Journal of Advanced Biomedical and Pharmaceutical Sciences

Journal Homepage: http://jabps.journals.ekb.eg

Optimization of azo-keratin hydrolysis by alginate-immobilized Keratinase produced from *Bacillus licheniforms*

Hesham M. El-Komy[1*], Sahar K. Al-Dosary[1], Mohamed A. El-Naghy[2], Mohamed A. A. Abdelhamid[2], Mohamed M. Immam[2]

[1] Department of Biology, Faculty of Science, Imam Abdulrahman Bin Faisal University, Saudi Arabia.
[2] Department of Botany and Microbiology, Faculty of Science, Minia University, 61519 Minia, Egypt

Received: November 23, 2018; revised: December 27, 2018; accepted: January 1, 2019

Abstract

Recently, more attention to Keratinolytic materials which can be converted into feed-stuffs, biofertilizers, glues, and foils or used for production of amino acids. Here, we determined the optimum conditions for keratin hydrolysis by alginate-immobilization of crude keratinase isolated from *Bacillus lichenoformis*. The results of this study indicated that the optimum pH for keratin degradation by immobilized keratinase enzyme was pH 8. Moreover, the crude enzyme was stable for 2 hours at pH 4, 7 and 10 and retained 55.5 %, 60 % and 53 %, of its activity respectively for immobilized enzyme while free enzyme retained only 40 %, 40 % and 61 %, respectively. The optimum temperature for keratinase activity was 50 ^{o}C when immobilized in alginate. At temperatures 30, 45 and 60 ^{o}C, keratinase was stable for 2 hours and retained 57 %, 66 % and 57 %, of its activity respectively for immobilized enzyme while free enzyme retained only 51 %, 62 % and 51 %, respectively. Furthermore, the effects of activators and inhibitors on the enzyme activity have been investigated.

Key words
Keratinase, Bacillus lichenoformis, immobilization, azo-keratin

1. Introduction

The resistance of keratinolaceous substances to hydrolysis by trypsin, pepsin, and papain could be attributed to the high degree of bisulphite linkage and its hydrophilic characteristics [1-3]. Different researchers are interesting in the recovery of protein from feather wastes [4,5]. Several microorganisms isolated from poultry wastes have the ability to produce keratinolytic enzymes [6,7]. Many bacteria, actinomycetes and fungal species can degrade keratin [8-10]. Effective Keratinase from *B. licheniformis and B. subtilis* is used in feather industries [9,11]. Previously, we reported the isolation, characterization of different *Bacillus* isolates with high activity of feather and azo-keratin degrading capacity [12,13].

Keratinase (E.C.no.3.4.99.11) is a group of proteinase used in hydrolysis of feather, hair, wool and in sewage water treatment [14]. While the nature of keratinase is still exactly unknown, it can effectively degrade feather insoluble keratin into useful meal [15], nitrogen fertilizers, biodegradable films, glues and foils [16, 17]. Keratinase is used effectively in traditional industries such as medicine and leather industries [1,18], as well as plastic, feather meal industries [17]. New fields of keratinase application are discovered as they degrade prions [19].

Immobilization of microbial cells and functional proteins becomes one of the most valuable tools in the field of biotechnology. Entrapment of microbial *Bacillus* spp. has been reported to improve the production of keratinolytic enzymes [13]. Moreover, immobilized enzymes have been used on various carriers e.g. sintered glass beads [20]. For industrial applications, enzyme immobilization may offer several advantages such as, reusability of the enzyme and improvement of enzyme stability [21]. In this study, we investigated the main characteristics of free and alginate-immobilized keratinase for azo-keratin hydrolysis.

2. Materials and Methods

2.1. Preparation of Crude Enzyme

The preparation of crude enzyme was performed according to [22], the microorganism used in this study was *Bacillus licheniformis* isolate No.3 isolated from poultry waste [12]. Cells were grown in sterile feather culture medium containing 0.5 g of NH_4Cl, 0.5 g of NaCl, 0.5 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.1 g of $MgCl_2.6H_2O$, 0.1 g of yeast extract, and 10 g of white chicken feathers per liter. The pH was adjusted to 7.5 using a pH meter. After 3 days of incubation at 40°C, the culture was centrifuged at 10,000 rpm for 20 min. The supernatant was filtrated through filter-sterilized (Millipore, 0.2 µm). Furthermore, the culture fluid was pre-concentrated twice per volume using an ultra-centrifugal filter (10kDa cutoff) and used as the crude enzyme.

2.2. Entrapping of Keratinase in Alginate

Sterilized crude keratinase was immobilized by entrapping in 2 % Ca-alginate by the method used at our laboratory [23]. The immobilization efficiency of the crude keratinase was calculated

**** Correspondence: Hesham M. El-Komy***
Tel.: *+966 50 387 2662* ***; Fax:*** *+20 862363011*
Email Address: *mfarrag83@yahoo.com*

by measuring the activity of enzyme before and after entrapping in alginate.

3. Factors affecting keratinase activity and stability

3.1. Effects of pH

The effect of pH on free and immobilized enzyme activity was assayed using keratin azure as a substrate. Keratinase activity was studied in the pH range from 3.0 to 10.0, using the following buffers (50 mM); potassium phosphate (KOH/KH_2PO_4) (pH=6-8), Tris-HCl (pH=9.0), Glycine/NaOH (pH, 10) [24].

3.2. The pH stability

Free and immobilized keratinase were pre-incubated at 45 ^oC and different pHs (pH 4,7and 10). Samples were taken at different time intervals (30, 60, 90 and 120 min) to measure the residual enzyme activities using the methods described before.

3.3. Effects of temperature

The optimum temperatures for free and immobilized keratinase activity were determined at different incubation temperatures between 25 and 65 ^oC at 5 ^oC intervals.

3.4. Thermal stability

The free and immobilized crude enzyme samples were incubated at several temperatures from 25 to 65 ^oC in 50 mM potassium phosphate buffer (pH 7.5). At different time intervals (30, 60, 90 and 120), the remaining activity was measured at the optimum conditions of enzyme assay.

3.5. The effects of some chemicals on keratinase activity

Crude and immobilized culture supernatant fluids were pre-incubated at room temperature for 5 min with different additives. These additives were prepared in two different concentrations prior to measurement of keratinase activity [24, 25]. These additives were tested at 5 mM/l concentration, except for NaCl, which in addition to this concentration was tested at 5 to 15 mM/1. These compounds were; EDTA; $FeSO_4$; $HgCl_2$; $ZnSO_4$; $CuCl_2$; CaCl2; $MnCl_2$; KCl; urea; $NiCl_2$; $MgSO_4$ and NaN_3.

3.6. Repeated use of immobilized enzyme

Reusability of the immobilized crude enzyme was tested by repeated measurement of the entrapped enzyme activity on fresh substrate contained in the buffer. The enzyme activity assay procedure was as previously described for each set.

3.7. Determination of soluble proteins

The protein content of culture filtrate was determined according to Lowery et al. [26].

3.8. Determination of keratinase activity

Keartinase activity was determined using azo-keratin as described by Saieb [13]. A unit of keratins activity was defined as a 0.01-unit increase in the absorbance at 450 nm as compared to the control after 15 minutes of reaction.

3.9. Statistical analysis

The triplicate sets of data for the various parameters evaluated were subjected to ANOVA (Analysis of variance) in accordance with the experimental design (Completely Randomized Design) using SPSS11 statistical packages to quantify and evaluate L.S.D values were calculated at P level of 0.05 %. All the tested samples are expressed as mean values with standard deviations of three replicates.

4. Results

4.1. Effect of different factors on the activities of free and immobilized crude keratinase enzyme

4.1.1. Effect of pH

(Figure 1) indicated that both free and immobilized keratinase showed different pH optima, both free and immobilized enzyme activities increased with increasing pH, up to pH 8-9. The maximum keratins activity of the free enzyme was at pH 8 and the maximum keratinase activity of immobilized enzyme was pH 7.5. **(Figure 1)** also indicated that immobilized keratinase enzyme (KE) showed high activity in the range pH 7-8 and that the activity decreased at higher or lower pH values. The free enzyme although showed lower activities at the different pH values than the immobilized one, it was more stable at pH 7-9.

4.1.2. The pH stability

Results in **(Figure 2)** showed that the free and immobilized enzymes were fairly stable at alkaline pH values than acidic pH values and the immobilized enzyme was more stable at pH 7.0 whereas the free enzyme was more stable at pH 10 in absence of substrate. At pH 4 the free enzyme retained only 40 % of its activity after 120 min while the immobilized enzyme retained 55.5 % after the same time of exposure whereas at pH 7 the free enzyme retained only 40 % of its activity after 120 min while the immobilized enzyme retained 60 % after the same time of exposure. On the other hand, at pH 10 the free enzyme retained 61.4 % of its activity after 120 min while the immobilized enzyme retained only 53.3 % after the same time of exposure.

4.1.3. Effects of temperature

(Figure 3) indicated that both free and immobilized keratinase showed different optimal temperature, and that the activities of both enzymes increased with increasing the temperature, up to 45-50 ^oC. Maximum keratinase activity for free enzyme was at 45 ^oC and that for immobilized enzyme was at 50 ^oC. (**Figure 3)** also showed that the immobilized enzyme was more active in the range of 50-60 ^oC and the free enzyme from 45-55 ^oC. The immobilized KE relatively was more stable than soluble KE at higher temperatures.

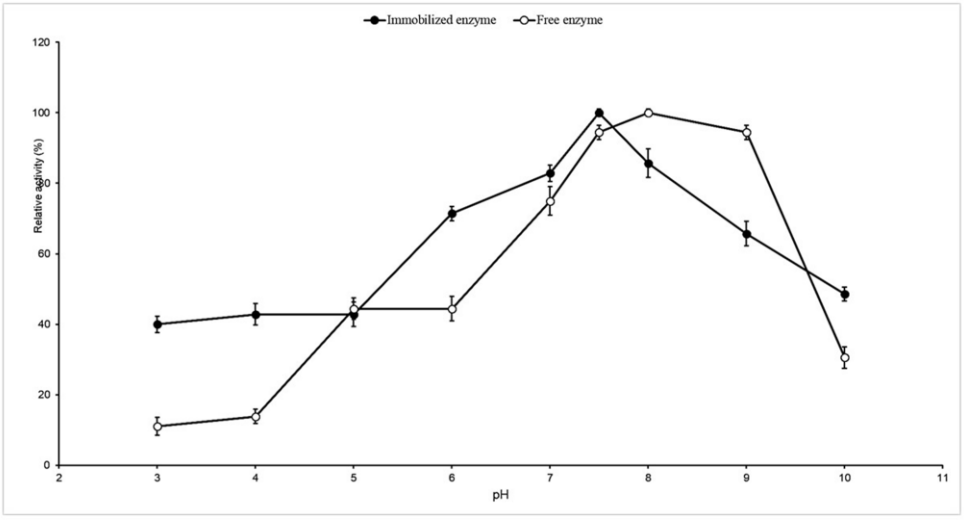

Figure 1: Effect of buffer pH on the activity of free and immobilized enzyme.

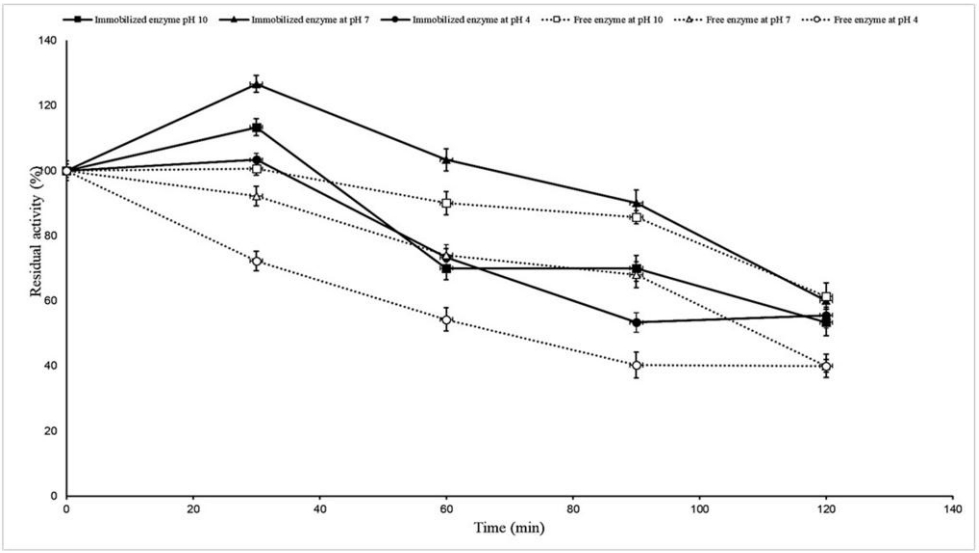

Figure 2: pH stability of free and immobilized enzyme.

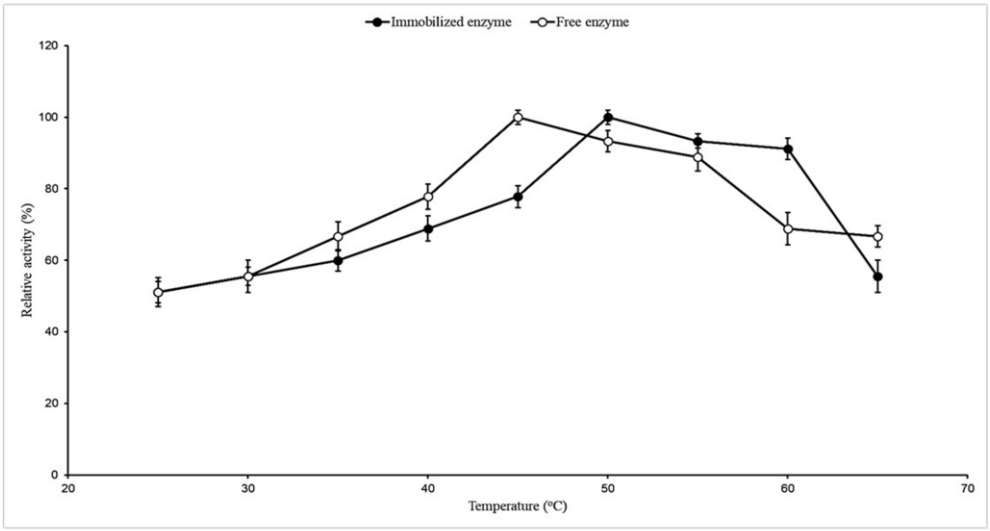

Figure 3: Effect of temperature on the activity of free and immobilized enzyme.

4.1.4. The thermal stability

Results presented **(Figure 4)** showed that free and immobilized enzymes were stable to heat treatment. At 45 ^{o}C, the immobilized enzyme was stable and retained 66.7 % of its activity after 120 min while the free enzyme retained 62 % after the same time of exposure. The free enzyme retained 51 % of its activity when treated at 30 and 60 ^{o}C for 120 min. The immobilized enzyme however retained 57 % of its activity when incubated at 30 and 60 ^{o}C for 120 min.

4.1.5. Effect of some chemicals on keratinase activity

The activity of the free enzyme was strongly inhibited by $NiCl_2$, Urea, $HgCl_2$, NaN_3 and KCl, while the inhibitory effect was less in case of immobilized enzyme. The highest activity of free crude enzyme appeared in presence NaCl of (10 mM) which recorded 150 % but was strongly inhibited by $NiCl_2$ (5 mM) and the activity decreased to 12 %. High activity of immobilized crude enzyme appeared with NaCl (5 mM) which recorded 130 %, and in case of $MgSO_4$, which recorded 117.9 % as well as NaCl (10 mM), KCl, $CaCl_2$, $CuCl_2$ and $HgCl_2$. Partial inhibition of immobilized crude enzyme activity was observed by the addition of $FeSO_4$ (5 mM) and Urea (5 mM) in presence of which the enzyme retained only 40 % and 47 % of its activity respectively. **(Table 1)** also showed that immobilized crude enzyme was more stable against the toxic effect of the added inhibitors than the free one. Addition of NaN_3, Urea or $FeSO_4$ exerted an inhibitory effect to the immobilized enzyme and were toxic to the free crude enzyme.

4.1.6. Repeated use of immobilized enzyme

The durability of the immobilized crude keratinase preparation was revealed by using the beads entrapping crude keratinase successfully for 9 successive times **(Figure 5)**. Results showed that about 55 % of initial activity was retained after 7 successive reuses and reached to about 42 % after the last cycle (9th reuse).

5. Discussion

The process of enzymes isolation and purification makes them highly expensive catalysts. Therefore, several approaches such as enzyme immobilization have been developed to enhance enzyme activity, resist environmental fluctuations, prolong its activity and enable enzyme recovery. The prolonged activity of bead-entrapped enzymes may be attributed to the partial release of the enzyme in the supernatant [27]. In the current study, an important industrial enzyme, keratinase, was efficiently immobilized into calcium alginate beads. Efficient immobilization using the same approach was achieved for other enzymes such as estrase [28] and β-1, 3-glucanase [21].

The sensitivity of both free and immobilized keratinases to different environmental factors such as temperature, pH, and metal ions were assessed. The activity of the free keratinase produced by *Bacillus licheniformis* isolate No. (3) was between pH values 7 and 9 with optimum at pH 8.0. For the immobilized keratinase, the activity was at pH values 7 and 8 with optimum pH at 7.5 (**Figure 1**). Keratinases isolated from different Bacillus strains showed somewhat different optimum pH values. The optimum pH for keratinase enzyme of *Bacillus pumilus* strain A1 was 9 [29], keratinase (KerQ7) of *Bacillus tequilensis* Q7, was 7.0 [30], *B. licheniformis* keratinase was at pH 7.5 [13] and *B. pumilis* keratinolytic alkaline serine protease optimum activity was at pH 10 [31]. Although these keratinases showed an optimum activity at a single pH value, other keratinases such as those isolated from *Kocuria rosea* displayed a full activity at a wide range of alkaline pH (10–11). The stability of this enzyme in extreme pH values makes it suitable for industrial activities such as leather industry and bioremediation processes [30].

Our immobilized keratinase enzyme was more sensitive to high alkaline pH than acidic one and was more stable than the free enzyme in acidic pH (**Figure 2**). This finding is in consistence with the findings of others. Anbu et al. [32] reported that the pH stability of immobilized crude keratinase was higher than the free enzyme at pH values of 4 and 7. Although bead-entrapped enzyme activity was reduced due to diffusion during the 2 h of storage in different buffers, the enzyme activity remained stable at pH values of 6.0–8.5 [32]. Usually the immobilized enzyme has a shift in its optimum temperature to higher values than the free one and this could result in a positive effect on its productivity [21]. In the current study, we also investigated the optimum temperatures of both crude free and bead-entrapped keratinases. The activity of both enzymes was increased with temperature with 45 °C optimum temperature for the free enzyme 50 °C for the immobilized one. Our results are in line with other keratinase preparations from *Bacillus subtilis* [33] and *Brevibacillus brevis* [34].

As in the case of pH stability, in comparison with the free enzyme, the beads entrapped-keratinase displayed heat stability even in the absence of its substrate (**Figure 3**). At higher temperature 60 ^{o}C the immobilized enzyme showed high activity in thermal stability (half-life) than free one (**Figure 4**). In addition, improvement in stability by immobilization is well known [35]. This higher stability could be attributed to the bead which may protect the enzyme from heat denaturation effect [36]. Another possible factor is that beads protect the immobilized enzyme from digestive protease associated with the crude sample [21].

In our study, in addition to urea, we also investigated the effect of metal ions such as $NiCl_2$, $HgCl_2$, NaN_3, and KCl **(Table 1)**. We found that the enzyme activity was completely inhibited in the presence of such ions. In contrast, the enzyme activity was enhanced in the presence of NaCl (5 mM, 10 mM, 15 mM), EDTA, $MgSO_4$ and $ZnSO_4$ **(Table 1)**. Enzyme encapsulation retained 68.5 %, 113 % and 79.3 % of its activity by the addition of NaN_3, $HgCl_2$ and $NiCl_2$, respectively. Regarding the ions tested, Su et al. [25] established that the presence of Ca^{2+} in the reaction mixture caused a 2.7-fold increase of the keratinase activity. Ca^{2+} as well as Mg^{2+} and Mn^{2+}, has been reported to be associated with metalloproteases and protect these enzymes against thermal denaturation [4, 37]. On contrast, the presence of Zn^{2+}, Al^{3+} and Cu^{2+} caused an inhibitory effect on keratinase

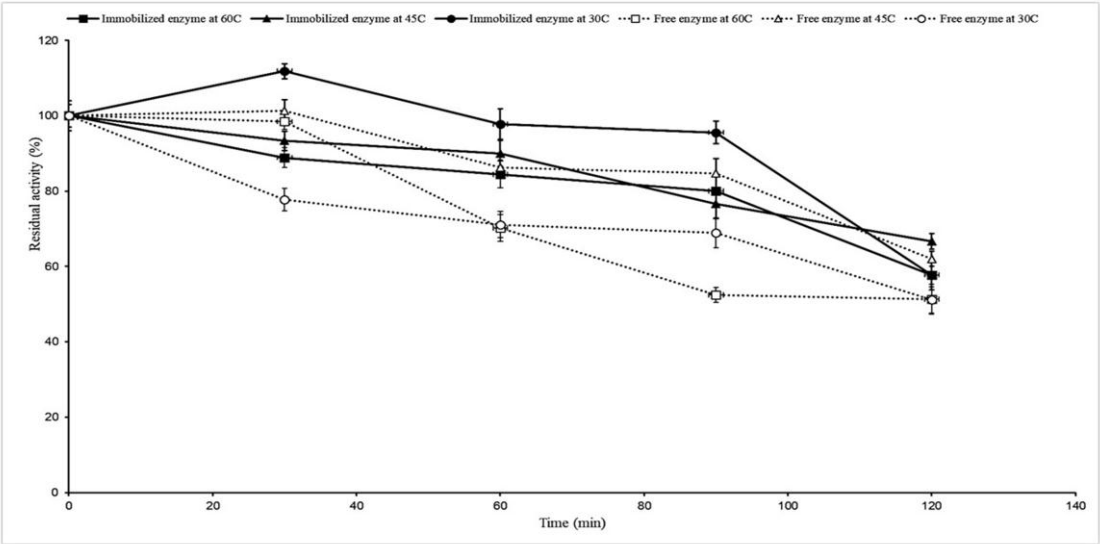

Figure 4: Thermal-stability of free and immobilized enzyme.

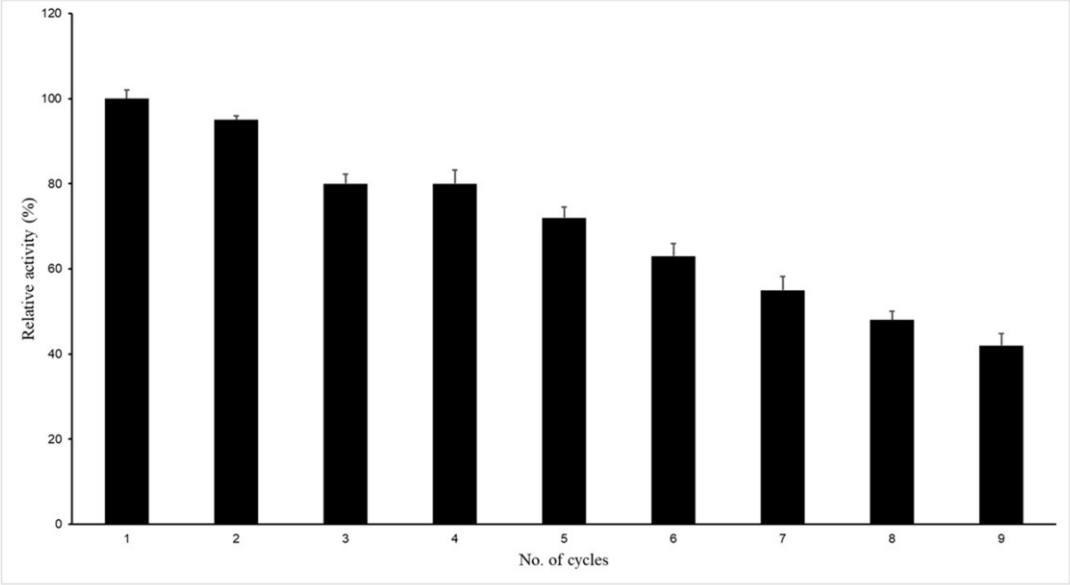

Figure 5: Reusability of immobilized keratinase through 9 cycles

Table 1: Effect of some chemicals on free and immobilized enzyme activity

Chemicals	Concentration	Free enzyme activity		Immobilized enzyme activity	
		(U/ml)	(%)	(U/ml)	(%) of control
None (control)	-	250	100	700	100
NaCl	5 mM	200	80	915	130.7
NaCl	10 mM	375	150	795	113.5
NaCl	15 mM	225	90	570	81.42
EDTA	5mM	235	94	630	90
$MgSO_4.7H_2O$	5 mM	175	70	825	117.9
$ZnSO_4.7H_2O$	5 mM	165	66	480	68.57
$MnCl_2.2H_2O$	5 mM	115	46	615	87.86
$CuCl_2.2H_2O$	5 mM	95	38	795	113.6
$FeSO_4$	5 mM	90	36	285	40.71
$CaCl_2.2H_2O$	5 mM	85	34	765	109.3
KCl	5 mM	65	26	735	105
NaN_3	5 mM	65	26	480	68.57
$HgCl_2$	5 mM	50	20	795	113.6
Urea	5 mM	50	20	330	47.14
$NiCl_2.6H_2O$	5mM	30	12	555	79.26
$LSD_{0.05}$	-	3.25	-	4.85	-
$LSD_{0.01}$	-	4.40	-	6.57	-

activity. Partial inhibition of most serine enzymes by EDTA indicating the importance of cations as stabilizing agents has been reported [4]. It was reported that keratinases are generally activated with divalent metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} [25], and inhibited with heavy metal ions such as Cu^{2+}, Hg^{2+}, Zn^{2+} [11], Ag^{+} and Pb^{+} [1].

References

[1] Farag A M, Hassan MA. Purification, characterization, and immobilization of a keratinase from *Aspergillus orizae*. *Enzyme Microb. Technol*. 2004; 34: 85–93.

[2] Jin H, Park SY, Kim K, Lee YJ, Nam GW, Kang NJ, Lee DW. Development of a keratinase activity assay using recombinant chicken feather keratin substrates. *PLoS ONE*. 2017; 12(2): e0172712.

[3] Wang B, Yang W, McKittrick J, Meyers MA. Keratin: Structure, mechanical properties, occurrence in biological organisms, and efforts at bioinspiration. *Progress in Materials Science*. 2016; 3:229–318.

[4] Oliveira CTD, Rieger TJ, Daroit DJ. Catalytic properties and thermal stability of a crude protease from the keratinolytic *Bacillus* sp. CL33A. *Biocatalysis and Agricultural Biotechnology*. 2017; 10: 270–277.

[5] Osman Y, Elsayed A, Mowafy AM, Elrazak AA, Fawzy M. Bioprocess enhancement of feather degradation using alkaliphilic microbial mixture. *British Poultry Science*. 2017; DOI: 10.1080/00071668. 1278627.

[6] Bohacz J. Biodegradation of feather waste keratin by a keratinolytic soil fungus of the genus *Chrysosporium* and statistical optimization of feather mass loss. *World J Microbiol Biotechnol*. 2017; 33:13. doi: 10.1007/s11274-016-2177-2

[7] Tamreihao K, Devi LJ, Khunjamayum R, Mukherjee S, Ashem RS, Ningthoujam DS. Biofertilizing potential of feather hydrolysate produced by indigenous keratinolytic Amycolatopsis sp. MBRL 40 for rice cultivation under field conditions. *Biocatalysis and Agricultural Biotechnology*. 2017; 10: 317–320.

[8] Bouacem K, Bouanane-Darenfed A, Zarai Jaouadi N, Joseph M, Hacene H, Ollivier B, Fardeau ML, Bejar S, Jaouadi B. Novel serine keratinase from *Caldicoprobacter algeriensis* exhibiting outstanding hide dehairing abilities. *Int J Biol Macromol*. 2016; 86:321–8.

[9] 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. *Int J Biol Macromol*. 87:256–62. doi: 10.1016/j.ijbiomac.2016.02.067 MID: 26940376 53.

[10] Verma A, Singh H, Anwar MS, Kumar S, Ansari MW, Agrawal S (2016) Production of Thermostable Organic Solvent Tolerant Keratinolytic Protease from *Thermoactinomyces* sp. RM4: IAA Production and Plant Growth Promotion. *Front Microbiol*. 2016; 7:1189. doi: 10.3389/fmicb..01189 PMID: 27555836

[11] Thys RC, Lucas FS, Riffel A, Heeb P, Brandelli A. Characterization of a protease of a feather-degrading *Microbacterium* species. *Lett. Appl. Microbiol*. 2004; 39: 181–6.

[12] Abdelhamid MAA. Degradation of keratin by some bacterial isolates. Dissertation, Minia University. Retrieved from 2009; *http://www.eulc.edu.eg/eulc_v5/Libraries/Thesis*;

[13] Saieb FM, Boaker SA, El-komy HM, Issa A. Production and estimation of keratinase by immobilized and free *B. licheniformis* (st. 24). *J. Appl Environ Microbiol*. 2015; 3: 119-122.

[14] Godfrey T. Protease in waste treatment. In: Godfrey T, editor. Industrial enzymology. 1996; London: Macmillan; p. 315–6.

[15] Riffel A, Lucas F, Heeb P, Brandelli A. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. *Arch. Microbiol*. 2003; 179: 258–65.

[16] De Toni CH, Richter MF, Chagas JR, Henriques JAP, Termignoni C. Purification and characterization of an alkaline serine endopeptidase from a feather-degrading *Xanthomonas maltophila* strain. *Can. J. Microbiol*. 2002; 48:342–348.

[17] Jin E, Reddy N, Zhu Z, Yang Y. Graft polymerization of native chicken feathers for thermoplastic applications. *J Agric Food Chem*. 2011; 59(5):1729–38.

[18] Villa ALV, Aragao MRS, dos Santos EP, Mazotto AM, Zingali RB, de Souza EP, Vermelho AB. Feather keratin hydrolysates obtained from microbial keratinases: effect on hair fiber. Bmc Biotechnology. 2013; 13:15

[19] Langeveld JPM, Wang JJ, Van de Wiel, DFM, Shih GC, GarssenGJ, Bossers A, Shih JCH. Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. *J Infect Dis*. 2003; 188:1782–1789.

[20] Wang JJ, Swaisgood HE, and Shih JC. Bioimmobilization of keratinase using *Bacillus subtilis* and *Escherichia coli* systems. *Biotechnol. Bioeng*. 2003; 81: 421–9.

[21] El-katatny M. The activity of β-1,3-glucanse from *Trichoderma harzianum* in native form and after immobilization on calcium alginate. *Archives of Phytopathology and plant protection*. 2008; 41: 175-186.

[22] Suh HJ, Lee HK. Characterization of a keratinolytic serine protease from *Bacillus subtilis* KS-1. *J Protein Chem*. 2001; 20:165–169.

[23] El Komy HM. Survival of and wheat root colonization by alginate encapsulation *Trichoderma* spp. *Folia microbiol*. 2001; 46: 25-30.

[24] Bressollier P, Letourneau F, Urdaci M, Verneuil B. Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. *Appl. Environ. Microbiol*. 1999; 65: 2570–2576.

[25] Su C, Gong JS, Zhang RX, Tao LY, Dou WF, Zhang DD, Li H, Lu ZM, Xu ZH, Shi JS. A novel alkaline surfactant-stable keratinase with superior feather-degrading potential based on library screening strategy. *International Journal of Biological Macromolecules*. 2017; 95:404–411

[26] Lowry OH, Rosenberg WJ, Farr AL, Randell RJ (1951) Quantitation of protein using Folin Ciocalteu reagent. *J. Biol. Chem*. 193: 265–75.

[27] Markvicheva EA, Kuptsova SV, Buryakov AV, Varlamova EA, Dugina TN. Proteases entrapped in polymer composite hydrogels: Preparation methods and applications. Vestnik Moskovskogo Universiteta. Khimiya. 2000; 41: 6.

[28] Lee S-Y, Min BH, Hwang SH, Koo YM, Lee CK, Song SW, Oh SY, Lim SM, Kim SL, Kim DI. Enantioselective production of levofloxin by immobilized porcine liver estrase. *Biotechnol. Lett*. 2001; 23: 1033-1037.

[29] Fakhfakh-Zouari N, Hmidet N, Haddar A, Kanoun S, Nasri M. A novel serin emetallokeratinase from a newly isolated *Bacillus pumilus* A1 grown on chicken feather meal: biochemical and molecular characterization, *Appl. Biochem. Biotechnol*. 2010; 162:329–344

[30] Jaouadi NZ, Rekik H, Ben Elhoul M, Rahem FZ, Hila CG, Ben Aicha HS, Badis A, Toumi A, Bejar S, Jaouadi B. A novel keratinase from *Bacillus tequilensisstrain* Q7 with promising potential for the leather bating process. *Int. J. Biol.Macromol*. 2015; 79: 952–964.

[31] Huang Q, Peng Y, Li X. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Curr Microbiol*. 2003; 43:169–173.

[32] Anbu P, Gopinath S, Hilda A, Lakshmipriya T, Annadurai G. Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis*. *Biores. Technol*. 2007; 98:1298–1303.

[33] Farhadian S, Asoodeh A, Lagzian M. Purification, biochemical characterization and structural modeling of a potential htrA-like serineprotease from *Bacillus subtilis* DR8806, *J. Mol. Catal. B-Enzym*. 2015; 115:51–58.

[34] Jaouadi NZ, Rekik H, Badis A, Trabelsi S, Belhoul M, Yahiaoui AB, BenAicha H, Toumi A, Bejar S, Jaouadi B. Biochemical and molecular characterization of a serine keratinase from *Brevibacillus brevis* US575 with promising keratin-biodegradation and hide-dehairing activities, *Plos One* 2013; 8(10) e76722.

[35] Korkomaz H, Hur H. Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste. *Annals of Microbiology*. 2004; 54: 201-211.

[36] Singh S, Kumar B, Bezbarush R. Calcium alginate as a support material for immobilization of L-amino acid oxidase from *A. fumigatus*. 2012, ISSN: 0976-3104.

[37] Bose A, Pathan S, Pathak K, Keharia H. Keratinolytic protease production by *Bacillus amyloliquefaciens* 6B using feather meal as substrate and application of feather hydrolysate as organic nitrogen input for agricultural soil. *Waste Biomass Valor*. 2014; 5, 595–605.