevis and Enterobacter cloacae bacteria were isolated in this study. Keratinase enzyme derived from Bacillus berevis at pH=7.5 and a temperature of 35 ° C and keratinase enzyme derived from Enterobacter cloacae at pH=7 and 37 ° C temperature showed the highest keratinase activity. By considering the great needs of the country in various industries to industrial enzymes, it seems that the use of local strains can be effective in making the country needless import this product.

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

Keratinase is an extracellular protease that decomposes Scleroprotein keratin. Most Keratinases reported are serine protease. Microbial keratinase are alkaline or neutral proteases that pH range is 7.5-9 and optimal temperature 40-85°C. Keratinases are produced only in the presence of substrates containing keratin and mainly attack to the disulfide bonds of keratin (1). The most important producers of keratinase enzyme 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, 3, 4, 5). By producing keratinase, these microorganisms are capable of hydrolysis of insoluble keratin.

Most of the keratinolytic strains are able to decompose feather keratin widely within 48 hours by producing keratinases enzyme and this show the ability of keratinase enzyme to bioconvert keratin wastes into valuable products (6, 3).

The huge increase of livestock wastes leads to production of a large amount of feathers, hair, wool, horns, nails, and body waste as byproducts. This keratinous waste is mass-produced by various industries such as dairy farms, poultry farms, tanneries, and slaughterhouses. Millions of tons of feathers as a byproduct (more than 90%) are producing by paltry farms. If measures are not taken to remove them from nature, it can be provided for bacteria growth especially anaerobic ones. Decomposing keratin lead to increase toxic substances such as hydrogen sulfide and ammonia in the environment and it causes health problems in human societies (8, 7). Thus, it is necessary that the process of research regarding the production of keratinase enzymes continue, so that localizing the field of enzyme extraction technology on an industrial scale be provided in the future. The aim of this research was isolation and molecular identification of keratinas-producing bacteria from the sludge in Qeshm Island.

MATERIAL AND METHODS

Samples including water and sludge were collected from separate beaches of Peyposht and Qeshm and transferred to research lab of Islamic Azad University of Falavarjan immediately in sterile containers. BOD and COD indicators were used to determine water quality. BOD was analyzed with BOD track method and COD was analyzed with classic method.

In this study, feather powder was used to isolate keratinase-producing bacteria as a source of carbon and energy. To prepare feather powder for main culture medium, raw feathers of chicken were cut into small pieces as long as 1-3 cm and rinsed with water several times. Then 500 grams of cut pieces were placed in a mixture of 1000 ml of chloroform and methanol in a ratio of (1:1v/v) for 2 days, and then in a mixture of chloroform, acetone, and methanol in a ratio of (4: 3: 1 v/v) for two days for degreasing. In order to remove residual solvents, small pieces of feather were washed with distilled water several times, rinsed and dried for 3 days at 60 °C. Finally, using home mills, feathers were turned into powder and kept in a capped sterile bottle for use in the original cultivation medium (2).

In order to isolate keratinase-producing bacteria, 10 ml of the sample (sludge water of Qeshm Island and Peyposht) with 2 to 3 feathers were added to 90 ml of distilled water and kept for 24 hours at a temperature 30 °C in a shaking incubator at 180 rpm. Serial dilution was prepared from samples. Then 100 ml of dilutions $10^{-5}$ and $10^{-6}$ was transferred to FMB medium (feather meal broth). This medium includes 0.1 g/l of yeast extract, 0.5 g/l of sodium chloride, 0.5 g/l of ammonium chloride, 0.3 g/l of dipotassium hydrogen phosphate, 0.4 g/l of potassium dihydrogen phosphate, 0.1 g/l of magnesium sulphate, 10 g/l of feather powder, and 0.0001 g/l of cyclohexamide. After transferring 1 ml from FMB to FMA that was solid feather culture medium with 2% agar (Merck, Germany), bacterial growth was
observed. To preserve the colonies, bacteria was cultured on the N.A medium (Merck, Germany) by linear method and then was placed at a temperature of 37°C for 24 hours (10, 9).

In order to examine the colonies, after Gram staining and ensuring its purity, characteristics of the colonies in terms of morphology, such as size, color, colony profile, and their states were examined, and each of the different colonies was purified using linear culturing on FMA medium (feather meal agar). Then they were moved to skim milk agar medium (Merck, Germany) and incubated for 24 hours at 37°C temperature, and bacteria that had created a clear halo on the medium were selected for the next stage (11).

For the measurement of enzyme activity, keratinase-generating isolates were cultured in liquid FMB medium and after 72 hours of incubation, feathers in the medium were taken out of the medium by Whatman paper and then the resulting culture medium containing the enzyme was centrifuged for 30 minutes at 3500 rpm. 1 ml of the supernatant from the centrifuge was mixed in 1 ml buffer 50 mM Tris–HCl (Merck, Germany) with diluted pH=8 and with 1 ml of soluble keratin 0.5% (Sigma, U.S.A) and then was heated for 10 minutes at 50°C in bain-marie. The reaction stopped by adding 2 ml of 0.4 M three chloroaetic acid (Sigma, U.S.A) and centrifuged at 3500 rpm for 10 minutes and 4°C. Then optical absorption or optical density (OD) of solution was read at 280 nm wavelength against a control. To prepare the control sample, 2 ml of 0.4 M TCA was added to 1 ml enzyme extract. Enzyme unit is defined as 0.01 unit increase per minute at 280 nm (280 A) compared to control (12).

To measure total protein, Lowry protein assessment kit (Taligene pars, Iran) was used and the standard curve was drawn by using different concentrations of BSA.

To determine proper pH for maximum growth of bacteria, FMB medium was prepared and pH =7.5 and 7.2, 7, 6.8, and 6.4 was set with pH meter for the internal environment of flasks. A colony of bacteria of nutrient agar medium was inoculated to each of the flask, and then placed in shaking incubator at 180 rpm per minute at 30°C. At intervals of 96, 72, 48, 24, 12 hours, incubation was prepared from dilution series medium. Six tubes containing 9 ml of sterile distilled water were numbered and 1 ml of the liquid cultivation medium was transferred to tube 1 and dilution 10⁻¹ was prepared. After vortex of tube 1, 1 ml sampler was transferred from tube 1 to tube 2 to build dilution 10⁻², and it was continued to dilution 10⁻⁶. Then 100 microliters of dilution 10⁻⁶ was cultured in grass way to NA medium with a glass rod and then incubated. After 24 hours of incubation at 30°C, the number of colonies was counted.

To determine the optimal temperature for maximum bacterial growth, FMB medium was prepared with pH=7.5 and a colony of bacteria of NA medium was inoculated into it. Then the flasks were placed in a shaking incubator at 180 rpm for 3 days at temperatures of 36, 34, 32, 30, 28 °C. At intervals of 96, 72, 48, 24, 12 hours, incubation was prepared from dilution series. 100 ml of 10⁻⁶ dilution was incubated to NA medium and cultured in grass form. After 24 hours of incubation at 30°C, the number of colonies was counted.

To determine the optimal temperature for maximum bacterial growth, FMB medium was prepared with pH=5, 6, 7, 8 and 9 were prepared. After inoculation of the culture medium was placed for 72 hours in a shaking incubator at a temperature of 37°C with 180 rpm aeration rate. Then the mediums were sampled and keratinase activity was assessed (12).

Temperatures studied in this study were 20, 25, 30, 37, 40 and 45°C. After 72 hours, each medium was sampled and keratinase activity in it was evaluated.

In this study, the universal primer (Taligene pars, Iran) was used. The forward primer sequences were 5’AGGAGGTTGATCCAACGCA 3’ for gram positive bacteria and 5’CCATTGTAGCAGCAGTGTTG3’ for gram negatives (Isman and et.al, 2014). The reverse primer sequence for g+ bacteria was
5’AACTGGAGGAAGTGTTGAT3’ and for g’ bacteria was 5’CCATTGTAGCACGTGTG3’ (Isman and et.al, 2014).

Time stages of PCR included initial denaturation temperature 95°C, 5 minutes, denaturation temperature 94°C, 30 seconds, fusing temperature 50°C, 30 seconds, extension temperature 72°C, 70 seconds, and final extension temperature 72°C, 5 minutes. After performing PCR colony, gel electrophoresis review, and interpretation of results, information was evaluated on NCBI genomic database.

RESULTS

In charts (3.1) and (3.2), physicochemical properties and biology of sludge of the waters studied such as: pH, temperature, BOD and COD were compared.

Table 3.1: Biological properties of the samples (sludge water)

| Sample     | Temperature °C | pH | BOD mg/lit | COD mg/lit |
|------------|----------------|----|------------|------------|
| Qeshm      | 33             | 5  | 760        | 1510       |
| Peyposht   | 31             | 6  | 930        | 1434       |

According to Table 3.1, samples are both in the acidic range with slight differences; their temperature is close to each other, both temperature. According to Table 3.1, biological oxygen demand of the sample of Peyposht is higher compared to Qeshm island that is indicative of further contamination.

Macroscopic and microscopic features of the isolated bacteria are presented in Table 3.2 below.

Table 3.2: Macroscopic and microscopic features of keratinase-producing bacteria

| Isolate name              | Macroscopic properties                                                                 | Microscopic characteristics                                      |
|---------------------------|----------------------------------------------------------------------------------------|------------------------------------------------------------------|
| *Bacillus berevis*        | Medium-sized colonies that are smooth, round, regular margins, opaque white color and consistency, with spores | Gram-positive spore bacilli with chain array                       |
| *Enterobacter Cloacae*    | Colonies with small to medium size, smooth, round, regular margins, opaque white color and no consistency | Gram-negative short bacilli with individual array                 |
For molecular identification of keratinase-producing bacteria, the colony of bacteria intended was considered as the template DNA and amplified by FD and RD in the thermocycler. Then PCR products were placed beside Molecular weight markers with the size 50bp.
The desired gene sequence were compared with sequences in the Genome Database using NCBI BLAST software. The obtained sequence from samples of Qeshm has the most similarity with Bacillus berevis sequence of 16S rRNA (99%) and Peyposht sample had the most similarity with 16S rRNA gene sequences of Enterobacter cloacae (96.5%).

Bacillus berevis bacteria at pH=7.2 and Enterobacter cloacae at pH=7.5 had more growth than any other pH values. These bacteria were able to grow in temperatures 28, 30, 32, 34 and 36°C. Nevertheless, the number of colonies formed in Bacillus berevis in FMB with 32°C and the number of colonies formed in Enterobacter cloacae in FMB with 34 °C indicated that the bacteria grew more at these temperatures compared to other temperatures.
Chart 3.2: Comparison of *Enterobacter cloacae* growth at different pH

Chart 3.3: Comparison of *Bacillus berevis* growth at different temperature
Keratinase enzyme activity was measured in medium with pH of 5, 6, 7, 5.7, 8 and 9, respectively, with 33.85 (U/ml), 36.20 (U/ml), 49.01 (U/ml), 71.24 (U/ml), 53.95 (U/ml), 40 (U/ml). Maximum production of keratinase occurred on *Bacillus berevis* at FMB medium with pH=7.5 and was equivalent to 71.24 (U/ml). About *Enterobacter Cloacae*, at the same amount of pH, level of activity that achieved, was measured as 37.01 (U/ml), 38.61 (U/ml), 52.29 (U/ml), 50.45 (U/ml), 31.8 (U/ml), and 30.5 (U/ml). Maximum keratinase production by *Bacillus berevis* took place in the medium FMB with pH=7 equivalent to 52.29(U/ml).

Chart 3.4: Comparison of *Enterobacter cloacae* growth at different temperature

Chart 3.5: Comparison of keratinase production by *Bacillus berevis* at different pH, temperature 37°C for 72 hours
Chart 3.6: Comparison of keratinase production by Enterobacter Cloacae at different pH, temperature 37°C for 72 hours

The activity of enzyme keratinase on bacillus brevis at temperatures 20, 25, 30, 35, 37 and 40°C was measured respectively 14.07 (U/ml), 28.27 (U/ml), 35.95 (U/ml), 59.4 (U/ml), 44 (U/ml), and 20.63 (U/ml). Maximum keratinase-production by Bacillus berevis took place at temperature 35 °C was equal to 59.4 (U/ml) and the minimum enzyme production at temperature 20 °C was equal to 14.07 (U/ml). Keratinase enzyme activity was measured by Enterobacter Cloacae at the same temperatures as 25.29 (U/ml), 36.08 (U/ml), 42.57 (U/ml), 60 (U/ml), 68.88 (U/ml), 55.4 (U/ml). Maximum production of keratinase by Enterobacter cloacae occurred at 37 °C, equivalent to 68.88 (U/ml) and the lowest production of the enzyme at temperature 20 °C, equivalent to 25.29 (U/ml).

Chart 3.7: Comparison of keratinase production by Bacillus berevis at different temperature, after 72 hours incubation
Chart 3.8: Comparison of keratinase production by *Enterobacter cloacae* at different temperature, after 72 hours incubation

The sample protein content was measured by using standard curve at a wavelength of 650 nm by *Bacillus berevis* 0.649 mg/ml and by *Enterobacter cloacae* 0.669 mg/ml.

| Density | 0   | 40  | 69  | 80  | 100 | 120 | 140 | 160  |
|---------|-----|-----|-----|-----|-----|-----|-----|------|
| The optical density (OD) | 0   | 0.276 | 0.448 | 0.536 | 0.751 | 0.815 | 0.964 | 0.986 |
Today, the increasing amount of industrial wastewater are pouring into water islands, such as the livestock industry, agriculture and fishing; moreover, environmental pollution problem show the need for proper management. Recycling keratin waste is a subject that has attracted much attention for feeding animals, and this is because of its potential as an alternative protein source and low price. Keratin-rich waste is considered as fertilizer for soil. Due to the insoluble nature of this protein, it is resistant against degradation by the proteolytic enzymes, normally such as pepsin, trypsin and papain. Keratin-degrading microorganisms usually grow in ecological and environmental conditions such as soil, air, forage, and waste. In this study, our effort was, isolation and molecular identification of bacterial strains from sludge water in two areas of Qeshm coast and Peyposht village that were unable to decompose keratin-rich waste.

In isolation and identification of bacterial strains producing keratinases, Treck et al. (2010) used 23 bacterial isolate with bright halos on skim milk, Agar ad selected 7 isolates as keratinase-producing. In this study, a gram-positive and a gram-negative bacilli with keratinolytic activity on the main culture medium containing feather powder was isolated, and in another study by Sangaly and Brandli (2000), the keratinolytic activities of Group Vibrio strains KR2 on medium containing raw feather was reported. Moreover, Maticoisin et al. (2009) collected keratin-degrading bacteria and declared that isolated bacterial strains are the bacteria that in the medium containing feather powder show extreme keratinolytic activity. In 2007, Jiogo et al. isolated three species of Bacillus keratinolytic from the Amazon River that, in terms of morphological and biochemical characteristics and sequence of 16S rRNA, were similar more than 90% to Bacillus subtilis, Bacillus amylobiquefaciens, and Bacillus velezensis. This is while some researchers sufficed to biochemical detection methods to identify and isolate bacteria such as Pandin et al. that reported colonies with high production of keratinases just by using reference books like Bergey’s manual. Isolated strains in this study were identified by microscopic and macroscopic examinations and finally based on determining gene sequence of 16S rRNA, where the isolates from Qeshm coast were identified with a probability of 96.5% as Bacillus berevis and isolates from Peyposht village were identified with a probability of 99% as Enterobacter cloacae. In another part of this study, the growth conditions of these strains in terms of pH and temperature were optimized. In 2000, Sangali and Brandeli reported keratinolytic activities of KR2 Group Vibrio strains on a medium containing raw feather and stated that the bacteria, under optimal conditions and temperature pH=7 and 30°C has shown growth. Moreover, Kim et al. (2001) isolated feather-degrading bacteria from poultry waste that from among the three strains isolated: Bacillus subtilis, Bacillus pumilus, and Bacillus cereus were able to grow at 55°C.

In this study, after optimizing the growth conditions for two strains Bacillus berevis and Enterobacter cloacae in terms of pH and temperature, the results showed that the highest growth of the bacteria was, respectively, at a temperature of 32°C 34 and pH=7.2 and pH=7.5. According to the results of optimizing growth conditions of the bacteria of interest, we can say that on bacilli isolated in five periods of 12, 24, 48, 72 and 96 hours at pH=7.2 more growth happens than other pH and Enterobacter isolated in the same period at pH=7.5 had the highest rate of growth. About optimizing temperature, it can be confirmed that the isolated bacteria grow at temperatures 28, 30, 32, 34 and 36°C but 32°C for Bacillus and 34°C for Enterobacter over 96 hours were more appropriate temperatures.

Overall, since BOD is determining the amount of oxygen needed that should be given to water so that aerobic bacteria oxidize materials in water and turn them into sustainable materials such as mineral salts, the amount of BOD water in the various times changes. These changes depend not only to the concentration of organic matter, but also on the activity of bacteria and temperature. BOD₃ is in fact, the
amount of oxygen that microorganisms use for biological decomposition of organic matter during the first 5 days after the sampling is taken (Rekhoii and Malardi, 2002).

Since COD is indeed total chemical oxygen demanded used in oxidation reactions and decomposition of organic matter in the acidic environment near oxidizing agent, so it is important. COD standard amount, depending on the type of industrial water evaluated in pollution control committees, has a minimum amount of 120 (mg/lit) and a maximum of 400 (mg/lit) (Pollution Control Section, 2014).

In this study, the amount of BOD and COD of sludge of water removed from Qeshm beach and Peyposht village is much higher than the standard value, which means severe and dangerous pollution. Higher level of COD compared to BOD is because when toxic compounds are present in the wastewater, the activity of organisms that destroy organic materials is prevented and, as a result BOD5 is reduced.

In the initial stage of this study, the effect of initial pH 5-9 on the production of keratinase by Bacillus berevis and Enterobacter cloacaee was examined, and as mentioned, maximum keratinases production by Bacillus berevis was observed at pH=7.5 equivalent to 71.24 (U/ml) and for Enterobacter cloacaee maximum keratinase production, was 52.29 (U/ml) at pH=7. According to the results, the activity of keratinase on Bacillus berevis in neutral pH to more alkaline is more than acidic, and keratinase activity of Enterobacter cloacaee in neutral pH has had its highest value.

In research conducted in 2013 by Nadia and Hatem, optimal pH for keratinase production has been reported 7.5, 8 and 9 for Bacillus berevis bacteria. In research conducted by Kim et al. (2001), the highest production of keratinase was obtained in Bacillus cereus at pH=7, while in Bacillus subtilis, maximum enzyme production was in the range pH=5-9, and in Bacillus pumilus in the range of pH=5-6.

While examining the effect of different temperatures in the production of enzymes, the most suitable incubation temperature for keratinase production by Bacillus berevis was obtained 35 C˚ equivalent to 59.4 (U/ml), and the results of this experiment showed that Bacillus brevis showed lowest activity at 20C˚. However, in the research conducted by Nadia and Hatem in 2013, the temperature has been reported 40 C˚. This temperature difference can be related to different physicochemical situations of the two studies. Moreover, the type of bacteria extracted by Nadia and Hatem in 2013 was different from the genus of bacteria isolated in this study.

In the study by Yu et al. in 2009 on Bacillus licheniformis K-19, in measuring the amount of soluble protein produced by this bacterium, reagent folin-phenol and bovine serum albumin were used as standard methods. During this study, concentration of soluble protein obtained after 36 hours of incubation at 37 C˚ was measured as almost 3 mg/ml.

In 2009, Matikoin et al. measured keratinases produced by Bacillus subtilis bacteria 11, Bacillus licheniformis 511, bacillus subtilis 717, and bacillus subtilis 103 with Lowry method and using bovine serum albumin after 72 hours of incubation at a wavelength of 565 nm as, respectively 1.23 mg/ml, 1.17 mg/ml, 1.16 mg/ml, and 1.05 mg/ml. However, in this study, the amount of enzyme produced by Bacillus brevis was measured using the standard curve at a wavelength of 650 nm, equivalent to 0.649 mg/ml and by Enterobacter cloacaee equivalent to 0.649 mg/ml at the same wavelength. Different results from the two studies can relate not only the difference of bacterial genera and species, but also wavelength used to measure keratinase protein that were different to some extent in the two studies.

CONCLUSIONS

Keratin waste includes waste, caused by the textile industry, tanning, farming and poultry as well as the coastal sludge, that is the habitat of sea birds and many creatures with keratin covers is a favorable environment for the growth of keratinase-producing bacteria. According to the studies conducted by researchers, most of these bacteria are of bacillus family because this group of bacteria, due to producing
spores, is able to handle adverse environmental conditions such as very high and low temperatures, high and low osmotic pressure, different pH, and bad weather conditions. Moreover, little research has been done on the sludge of coastal waters in this area.

Samples studied in this study have BOD and COD with small differences reflecting the differences in microbial population and their activity. According to the studies conducted, suitable pH for growth of keratinase-producing bacteria is in the range of neutral to acidic pH range, and optimal pH range for keratinase activity was neutral to alkaline. In terms of temperature, due to temperature changes in the sea, the best temperature range for growth of isolated bacteria, is 32-34°C and the best temperature range for keratinase activity is 35-37°C.

Bacteria isolated in this study were gram-negative *Bacillus* and spore-forming gram-positive *Bacillus* whose spore shows its robustness of the bacteria in difficult conditions. Moreover, these two bacteria are very rich in terms of enzyme production, and in the future, we hope to be able to use it by optimizing the conditions and purification of the enzyme used in various industries.

**CONFLICT OF INTEREST**
The authors declare no conflicts of interest regarding this manuscript.
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