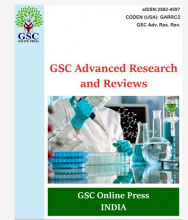
GSC Advanced Research and Reviews

eISSN: 2582-4597 CODEN (USA): GARRC2

Cross Ref DOI: 10.30574/gscarr

Journal homepage: https://gsconlinepress.com/journals/gscarr/



(RESEARCH ARTICLE)

# Stain-removal potential of keratinase produced by *Bacillus* species isolated from feather waste

Michael Tunde DADA * and Sherifah Monilola Wakil

*Department of Microbiology, University of Ibadan, Nigeria G. P. O. Box 15372.*

GSC Advanced Research and Reviews, 2022, 11(03), 013–019

Publication history: Received on 25 April 2022; revised on 31 May 2022; accepted on 02 June 2022

Article DOI: https://doi.org/10.30574/gscarr.2022.11.3.0119

## Abstract

The aim of this study was to determine the potential of keratinases produced by *Bacillus* species isolated from poultry feather waste in stain removal**.** The effect of individual and combined detergent components on the keratinase activity of EZYKer-51, EZYKer-50 and EZYKer-53 enzymes produced from keratin degrading *Bacillus licheniformis*-K51, *Bacillus subtilis*-K50 and *Bacillus* sp.-K53 respectively was determined. EZYKer-51, which had the least enzyme inhibition in the presence of the detergent component was used as the enzyme component in detergent formulation and a wash performance analysis test was carried out to compare the stain removal capacity of DETKER 51 (detergent formulated using EZYKer-51 as enzyme component) with a commercial enzyme. Data were analyzed using descriptive statistics. EZYKer-51 showed a significantly higher ($P \leq 0.05$) enzyme activity in the presence of the detergent ingredients for both the crude and purified forms of the enzyme compared with the activities of EZYKer-50 and EZYKer-53, except for the activities ($8.02 \pm 1.32$ U/mL and $7.94 \pm 0.63$ U/mL) in the presence of 15% LAS and 5% liquid paraffin. Visually, better stain removal was achieved when endogenous protease of commercial enzyme was replaced with EZYKer-51.

These results suggest that keratinase EZYKer-51 may be a useful alternative for applications in detergent formulation.

**Keywords:** *Bacillus* keratinase; Detergent; Keratin-degrading bacteria; Stain-removal

## 1. Introduction

Enzymes are macromolecular biological catalysts [1]. Their utilization has a wide scope in basic and applied research and also manufacturing processes involving food, beverage, pharmaceuticals and industries involving processing of leather and synthesis of peptides [2]. Enzymes are no longer a minor additive and have become an essential ingredient in modern detergents. The major classes of detergent enzymes include proteases, lipases, amylases, and cellulases. Proteases are the first used and the most common detergent enzymes, therefore, presently have many commercial forms in the market [3].

Laundry detergents are becoming more and more popular because of their increasing use in washing machine, where it is soft, resilient to fabrics, antistatic, dispersible in water and mild to skins [4]. There are concerns over persistence of detergent chemicals in the environment and its possible contamination of ground water. Their subsequent health related issues have raised speculation over biodegradability. This has brought about an increasing preference for greener detergents and inadvertently, the need for greater consumption of enzymes as a detergent component [4].

Enzymes can reduce the environmental load of detergent products as the chemicals used in conventional detergents are reduced; they are biodegradable, non-toxic and leave no harmful residues [4]. More focus has been drawn to keratinases

* Corresponding author: Michael Tunde DADA
Department of Microbiology, University of Ibadan, Nigeria G. P. O. Box 15372.

Copyright © 2022 Author(s) retain the copyright of this article. This article is published under the terms of the Creative Commons Attribution Liscense 4.0.

produced from bacteria because of their actions on both insoluble keratin substrates as well as a wide range of other protein substrates [5]. Keratinases have represented the only group of proteases with a wide temperature and pH range that allow complete degradation of complex and recalcitrant proteins [6]. The unique characteristic that distinguishes keratinases from other proteases is the ability to bind to complex and insoluble substrates (feathers, wool, silk, collagen, elastin, horns, stratum corneum, hair, azokeratin and nails) [7]. This work is therefore aimed at investigating the potential of keratinases produced by *Bacillus* species isolated from poultry feather waste in stain removal.

## 2. Material and methods

### 2.1. Material

All chemicals used were of analytical grade obtained from Sigma-Aldrich (United States). Keratinases; EZYKer-51, EZYKer-50 and EZYKer-53 from keratin degrading *Bacillus licheniformis*-K51, *Bacillus subtilis*-K50 and *Bacillus* sp.-K53 respectively were obtained from a previous work carried out at the Industrial Microbiology of the University of Ibadan [8].

### 2.2. Preparation of soluble keratin

Keratinase activity was assayed using soluble keratin substrate (0.5% g/v) as substrate and was prepared by the modified method [9]. Ten grams of white chicken feathers was heated in 500 mL of 50% dimethyl sulfoxide (DMSO) at a temperature of 100 °C for two hours. The resulting soluble keratin was obtained by precipitation with 1 L acetone at freezing temperature for 6 h. the precipitate was obtained by undergoing centrifugation at 10,000 x g for 10 min. The resulting precipitate was washed three times with water and dried at 70 °C for 24 h in an oven dryer. One gram of the obtained precipitate was dissolved in 20 mL of 0.05M NaOH. Using 0.1M Tris and 0.1M HCl the pH was adjusted and stabilized at 8.0 and the solution was made up to 200 mL with the same buffer.

### 2.3. Determination of keratinolytic activity

The keratinolytic activity of the bacterial enzyme was assayed as follows: One milliliter of the purified enzyme which has been properly diluted in Tris-HCl buffer (0.05M pH 8.0) was incubated with 1 mL keratin solution at a temperature of 50 °C for ten min in a water bath. The reaction was terminated by the addition of 2.0 mL of 0.4 M Trichloroacetic acid (TCA). After centrifugation at speed of 1450 xg for 30 min, the absorbance of the supernatant was determined at a wave length of 280 nm against a control which was prepared by incubating the enzyme solution with 2.0 mL TCA without the addition of keratin solution.

One unit (U/mL) of keratinolytic activity was defined as an increase in corrected absorbance of 280 nm ($A_{280}$) [10] with the control for 0.01 per minute and calculated by the following equation:

$$U = \frac{4n \times A_{280}}{(0.01 \times 10)}$$

Where; n=dilution rate, 4=final reaction volume (4 mL), 10 = incubation time (min)

### 2.4. Application of Keratinase in Detergent Formulation

#### *2.4.1. Effect of Detergent Components on Keratinase activity*

The effect of the individual detergent components on the keratinase activity of both crude and purified enzyme was carried out by a modified method of [11] by incubating 2% Keratinase with the keratin preparation containing, separately, the following detergent components; linear alkylbenzene sulfonate LAS (15%), KOH (0.5%), $H_2O_2$ (7%), Liquid Paraffin (5%), $Na_2CO_3$ (50%), and Fragrance (0.6%) then assayed for enzyme activity.

#### *2.4.2. Effect of Combined Detergent Ingredients on Keratinase Activity*

The effect of the combined detergent components on the keratinase activity of both crude and purified enzyme was carried out by incubating the 2% Keratinase with the keratin preparation containing the combined detergent components; linear alkylbenzene sulfonate LAS (15%), KOH (0.5%), $H_2O_2$ (7%), Liquid Paraffin (5%), $Na_2CO_3$ (50%), and Fragrance (0.6%) then assayed for enzyme activity.

*2.4.3. Detergent Formulation*

The ingredients for liquid detergent formulation include the following: Linear alkyl benzene sulfonate LAS (15%), Potassium hydroxide (4.7 Molar) KOH (0.5%), Hydrogen peroxide $H_2O_2$ (7%), Liquid Paraffin (5%), Sodium carbonate $Na_2CO_3$ (50%), Fragrance (0.6%) and Enzyme (2%).

*2.4.4. Soap-mix manufacture and Detergent Ingredient mixing*

The aqueous base solution was made by slowly adding 4.7 Molar KOH to water while stirring with a glass stirring rod until dissolved and allowed to cool to 33-43 °C. The olive oil is placed on a beaker and heated on a hotplate to 33-43 °C. The oil and the base are mixed at similar temperature while slowly pouring the base solution into oil. The mixture is further heated and linear alkyl benzene sulfonate LAS, $H_2O_2$, Liquid Paraffin, $Na_2CO_3$, and Fragrance are added at 75-80 °C and mixed gently. The mixture is cooled, and Keratinase is added.

*2.4.5. Inactivation and Replacement of Endogenous proteases of commercial detergent*

To replace the endogenous enzyme in the commercial detergent with Keratinase, the commercial detergent was heated for 1h at 65 °C prior to the addition of Keratinase

*2.4.6. Wash Performance analysis test of Keratinase*

Clean white cloth was cut to small sizes of about 10-15 $cm^2$ in area and placed in Petri-dishes. The cloth pieces were each stained with 0.5 mL of fresh blood and allowed to stay for 24 h. The cloth pieces were treated separately with 2% Keratinase only, commercial detergent only, formulated detergent (containing 2% Keratinase), commercial detergent (with replaced enzyme) and ordinary tap water (control). The treatment was observed for 24 h without agitation after which it was gently drained, rinsed and allowed to dry.

### 2.5. Statistical analysis

The data obtained from this experiment were analyzed by one way analysis of variance (ANOVA) and means of differences among treatment were examined using Duncan's multiple rage test at $p<0.05$.

## 3. Results

### 3.1. Effect of Detergent Ingredients on Keratinase Activity

The effect of individual detergent ingredients (applied at formulation concentrations) on keratinase activity for crude and purified enzyme is presented in Table 1.

EZYKer-51 showed a significantly higher ($P\leq 0.05$) enzyme activity in the presence of the detergent ingredients for both the crude and purified forms of the enzyme compared with the activities of EZYKer-50 and EZYKer-53 except for the activities ($8.02\pm1.32$ U/mL and $7.94\pm0.63$ U/mL) in the presence of 15% LAS and 5% liquid paraffin where there was no significant difference ($P\leq 0.05$) in the enzyme activities of EZYKer-50 for their respective crude and purified forms. EZYKer-53 showed lowest keratinase activities with all the detergent ingredients for both crude and purified forms of the enzymes compared to EZYKer-50 and EZYKer-51 except in the presence of 0.5% KOH and 50% $Na_2CO_3$ where the purified form of the EZYKer-50 showed lowest activities of $3.11\pm0.87$ U/mL and $7.02\pm0.46$. U/mL.

From the table, the purified form of EZYKer-50 showed significantly higher ($P\leq 0.05$) enzyme activities than the crude form in the presence of 15% LAS and 50% $Na_2CO_3$ while activities were significantly higher in the crude than purified forms in the presence of 0.5% KOH and 7% $H_2O_2$. The observed difference in the enzyme activities for both crude and purified forms in the presence of 5% liquid paraffin and 0.6% Fragrance was not significant ($P\leq 0.05$).

Except in the presence of 50% $Na_2CO_3$ and 0.6% Fragrance, where enzyme activity of the purified form of EZYKer-51 enzyme was significantly higher ($P\leq 0.05$) than the crude form, and 0.5% KOH where the observed difference in activity for crude and purified enzyme forms was not significant ($P\leq 0.05$), the crude form of EZYKer-51 showed significantly higher ($P\leq 0.05$) keratinolytic activity than the purified form. For EZYKer-53, the purified form of the enzyme gave significantly higher ($P\leq 0.05$) enzyme activities in the presence of 0.5% KOH, 50% $Na_2CO_3$ and 0.6% Fragrance than the crude form of the enzyme, while, significantly higher ($P\leq 0.05$) keratinolytic activities were obtained for crude form in the presence of 15% LAS and liquid paraffin. The observed difference in the enzyme activities in the presence of 7% $H_2O_2$ was not significant ($P\leq 0.05$).

The result also shows that EZYKer-50, EZYKer-51 and EZYKer-53 showed lower keratinase activities in the presence of detergent ingredients than with the control for both crude and purified enzymes. Generally, of the six ingredients, 0.5% KOH gave lowest keratinase activities for both crude and purified forms of the enzymes with EZYKer-50, EZYKer-51 and EZYKer-53 showing activities of 4.40 U/mL and 3.11 U/mL, 5.63 U/mL and 5.09 U/mL, 3.33 U/mL and 4.60 U/mL for crude and purified forms respectively. Except for the crude form of EZYKer-53 enzyme which showed highest enzyme activity in the presence of 7% $H_2O_2$, the enzymes showed highest enzyme activities in the presence of 50% $Na_2CO_3$ compared to other components for both crude and purified forms of the enzymes

### 3.2. Combined Effect of All Detergent Ingredients on Keratinase Activity

The effect of combining detergent ingredients on keratinase activity of the crude and purified forms of the enzymes is shown in figure 1. For the three enzymes, keratinase activity for both crude and purified enzyme was lower on addition of all detergent ingredients than in the control (without any of the detergent ingredients). The purified form of the three enzymes gave higher keratinase activity than the crude form. EZYKer-51 gave highest keratinase activity of 13.88 U/mL and 10.35 U/mL for purified and crude enzymes respectively, while EZYKer-53 gave least keratinase activity of 11.80 U/mL and 9.15 U/mL for purified and crude enzymes respectively.

### 3.3. Wash performance Analysis Test of Keratinase

**Table 1** Effect of Detergent Ingredients (at Formulation Concentration) on Keratinase Activity for Purified and Crude Keratinases

| Enzyme | Form | Ingredient/ Keratinolytic Activity (U/mL) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | LAS (15%) | KOH (0.5%) | $H_2O_2$ (7%) | Liquid Paraffin (5%) | $Na_2CO_3$ (50%) | Fragrance (0.6%) | Control |
| EZYKer-50 | Crude | $8.39\pm0.11^{d}$ | $4.60\pm1.09^{a}$ | $9.15\pm1.47^{e}$ | $7.82\pm1.17^{c}$ | $6.13\pm0.22^{b}$ | $11.31\pm0.41^{g}$ | $19.02\pm0.74^{h}$ |
| | Purified | $5.10\pm1.38^{b}$ | $3.11\pm0.87^{a}$ | $8.57\pm0.40^{d}$ | $7.94\pm1.09^{c}$ | $7.02\pm0.46^{c}$ | $11.02\pm0.15^{e}$ | $20.64\pm0.4^{f}$ |
| EZYKer-51 | Crude | $8.02\pm1.32^{b}$ | $5.63\pm0.99^{a}$ | $12.16\pm0.57^{c}$ | $8.72\pm1.10^{b}$ | $8.63\pm0.65^{b}$ | $12.93\pm1.38^{c}$ | $24.76\pm0.9^{d}$ |
| | Purified | $6.86\pm0.57^{b}$ | $5.09\pm0.63^{a}$ | $9.27\pm1.62^{c}$ | $7.94\pm0.63^{b}$ | $12.04\pm1.19^{d}$ | $15.40\pm0.23^{e}$ | $26.31\pm0.64^{f}$ |
| EZYKer-53 | Crude | $7.04\pm0.96^{c}$ | $3.33\pm1.90^{a}$ | $7.82\pm0.41^{c}$ | $7.70\pm1.54^{c}$ | $6.37\pm1.72^{b}$ | $6.01\pm0.77^{b}$ | $18.41\pm0.60^{d}$ |
| | Purified | $5.02\pm0.27^{b}$ | $4.60\pm0.44^{a}$ | $7.05\pm2.23^{d}$ | $6.07\pm0.72^{c}$ | $8.80\pm0.43^{e}$ | $7.40\pm0.77^{d}$ | $23.57\pm019^{f}$ |

LAS: Linear alkylbenzene sulfonate; Values are in means ± standard deviation; at 95% confidence level, means with different / similar superscripts along the same row are significantly different / not significantly different from one another

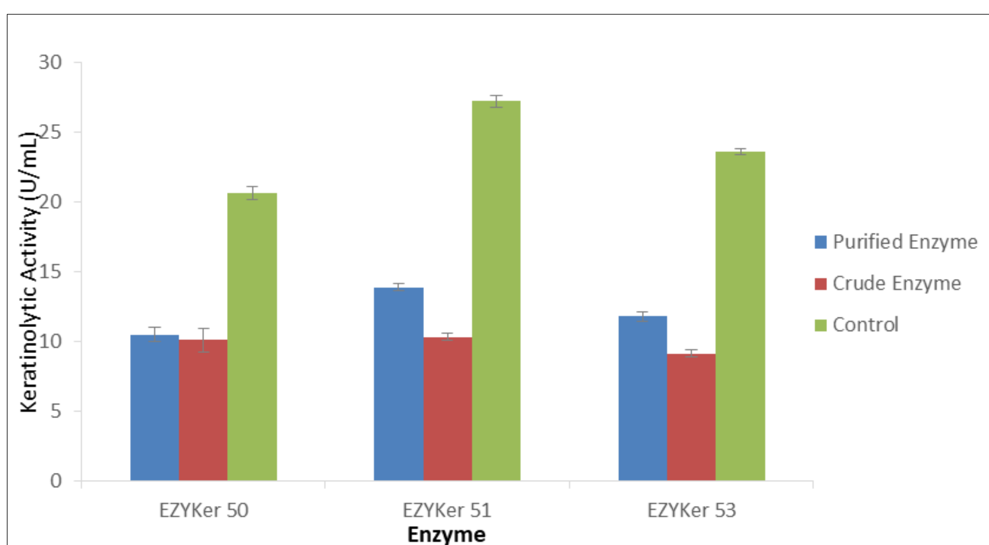



**Figure 1** Keratinase activity of purified and crude enzymes in the presence of all detergent ingredients (at Formulation Concentration)

The result of the wash performance analysis test of purified EZYKer-51 is shown in Figure 1. The blood-stain piece of cloth in Figure 1 A was treated with tap water (control) and the stain remained after 24 h of treatment. When stained cloth was treated with purified EZYKer-51 only (Figure 1 B), the stain immediately started spreading from the point of application and had diffused to the entire piece of cloth after 24h. The stain remained even after rinsing. A nearly complete stain removal is shown in Figure 1 C with treatment of stained cloth piece with commercial detergent (whose endogenous protease has been replaced with EZYKer-51) In Figure 1 D, the cloth was treated with DETKER 51 (formulated detergent containing EZYKer-51) and the result shows that stain was removed partially from the point of application on cloth piece. Treatment of the stained piece of cloth with commercial detergent only (positive control) (Figure 1 E) shows removal of blood stain after 24h of application with little portion of the stain left after rinsing with water.

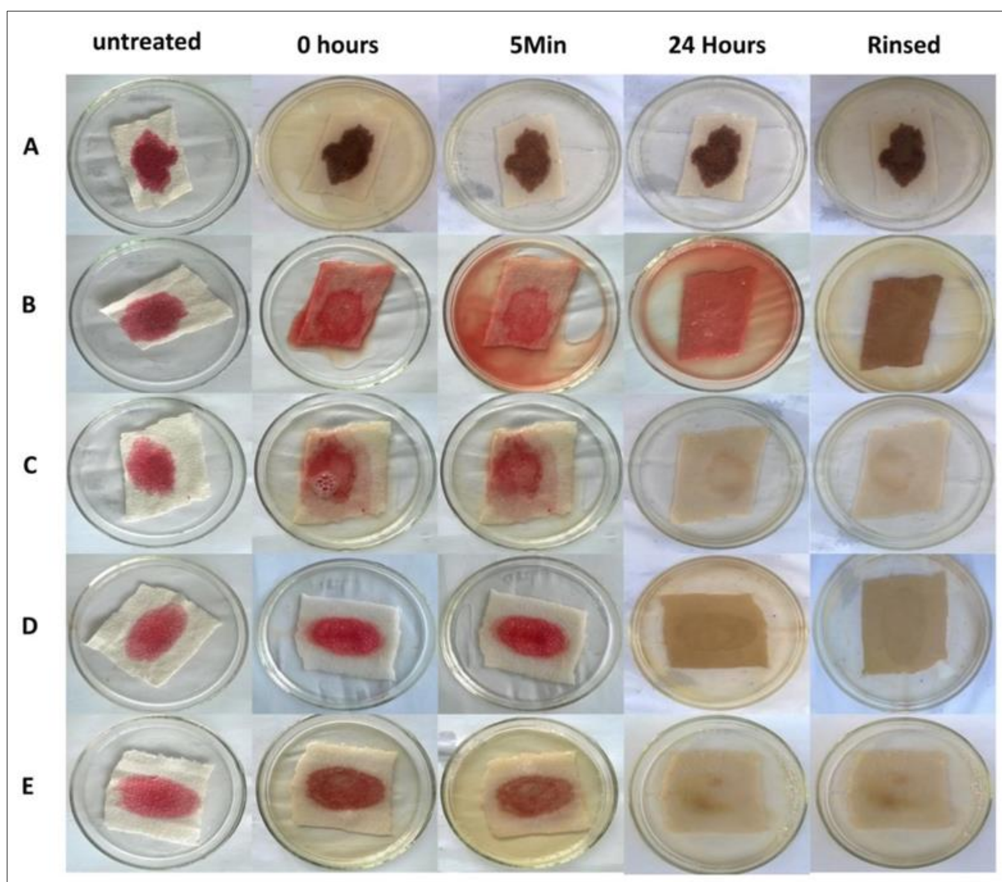



**Figure 2** Wash performance analysis test of Purified EZYKer-51. Blood-stained cloth pieces treated with: (A) tap water: Control, (B) EZYKer-51 only, (C) EZYKer-51 + commercial detergent, (D) DETKER 51 (E) Commercial detergent only

## 4. Discussion

The presence of hydrogen peroxide as a bleaching agent creates an oxidizing environment which may have caused inhibitory effects in the enzyme activity, except in some cases where bleach stability is attained through protein engineering [12]. However, [13] reported wild-type keratinase enzyme with high relative enzyme activities of 155% and 120% in the presence of $H_2O_2$ and LAS respectively when compared to the activities of the non-incubated enzyme. He also reported a low relative enzyme activity of 50% with an alkaline protease in the presence of $Na_2CO_3$, which is in line with the observation made from this study.

Comparatively, in the combined form of the detergent components, the resultant keratinase activities for keratinases EZYKer-50, EZYKer-51 and EZYKer-53 were observed to be higher than most of the activities on the individual detergent components. This observation can be presumably correlated to the resultant interactions between the components of the detergent when combined.

The performance of EZYKer-51 (which performed better compared to keratinase EZYKer-50 and EZYKer-53) in terms of the ability to remove stains revealed that the blood-stain cloth piece was found to be cleaner when soaked with commercial detergent whose indigenous protease has been replaced with EZYKer-51 (Figure 1 c) than when soaked with commercial detergent with indigenous protease intact (Figure 1 e) under the same conditions. This shows that the presence of EZYKer-51 exhibited a better proteolytic ability which translated to a better digestion of the blood stain from the cloth than the indigenous protease present in the commercial enzyme. However, soaking the cloth in EZYKer-51 alone (Figure 1 b) resulted in immediate spreading of the stain from the point of application and diffusion to the entire piece of cloth after 24h, the stain remained even after rinsing. This shows that the enzyme was able to digest the blood but the absence of other detergent components (such as Linear alkylbenzene sulfonate which serves as a surfactant; the active cleaning ingredient which serves as a wetting agent and allows formation of micelle around dirt particles keeping the stains suspended in polar solution, $Na_2CO_3$ which creates the alkaline conditions for alkaline protease to breaks down proteins, and $H_2O_2$ which serves as a bleaching agent, helping to remove stains), which work in synergy with the enzyme may have caused the stain to remain. Similarly, in the bioengineering and industrial application of keratinase from *Bacillus pumilus* by [13], blood and chocolate stain removal achieved with the enzymes alone were more effective than the ones obtained with detergent alone. Furthermore, the combination of the enzyme and the detergent resulted in complete stain removal. In the removal of blood stains from cotton cloth, alkaline protease from *B. brevis* [14] and *Spilosoma obliqua* [15] was observed to be more effective than with detergent.

## 5. Conclusion

Purified keratinases from *Bacillus licheniformis*-K51, *Bacillus subtilis*-K50 and *Bacillus* sp.-K53; EZYKer-51, EZYKer-50 and EZYKer-53 respectively were significantly active in the presence of detergent ingredients. EZYKer-51 demonstrated a more effective wash performance analysis test than the commercial detergent. Overall, the findings provide support for the potential of this enzyme in detergent formulation.

## Compliance with ethical standards

### *Acknowledgments*

Authors wish to thank the University of Ibadan for providing research facilities for the accomplishment of this work.

### *Disclosure of conflict of interest*

The authors declare that they have no competing interests.

## References

[1] Stryer L, Berg JM, Tymoczk, JL. Biochemistry. 5th ed. New York: Freeman; 2002.

[2] Gupta RQ, Beggs, Lorenzo P. Bacteria alkaline protease: Molecular approach and industrial application. Applied Microbiology and Biotechnology. 2002; 59: 16-33.

[3] Gurkok S. Microbial Enzymes in Detergents: A Review Sumeyra GÜRKÖK". International Journal of Scientific and Engineering Research. 2019; 10: 75-81.

[4] Hasan F, Shah AA, Javed S, Hameed A. Enzymes used in detergents: Lipases. African Journal of Biotechnology. 2010; 9: 4836-4844.

[5] Lin HH, Yin LJ, Jiang ST. Functional expression and characterisation of keratinase from *P. aeruginosa* in *Pichia pastoris*. Journal of Agricultural and Food Chemistry. 2010; 57: 5321-5325.

[6] Vidmar B, Vodovnik M. Microbial Keratinases: Enzymes with Promising Biotechnological Applications. Food technology and biotechnology. 2018; *56*: 312–328.

[7] Gupta R, Ramnani P. Microbial keratinases and their prospective applications: an overview. Applied Microbiology and Biotechnology. 2006; 70: 21–33.

[8] Dada MT, Wakil S. Production, Purification and Characterisation of Keratinases from *Bacillus* species Isolated From Poultry Feather Waste. Scientific Research Journal. 2020; 8: 3-98.

[9] Wawrzkiewiez K, Wolski T, Lobarzewski J. Screening the keratinolytic activity of dermatophytes *in vitro.* Mycopathologia. 1987; 114: 1-8.

[10] Gradisar H, Kern S, Friedrich J. Keratinase of *Doratomyces microspores*. Applied Microbiology and Biotechnology. 2000; 53: 196-200.

[11] Jaouadi NZ, Rekik H, Badis A, Trabelsi S, Belhoul M, Yahiaoui AB, Ben Aicha H, Toumi A, Bejar S, Jaouadi B. Biochemical and molecular characterisation of a serine keratinase from *Brevibacillus brevis* US575 with promising keratin-biodegradation and hide-dehairing activities. Public Library of Science. 2013; 2: 72-726.

[12] Radha S, Gunasekaran P. Cloning and expression of keratinase gene in *Bacillus megaterium* and optimization of fermentation conditions for the production of keratinase by recombinant strain. Journal of Applied Microbiology. 2012; 103: 1301-1310.

[13] Jaouadi B, Abdelmalek B, Fodil D, Ferradji FZ, Rekik H, Zaraî N, Bejar S. "Purification and characterisation of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. *Bioresource Technology*. 2012; 101: 8361-8369.

[14] Banerjee U, Sani R, Azmi W, Soni R. Thermostable alkaline protease from *Bacillus brevis* and its characterisation as a laundry detergent additive. Journal of Process Biochemistry. 1999; 35: 213-219.

[15] Anwar A, Saleemuddin M. Alkaline protease from *Spilosoma obliqua*: Potential applications in bio-formulations. Biotechnology and applied biochemistry. 2000; 31: 85-89.