Journal of Applied Biological Sciences
E-ISSN: 2146-0108 15(2): 185-195, 2021
Research Article

BIODEGRADATION OF KERATINOUS WASTES BY ALKALINE-KERATINASE PRODUCED FROM *BACILLUS TEQUILENSIS* BK206 AND OPTIMIZING THE CULTURAL CONDITIONS FOR INCREASED KERATINOLYTIC ACTIVITY

Roqyeh Moridshahi[1], Mohammad Reza Sharifmoghadam[1], Masoumeh Bahreini[1*]

[1]*Ferdowsi University of Mashhad, Faculty of Science, Department of Biology, Mashhad, Iran*

**Corresponding Author:*
E-mail: mbahreini@um.ac.ir

(Received 07th September 2020; accepted 03rd January 2021)

ABSTRACT. Keratinase-producing microorganisms can serve as a safe, environment-friendly, and cost-effective means for recycling and hydrolysis of keratin wastes and therefore play a substantial role in biotechnological applications. This study aimed to investigate the ability of *Bacillus tequilensis* BK206 to degrade keratin wastes, especially chicken feathers, and to measure its proteolytic and keratinolytic activity. The proteolytic and keratinolytic activity of the strain in different substrates was measured qualitatively and quantitatively. Then, the one-factor-at-a-time method was used to optimize the enzyme production. Optimization of the conditions (temperature 37°C, pH 10.5, feather concentration 2%, and ammonium chloride as a nitrogen source) increased the keratinolytic activity of the isolate by 2.3-fold. The alkaline pH was found to have the highest effect on the keratinolytic activity. The isolate was able to degrade chicken feathers completely and breakdown sheep wool, and hoof to a large extent. The results demonstrated the potential of *Bacillus tequilensis* BK206 is a potent keratinase-producing bacterium in treating various keratinous wastes in a safe, inexpensive, and easy-to-implement ecological process. Also, our alkaline keratinase is a good candidate for use in various industrial processes, especially in detergent and leather industries.

Keywords: *Bacillus* sp., *feather, keratine; keratinase, keratinous wastes.*

INTRODUCTION

In recent years, researchers have shown a growing interest in keratinases as enzymes with remarkable potentials and properties. Keratinases not only have extensive use in industry, but are also greatly important from the environmental perspective because of their role in the management and disposal of keratin wastes [1]. These wastes are mostly produced by the poultry and livestock industries, which are among the steadily growing segments of the food industry. The growth, which can be attributed to ever increasing demand for white and red meat, has also led to rising production of by-products and wastes [2]. Feather is one of the most abundantly produced wastes of the poultry industry, with annual production volumes reaching up to millions of tons [3].

Keratin is the third most abundant natural polymer and consists of fibrous proteins rich in cysteine amino acids. Due to the high number of disulfide bonds in the structure, keratin has a

rigid and durable matrix that makes it resistant to common proteases, water, weak acids and bases, and organic solvents [4, 5].

The slow degradation of keratin makes it a hazardous waste with the potential to cause various diseases and environmental and health problems [6]. Biological methods and specifically microbial keratinases can accelerate the degradation process and ensure the complete breakdown of keratin wastes [7]. Keratinases are a group of modern proteases that are used in various industries to produce food supplements, organic fertilizers, animal feed, biogas production, and also as biosorbent in bioremediation processes [8, 9, 10]. Most of the commercial proteases are produced from various bacteria, especially *Bacillus* spp. Two well-known species used in the industrial production of this group of proteases are *B. licheniformis* and *B. subtilis* [10, 11].

The present study aimed to evaluate the proteolytic and keratinolytic activity of *Bacillus tequilensis* BK206 isolated from poultry waste for biodegradation of keratin wastes, including alpha, and beta keratin, and to optimize the culture conditions to increase keratinase production.

MATERIALS AND METHODS

Microorganism and Cultural media

In this study, *Bacillus tequilensis* BK206 (accession number KY810609) was isolated and identified from a poultry feather, Mashhad, Iran in pervious study (unpublished), was used for evaluation of the proteolytic and keratinolytic activity. The media were used as follows ($g.L^{-1}$): Feather meal broth (FMB) containing minimum salt medium: NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgSo_4.7H_2O$, 0.1; with 1% feather (pH 7.5). Feather meal agar (FMA) prepared by adding 1.5% agar to FMB. Skim Milk Agar (SMA) containing NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgSo_4.7H_2O$, 0.1; lean milk (Skim milk) 1% and agar 1.5% (pH 7.5). Poultry feathers were purchased from a local poultry plant, prepared according to Mazotto et al. [12] method, and then powdered by mixer mill (Amin Asia Co., Iran).

Sample Population

Qualitative analysis of protease and keratinase activity of the isolate was performed using SMA and FMA media. The overnight culture of the isolate was inoculated on SMA and FMA media and incubated at 20, 30, 37, and 50 °C. The proteolytic activity and the keratinolytic activity were investigated with the formation of hydrolysis zones around the colonies [13]. Quantitative analysis of protease and keratinase activity of the enzyme was measured using casein substrate for the proteolytic activity, azokeratin (beta-keratin) and keratin azure (alpha-keratin) substrates for keratinolytic activity as follows: the isolate was inoculated (2%) in FMB medium with 1% feather as the sole source of carbon and nitrogen and incubated at 37 °C with shaking at 150 g. The enzyme activity at 24 h intervals was measured as follows: 1 ml of the culture medium was centrifuged at 10000 g for 5 min, and the supernatant was used for assay as raw enzyme [14].

The protease activity was investigated using Ionata et al., [15] protocol with some modification. Casein, 0.5% (w/v), was dissolved in Tris-HCl buffer (50 mM) on a magnetic stirrer and adjusted to pH 8. The crude extract of the enzyme (50 µl) was mixed with 450 µl of casein solution, incubated (60 min) in a hot bath (50 °C), 500 µl of 10% trichloroacetic acid (TCA) was added to stop the reaction. Then, the solution was centrifugated for 10 min at 12,000 rpm and the absorption of the supernatant was measured at 280 nm. Enzyme solution without casein was used as a control sample.

Finally, using the standard curve of tyrosine (the rate of enzyme production and activity was evaluated on the obtained equation), the equation of production and activity of the enzyme was evaluated. One unit of protease activity was considered as the amount of enzyme required to release one micromole of tyrosine in one minute. This value is defined in U/ml and calculated according to the following Eqn. 1.

*Unit/ml=(X) concentration of the product/Volume of the enzyme (ml) * Time (min)*

Eqn. 1

Azokeratin was synthesized according to the Herzog et al. [16] method and used as substrate (beta-keratin) to measure azokeratinolytic activity. 100 µl of crude enzyme were added to 900 µl of Tris-HCl buffer (50 mM) containing 10 mg of azokeratin at pH 8. The tubes were incubated at 37 °C, 150 rpm for 60 min, and then placed on ice for half an hour to stop the enzymatic activity. In the end, the optical absorption was read at 450 nm against the control sample. Where one unit of azokeratinolytic activity is defined as the amount of the enzyme that causes a 0.01-fold increase in the absorbance unit at 450 nm against the control sample over 60 minutes under standard conditions [17].

Keratin azure was used as an alpha-keratin (wool) substrate (Sigma-Aldrich, USA) according to the Daroit et al. method [13]. Crude enzyme solution, 100 µl, was added to 900 µl Tris-HCl buffer containing 5 mg of keratin azure and incubated at 50 °C for 60 min at 150 rpm. To stop the reaction, the solution was placed on ice for half an hour, and the optical absorption of the sample was read at 595 nm. A keratinase activity unit using keratin azure is defined as the amount of enzyme that causes a 0.01-fold increase in absorbance unit at 595 nm against the control sample over 60 min under standard conditions. In all three assays, the medium without inoculation was used as control. The experiments were repeated twice. Next, the absorption results at 450 and 595 nm were converted to the enzyme unit using Eqn. 2.

*Unit/ml= (N) Volume of solution*A (450 or 595) Absorption rate/0/01 *Time (min)*

Eqn. 2

Optimization of keratinase production

Optimization of the culture conditions was performed using *one factor at a time* method. In this study, temperature (20, 30, 37, 40, 45, 50, 55 °C), pH (6, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5 and 12) , different concentration of feather substrate (0.2, 0.5, 1, 2, 3, 4, 5 and 6%), different carbon sources (glucose, sucrose, lactose, fructose, mannitol and starch at 1% concentration), different nitrogen sources (Organic sources including peptone, yeast extract, urea and mineral sources including ammonium nitrate, ammonium chloride and potassium nitrate with concentration of 0.4%) and inoculation size (1, 2, 3, 4, 5, 6, 8 and 10%) was studied.

Degradation ability of various keratinous wastes

The biodegradation ability of the isolate was investigated in the presence of four types of substrates, chicken feather (β-keratin), sheep wool, sheep hoof, and human hair (α-

keratin). One percent of each substrate (w/v) was added to the minimal salt medium (MSM (g/L): NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgSo_4.7H_2O$, 0.1 with pH 7.5), and incubated at 37°C for 48 h at 150 rpm. The enzyme activity was measured using azokeratin substrate at 450 nm for 60 min.

The rate of degradation of different keratin wastes by the isolate was evaluated by measuring biomass and quantifying enzyme activity in terms of enzyme unit. Feather and sheep wool were added as a sample substrate of α- keratin and β-keratin to the MSM before and after optimization conditions. After 48 h of incubation, the culture medium was filtered using Whatman filter paper No. 1 and washed with 70% alcohol to remove the remaining bacterial cells. The filter paper was placed in an oven at 60 ° C for 48 h to achieve a constant weight. The weight of the filter paper was expressed as a percentage of the total weight and substrate residue, and the percentage of substrate degradation was determined by weight loss [18]. Experiments were performed in duplicates, and the results were averaged.

Statistical analysis

To investigate the significance of each of the factors tested, statistical analysis of variance (ANOVA) was performed using SSPS software (version 18) at 95% confidence level.

RESULTS AND DISCUSSION

Qualitative and Quantitative assessment of proteolytic and keratinolytic activity

The effect of temperature on the proteolytic and keratinolytic activity of *Bacillus tequilensis* BK206 is shown in Table 1. The results showed that the isolate was able to have hydrolysis activity at temperatures of 20, 30, and 37°C. The highest proteolytic and keratinolytic ability were observed at 37°C. The activity of the isolate was measured using three substrates: casein (proteolytic), azokeratin and keratin azure (keratinolytic) in terms of enzyme unit. The results showed that the highest protease activity occurred on the second day of incubation (48h), which matched the highest keratinase activity on the azokeratin. The highest keratinase activity on keratin azure was observed after 96 h of incubation, which indicates that it takes more time for enzymatic hydrolysis to take place in this substrate (Table 2). Enzymatic activity was assessed based on colorimetric method in which the intensity of substrate degradation was shown with the intensity of the released dye (Fig. 1).

Table 1. *The diameter of the clear zone around the colonies (mm), (+) colony growth but lack of hydrolysis zone, and (-) not growth and hydrolysis zone*

Temperature (°C)	Diameter of the clear zone of Skim milk (mm)	Diameter of the clear zone of Feather meal
20	12	3
30	20	5
37	35	8
50	+	-

Table 2. *Investigation of enzymatic activity of Bacillus tequilensis* BK206 *using different substrates over 120 hours of incubation*

Incubation time (h)	Enzymatic activity (U/ml ±SD)		
	Casein (Protease activity)	Azokeratin: β-keratin (Keratinase activity)	Keratinazure: α-keratin (Keratinase activity)
24	51.25 ± 2.21	40.09 ±1.13	1.45 ± 0.45
48	98.86 ± 1.9	46.35 ± 1.47	3.521 ± .05
72	74.54 ± 1.12	41.81 ±0.42	9.88 ± 0.3
96	33.65 ±3.11	24.5 ± 0.81	10.12 ± 0.14
120	1.98± 10.2	19.1 ±2.45	7.41 ±1.11

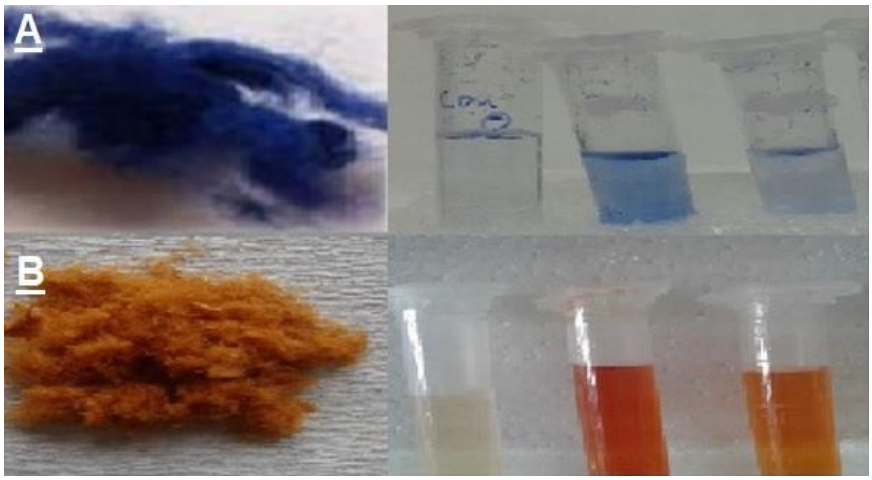

Fig. 1. *Effect of enzymatic activity on (A) keratin azure and (B) azokeratin substrates based on colorimetric method (the evaluation of enzymatic activity was performed based on colorimetric method and the intensity of the released dye indicates the severity of substrate degradation)*

Optimization of enzyme production

The results showed that the isolate had high keratinase activity in the temperature range of 20-45°C with the highest keratinolytic activity occurring at 37°C. There was no significant difference between activity levels in temperatures of 20, 30, and 45°C. At 50°C, enzyme production decreased drastically and at 55°C bacterial growth stopped completely (Fig. 2A). The results also showed that the isolate was active in a wide range of pH. Keratinase activity increased significantly in pHs of 9-11.5, reaching its maximum value (2x) at pH:10.5, where there was an 86.8% increase in keratinolytic activity. At pH:11.5, keratinolytic activity decreased, and at pH:12, the isolate stopped growing (Fig. 2B). Investigation of the isolate's ability to decompose different amounts of feather showed that the 2% feather concentration resulted in the highest keratinolytic activity. Statistical analysis revealed no significant change in the amount of enzyme produced in

feather concentrations of 2, 3, and 4% (Fig. 2C). Our results showed that using 1% lactose as a carbon source led to increased keratinolytic activity, but the difference from the base medium was not statistically significant. Using other carbon sources significantly reduced the keratinase activity. The best improvement in enzyme production (12%) was observed in the media containing ammonium chloride and ammonium nitrate. Therefore, subsequent phases of the study were performed using ammonium chloride (Fig. 2D, 2E). Since there was no significant difference between bacterial inoculum levels at 2, 4, 6, and 8% concentrations, the 2% concentration was selected as the optimal inoculum ratio (Fig. 2F).

Evaluation of substrate degradation and degradation ability against different keratinous substrates

The percentage degradation of the substrate was evaluated based on the dry weight of keratin that remained in the base and optimized media. In this stage, the amount of keratin was measured as a percentage of the initial dry weight of keratin. Complete degradation was defined as having a residual feather concentration of less than 5% (i.e. more than 95% decrease from the initial feather concentration) (Table 3). The results showed that *Bacillus tequilensis* BK206 was able to degrade chicken feathers completely, and had a good performance in sheep wool. The isolate also showed good hydrolysis and keratinase activity in sheep hoof substrate, but was not able to produce the enzyme in the presence of human hair (Fig .3).

Determination of keratinase: protease activity rate (K/C)

In this study, the keratinolytic activity of the isolate was studied on the chicken feather substrate and the caseinolytic activity on the casein substrate. The K/C ratio was 0.951, indicating the high ability of this isolate to produce the efficient and functional keratinase enzyme.

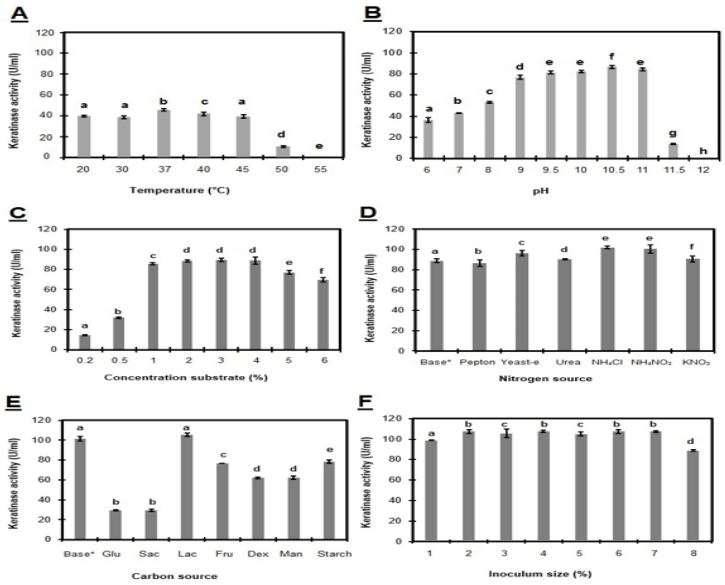

Fig. 2*. Charts related to the optimization of various parameters. The sample (con *) is the same as the base medium of the previous stage. (The same letters indicate no significant difference)*

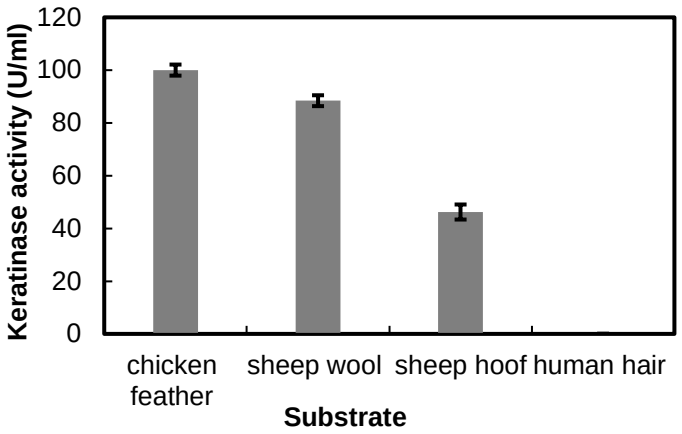

***Fig. 3**. Investigation of enzyme activity of Bacillus tequilensis* BK206 *in the presence of different substrates*

In the qualitative study of the proteolytic and keratinolytic activity of the isolate, the highest growth rate and hydrolysis were observed at 37°C. It was deduced that the isolate is mesophilic, which is an important feature for the hydrolysis of keratinolytic bacteria with low energy consumption. In line with the present study, Agrawal and Dalal, Riffel and Brandelli [19, 20] reported that 37°C is the optimum temperature for the keratinolytic activity of their isolates.

Protease and keratinase activity of the isolate reached its highest level after 48 h of incubation and started to decline on the 72 h. In the keratin azure substrate, keratinase activity peaked at 96 h, which indicated that the enzyme needed more time to hydrolysis in the presence of this substrate. The molecular structure of keratin azure, which is alpha-keratin with high amounts of cysteine and large numbers of disulfide bonds, makes it more rigid than beta-keratin and casein soluble protein, which means it takes longer for the bacteria to cause hydrolysis [13]. In this study, the keratinolytic activity of the isolate was increased at the temperature of 37°C and pH of 10.5 after 48 h of incubation. Similar to this study, Prakasham et al. [21] reported maximum keratinase production in *B. halodurans* PPKS-2 after 48 h incubation. Most microbial keratinases are neutral to alkaline proteases with an optimum pH of 7.5 to 9, although some enzymes are more active at alkaline or acidic pHs [10]. The study conducted by Gupta and Singh [22], also observed maximum keratinase activity at pH 10.5. The high stability of many proteases especially keratinase, over a wide range of pH, is a remarkable feature that makes them a suitable option for biotechnological applications, especially in detergent and leather industries [23, 24, 25].

Investigating the effect of substrate quantity on enzyme production showed that changing the substrate concentration in the range of 2 to 4% had no impact on keratinase activity. Using more than 4% substrate decreased the enzyme production, a change that perhaps can be attributed to the saturation of the active sites of the enzyme by the substrate or the reduced aeration due to increased substrate density [14]. In this study, adding any carbon source -except lactose- reduced the keratinolytic activity of the isolate. This means that in the presence of other easier to access carbon sources, the isolate will use those

sources rather than feather meal, which will sharply decrease its keratinolytic effectiveness. Tiwary and Gupta [26] showed that the excess carbon source had a positive effect on proteolytic activity; a result that contradicts the findings of the present study and many other studies that have identified glucose and other sugars as inhibitors of proteolytic enzymes (catalytic suppressors) [27, 28]. Also, nitrogen source is an essential factor in the microbial growth and production of microbial products. Different bacteria react differently during growth and keratinolytic activity in the presence of organic and inorganic nitrogen. And different bacteria may prefer and use different sources of nitrogen, according to nitrogen availability and simplicity. Depending on the nitrogen source used by the bacteria, the optimization results may be affected. Brandelli et al. believed that the additional nitrogen sources might act as inducers of keratinase production (2). In the study conducted by Tiwary and Gupta [26] on keratinolytic activity of *B. licheniformis* ER-15 isolate, soybean flour and yeast extract were identified as nitrogen sources inducing keratinolytic activity; in contrast, Bernal et al. and Ramnani and Gupta [29, 30] identified tryptone and peptone as useful nitrogen sources for the keratinolytic activity of isolates of *Kocuria rosea* and *B.licheniformis* RG1, respectively. In this study, the addition of ammonium chloride and ammonium nitrate enhanced the keratinolytic activity of the isolate and increased keratinase production by 14 units which is in line with the study conducted by others (2, 26, 29, 30).

Optimizing the culture conditions increased the enzyme production from 46.3 ($U.ml^{-1}$) to 107.6 ($U.ml^{-1}$), a 2.3-fold increase. This study showed that pH has the greatest effect, and carbon source and inoculum ratio had the least effect on the process by the isolate.

The rate of degradation of various keratinous wastes by the isolate was evaluated by measuring biomass and quantifying enzyme activity in terms of enzyme unit. The results showed that the isolate has high potential in degrading a variety of keratin substances, including alpha and beta keratin, and in the hydrolysis of significant amounts of chicken feather (100%), sheep wool (88%), and sheep hoof (46%) but is unable to degrade human hair. In a study by Son et al. [31] up to 75% of the chicken feather, 10% of sheep wool, and 9% of human hair was broken down by *B. pumilis* F3-4. A study by Park and Son [32] on the keratinolytic ability of *B. megaterium* F7-1 showed that it managed to break down 100% of chicken feathers but only 19% of sheep wool and 18% of hair.

Substrate specificity is one of the most essential criteria for designating a protease as keratinase. Any protease with Keratinolytic; caseinolytic K:C ratio of more than 0.5 can be considered a keratinase with potential industrial and environmental value [4]. In this study, the K:C ratio was measured to 0.95, which signifies the excellent keratinolytic ability of *Bacillus tequilensis* BK206 and the high potential in the use of its keratinase as a catalyst for environmental purposes in various industries [25, 33].

CONCLUSION

While there are many microbial sources for producing enzymes, such as keratinase, only a few of them are capable of producing a suitable keratinase for biotechnological purposes. The ability of the isolate to grow at moderate temperatures, alkaline pH, and high substrate contents makes this strain an excellent candidate for keratinase production in various industrial processes. Given the unique ability of this isolate to degrade feathers completely and breakdown sheep wool and other keratin substances to a large degree, it is an excellent option for bioremediation processes and the production of waste derivatives. The results of this study suggest that *Bacillus tequilensis* BK206can serve as

an easily accessible, cost-effective, and environment-friendly means of keratinase production for various biotechnological purposes.

Acknowledgements

From Ferdowsi University of Mashhad for Funding for this research (Grant No. 3.40942) thanks and gratitude.

REFERENCES

[1] Lasekan A, Bakar FA, Hashim D, (2013): Potential of chicken by-products as sources of useful biological resources. Waste Management 33(3): 552-565.

[2] Brandelli A, Sala L, Kalil SJ, (2015): Microbial enzymes for bioconversion of poultry waste into added-value products. Food Research 73: 3-12.

[3] Reddy N, Yang Y, (2007): Structure and properties of chicken feather barbs as natural protein fibers. Journal of Polymer Environment 15(2): 81-87.

[4] Gupta R, Sharma R, Beg QK, (2013): Revisiting microbial keratinases: next generation proteases for sustainable biotechnology. Journal of Critical Reviews in Biotechnology 33(2): 216-228.

[5] Purchase D, (2016): Microbial keratinases: characteristics, biotechnological applications and potential 634-674.

[6] Thyagarajan D, Barathi M, Sakthivadivu R, (2014): Risk mitigation of poultry industry pollutants and waste for environmental safety. Global Journal of Science Frontier Research D: Agriculture Veterinary 14(1): 49-56.

[7] Verma A, Singh H, Anwar S, Chattopadhyay A, Tiwari KK, Kaur S, Dhilon GS, (2017): Microbial keratinases: industrial enzymes with waste management potential. Journal of Critical Reviews in Biotechnology 37(4): 476-491.

[8] Tesfaye T, Sithole B, Ramjugernath D, Chunilall V, (2017): Valorisation of chicken feathers: characterisation of chemical properties. Waste Management 68: 626-635.

[9] Desai SS, Hegde S, Inamdar P, Sake N, Aravind MS, (2010): Isolation of keratinase from bacterial isolates of poultry soil for waste degradation. Engineering Life Science 10(4): 361-367.

[10] Brandelli A, Daroit DJ, Riffel A, (2010): Biochemical features of microbial keratinases and their production and applications. Applied Microbiology and Biotechnology 85(6): 1735-1750.

[11] Sanghvi G, Patel H, Vaishnav D, Oza T, Dave G, Kunjadia P, Sheth N, (2016): A novel alkaline keratinase from *Bacillus subtilis* DP1 with potential utility in cosmetic formulation. International Journal of Biological Macromolecules 87: 256-262.

[12] Mazotto AM, Couri S, Damaso MC, Vermelho AB, (2013): Degradation of feather waste by Aspergillus niger keratinases: comparison of submerged and solid-state fermentation. International Biodeterioration and Biodegradation 85: 189-195.

[13] Daroit DJ, Corrêa AP, Segalin J, Brandelli A, (2010): Characterization of a keratinolytic protease produced by the feather-degrading Amazonian bacterium *Bacillus sp*. P45. Biocatalyst 28: 370-379.

[14] Pereira JQ, Lopes FC, Petry MV, da Costa Medina LF, Brandelli A, (2014): Isolation of three novel Antarctic psychrotolerant feather-degrading bacteria and partial purification of keratinolytic enzyme from *Lysobacter sp.* A03. International Biodeterioration and Biodegradation 88: 1-7.

[15] Ionata E, Canganella F, Bianconi G, Benno Y, Sakamoto M, Capasso A, Rossi M, La Cara F, (2008): A novel keratinase from *Clostridium sporogenes bv. pennavorans bv. nov*., a thermotolerant organism isolated from solfataric muds. Microbiology Research 163(1): 105-112.

[16] Herzog B, Overy DP, Haltli B, Kerr RG, (2016): Discovery of keratinases using bacteria isolated from marine environments. Systematic Applied Microbiology 39(1): 49-57.

[17] Martínez YN, Cavello I, Hours R, Cavalitto S, Castro GR, (2013): Immobilized keratinase and enrofloxacin loaded on pectin PVA cryogel patches for antimicrobial treatment. Bioresource Technology 145: 280-284.

[18] Liu Q, Zhang T, Song N, Li Q, Wang Z, Zhang X, Lu X, Fang J, Chen J, (2014): Purification and characterization of four key enzymes from a feather-degrading *Bacillus subtilis* from the gut of tarantula *Chilobrachys guangxiensis*. International Biodeterioration and Biodegradation 96: 26-32.

[19] Agrawal BH, Dalal MI, (2015): Screening and characterization of keratinase enzyme obtained from keratin degrading microorganism isolated from Sanjan poultry waste dumping soil. European Academic Research 2(11): 13986-13994.

[20] Riffel A, Brandelli A, (2006): Keratinolytic bacteria isolated from feather waste. Brazilian Journal of Microbiology 37(3): 395-399.

[21] Prakasham RS, Rao CS, Sarma PN, (2006): Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus sp*. in solid-state fermentation. Bioresource Technology 97(13): 1449-1454.

[22] Gupta S, Singh R, (2014): Hydrolyzing proficiency of keratinases in feather degradation. Indian Journal of Microbiology 54(4): 466-470.

[23] Gupta R, Ramnani P, (2006): Microbial keratinases and their prospective applications: an overview. Applied Microbiology 70(1): 21.

[24] Saraçoğlu N. Z., Tanrisever D., Arikan B., Korkmaz Güvenmez H., (2013): Characterization of an oxidant and detergent stable alkaline protease produced from a novel isolate *Bacillus* sp. strain, Journal of Applied Biological Sciences 7 (2): 4-9.

[25] Moridshahi R, Bahreini M, Sharifmoghaddam MR, Asoodeh A, (2020): Biochemical characterization of an alkaline surfactant-stable keratinase from a new keratinase producer, *Bacillus zhangzhouensis*. Extremophiles 24: 693–704

[26] Tiwary E, Gupta R, (2010): Medium optimization for a novel 58 kDa dimeric keratinase from *Bacillus licheniformis* ER-15: biochemical characterization and application in feather degradation and dehairing of hides. Bioresource Technology 101(15): 6103-6110.

[27] Hossain MS, Azad AK, Sayem SA, Mostafa G, Hoq MM, (2007): Production and partial characterization of feather-degrading keratinolytic serine protease from *Bacillus licheniformis* MZK-3. Journal of Biological Science 7(4): 599-606.

[28] Vidyasagar M, Prakash S, Jayalakshmi SK, Sreeramulu K, (2007): Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium *Chromohalobacter sp.* TVSP101. World Journal of Mirobiology and Biotechnology 23(5): 655-662.

[29] Bernal C, Diaz I, Coello N, (2006): Response surface methodology for the optimization of keratinase production in culture medium containing feathers produced by *Kocuria rosea*. Candian Journal of Microbiology 52: 445-450.

[30] Ramnani, P. and Gupta, R, (2004): Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG1 using statistical methods involving response surface methodology. Applied Biochemistry 40: 191-196.

[31] Son HJ, Park HC, Kim HS, Lee CY, (2008): Nutritional regulation of keratinolytic activity in *Bacillus pumilis*. Biotechnology Letters 30(3): 461.

[32] Park GT, Son HJ, (2009): Keratinolytic activity of *Bacillus megaterium* F7-1, a feather-degrading mesophilic bacterium. Microbiology Research 164(4): 478-485.

[33] Gong JS, Wang Y, Zhang DD, Zhang RX, Su C, Li H, Zhang XM, Xu ZH, Shi JS, (2015): Biochemical characterization of an extreme alkaline and surfactant-stable keratinase derived from a newly isolated actinomycete *Streptomyces aureofaciens* K13. RSC Advances *5*(31): 24691-24699.