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Original Article

Efficient Keratinolysis of Poultry Feather Waste by the Halotolerant Keratinase from *Salicola Marasensis*

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Abstract

Sustainable development in the bio-treatment of large-scale biomass bulks requires high performance enzymes adapted to extreme conditions. An extracellular keratinolytic extract was obtained from the culture broth of a halotolerant strain of *Salicola marasensis*. Keratin hydrolyzing activity of the concentrated enzyme extract was observed on a 100 mg of pretreated feather waste. The concentrated enzyme was able to hydrolyze the poultry feathers by 25% after 12 h incubation. The bio-waste material was optimally hydrolyzed at pH 9 and temperature of 40 °C. Among reductants, 1,4-dithiothreitol, L-cysteine, 2-mercaptoethanol, glutathione, and sodium sulfate showed the most remarkable effect on the bio-waste keratinolysis, while the tested surfactants and urea had no significant effect on the keratinolytic activity. Hexane and hexadecane indicated strong effect on keratinase activity and bio-treatment in the presence of 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) as a hydrophobic ionic liquid resulted in a maximal of 80% extraction yield of soluble proteins from feathers. Considering the stability of the extracellular keratinolytic content in [BMIM][PF_6], the observed keratinase activity was noteworthy suggesting that the secreted enzyme may contribute to the bioconversion of feather wastes.

Keywords: *Salicola marasensis*; Halotolerant; Keratinolytic activity; Feather wastes; Ionic liquid.

Introduction

With the ever-increasing of industries based on the recycling of natural wastes such as keratin containing materials, great interest has been drawn on extremozymes during the recent decades, because the adjustment of industrial harsh conditions needs the activity and stability of the applied enzymes in such conditions (1, 2). Therefore, it would be critical to have enzymes which optimally work at extreme pHs, or temperature, as well as high concentrations of salts, organic solvents, surfactants, and oxidizing/reducing agents (3, 4). In addition to being resistant to salt, it has been found that enzymes from halophiles are usually able to survive and carry out reactions efficiently under a variety of other extreme conditions as well (5, 6).

Because of their action on insoluble and recalcitrant protein substrates, the keratinolytic proteases of bacterial origin have been widely employed in industrial processes of detergent, leather, pearl, pharmaceuticals, cosmetics, and textile manufacturing, as well as biofuel,

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biofertilization, bioremediation, and drug delivery, etc. (7, 8). Specifically, keratinolytic proteases offer considerable opportunities for an eco-friendly and low-energy consuming method to convert keratinous materials (one of the main by-product of poultry industry) to valuable products, especially in the form of protein- and amino acids-rich feed and organic soil fertilizer (9, 10).

Halophilic proteases with keratinolytic activity not only carry out the identical enzymatic roles as their non-halophilic counterparts but also adapt to the extreme environments and are unusually stable. This property makes them suitable candidates to catalyze under the harsh conditions (11-13). As the biodegradation of keratins with high disulfide content is hampered by hindering the access of protein-degrading enzymes to the peptide bonds, the coupling of disulfide bonds reduction using the chemical reductants with enzymatic hydrolyzing of peptide bonds could be an effective method to recycle from the keratinous wastes (9, 10). To solve the environmental problems of the chemical materials, a number of desirable green salts called ionic liquids (ILs) have been recently recognized as solvents to dissolve the recalcitrant and insoluble biopolymers (14). However, it was found that ILs can only dissolve the biopolymers at high temperatures and in a relatively long period of time (15). Therefore, to develop an effective process to employ keratinolytic proteases under the suitable and cost-effective conditions, a combination of the stable keratin degrading enzymes and the room temperature ILs on the pretreated feathers might be applied (15). Considering that ILs are expensive and the separation of protein extract from the hydrophilic ILs is a challenging issue, applying the hydrophobic ones could be a more interesting target because the hydrophobic ILs can be easily recovered and reused from the aqueous phase containing the soluble proteins (11, 13 and 16). As the halophilic enzymes are hypothesized to be suitable candidates for screening the IL-resistant enzymes, the search for new promising halophilic proteases with raised stability against salts (such as ILs) is a continuing process aimed at improving their adoption in the special processes (17).

The current study aimed to apply a recently isolated halotolerant strain of *Salicola marasensis* capable to produce keratinolytic enzyme. The extracellular keratinase was then concentrated, characterized, and its stability against pH, temperature, salts, metal ions, chemicals, surfactants, and solvents was examined. Considering the high protein content of the keratin wastes and stability of the keratinolytic extract in a hydrophobic IL, the preliminary experiments were performed to evaluate the keratinolytic activity of the bacterial culture concentrate in [BMIM][PF_6] compared with the other chosen extreme conditions.

Experimental

Chemicals

Folin-Ciocalteu reagent (FCR), culture media and buffer components, surfactants, and solvents were provided by Merck chemicals (Darmstadt, Germany). Tyrosine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Keratin was provided by J and K Company (Shanghai, China).

Bacterial strain, culture conditions, and enzyme production

Growth and keratinase production of *Salicola marasensis (S. marasensis)* SR-081 Halo [previously isolated from Golestan Salt Lake, Iran (37°24′3″N 54°38′44″E), GenBank accession no. KF859984] were done in an optimized basal medium (18) consisting of (g/L): peptone, 3.0; glucose, 12.5; $MgCl_2.6H_2O$, 5.5; K_2SO_4, 3.0; $CaCl_2.2H_2O$, 2.8; $MnSO_4$, 1.5 g; KNO_3, 5.5; and NaCl, 200. Fifty millilitres of the basal medium were transferred to 250-mL Erlenmeyer flasks and the pH was adjusted to 7.0 before being autoclaved. To prepare the bacterial inoculum, into a modified LB broth medium a loopfull of the stock slant culture was added and incubated for 48 h at 40 °C to reach the absorbance of 1.0 at 600 nm. The prepared flasks were then inoculated with 1 mL of a two-day-old inoculum culture of *S. marasensis* and incubated at 40 °C under continuous shaking (150 rpm, 120 h). The samples were withdrawn periodically at the 24-h intervals and analyzed for the keratinase production and the growth

estimation. Bacterial growth was estimated by determination of viable colony count. To assay the keratinolytic activity, the withdrawn samples were centrifuged at 8,000 × g for 10 min and the cell-free supernatant was assayed for the extracellular keratinase activity. The bacterial strain was grown on agar slants containing the above mentioned medium, incubated at 40 °C for 48 h and then stored at 4 °C. Sub-culturing was performed monthly.

Preparation of concentrated keratinolytic extract

In order to produce the enzyme stock, Erlenmeyer flask (2 L) containing 500 mL culture medium was inserted by 2% (v/v) bacterial inoculum and incubated at 40 °C under shaking (150 rpm) for 48 h. Thereafter, the produced cell mass was removed from the culture media using centrifugation (8,000 × g, 15 min) followed by sequential precipitation steps for preparation of crude keratinase as follows. A gradual addition of pre-cold ethanol to the supernatant up to 80% (v/v) saturation and stirring for 2 h was firstly performed. The precipitate was then recovered by centrifugation at 18,000 × g for 15 min and suspended in a minimal volume of 50 mM phosphate buffer (pH 8.0) including 20% (w/v) NaCl. Dialysis of the collected sample was then performed using the similar buffer and the obtained concentrated sample was centrifuged (8,000 × g for 10 min) followed by determination of keratinase activity under assay conditions. All the concentration steps were done at 4 °C.

Keratinase assay and protein determination

The keratinolytic activity of the obtained concentrated bacterial culture extract (section 2.3) was determined following the method described by Zhang *et al.* with some modifications (19). The reaction mixture consisted of 1 mL phosphate buffer pH 8.0 containing 0.5% (w/v) keratin and 20% (w/v) NaCl and 1 mL enzyme solution was firstly incubated at 40 °C for 30 min. The reaction was then stopped by addition of 0.5 mL trichloroacetic acid (TCA) solution (10%, w/v) followed by standing at room temperature for 5 min. The mixture was consequently centrifuged at 18,000 × g for 10 min to remove the precipitate and the supernatant was used to estimate the amount of free tyrosine according to the following method (20). Briefly, one mL of Na_2CO_3 (0.5 M, to neutralize the supernatant) and 250 µL of the FCR (1 N) were added to 0.5 mL of the obtained supernatant followed by keeping the mixture at 40 °C for 15 min and reading the absorbance at 660 nm against the corresponding blank. As a blank, the enzyme was inactivated (using TCA 10%, w/v) at the beginning of the incubation period. The liberated tyrosine was determined by a calibration curve obtained from a standard solution of tyrosine (0.1−100 mM). One unit of keratinolytic activity was defined as the enzyme quantity that releases 1 µmol of tyrosine min^{-1} mL^{-1} under the standard assay conditions (20). Total protein content in the enzyme solution was measured by dye binding assay suggested by Bradford, using bovine serum albumin (BSA) as the standard (21).

Evaluation of poultry feathers degradation using S. marasensis extracellular keratinase

Substrate preparation

In order to prepare keratinase substrate, the chicken features were firstly provided by a local poultry industry (Islamkish, Tehran, Iran) which were then carefully washed and degreased by warm tap water and methanol-chloroform (1:1), respectively. The washed features were then dried at 45 °C for 48 h followed by autoclaving at 121 °C for 30 min and storing at room temperature for further studies.

Feather treatment

An amount of 100 mg of the dried and raw feathers were mixed with 10 mL of the concentrated enzyme sample (~200 U) in phosphate buffer (pH 8.0). The reaction mixture was shaken (120 rpm) at 40 °C for 24 h and the reaction was quenched by adding 2.5 mL of TCA (10%, w/v) and centrifuged at 12,000 × g for 10 min. The released protein was then determined using the Bradford method and the amount of the dissolved protein was expressed as the percentage of the total weight of the used dried feathers (21). All the experiments were carried out in triplicates and the average values were taken. In addition, the reaction mixture

containing equal amount of the inactivated enzyme was used as the control.

The effects of additives on soluble protein extraction efficiency

To test the effect of additives on the enzymatic keratin hydrolysis, the reaction mixtures containing 100 mg feather and 200 U crude enzyme were prepared in the desired conditions and the soluble protein was measured after 12 h incubation. The chosen additives were surfactants of cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), Triton X-100, and Tween 80, chemical reductants of 2-mercaptoethanol (2-ME), 1,4-dithiothreitol (1,4-DTT), urea, sodium sulfite, sodium thiosulfate, glutathione, oxalic acid, formic acid, L-cysteine, and sodium borohydride, and solvents of acetone, butanol, chloroform, cyclohexane, cyclohexanol, dodecane, heptane, hexadecane, hexane, and nonane as well as IL of [BMIM][PF_6]. [BMIM][PF_6] (20%, v/v) which was prepared and purified by vacuuming at 70 °C for 6 h. Each mentioned additive was separately added to the reaction mixture and its effect was evaluated. In the presence of [BMIM][PF_6] and the hydrophobic solvents, a three-phase system containing [BMIM][PF_6] or solvents, insoluble residual of feathers, and aquatic solution of the protein released from feathers was formed. After centrifugation, the aqueous extract was used for protein assay. The IL phase was used to recover the [BMIM][PF_6] by the method described above for preparing and purifying the IL and reused for the next times.

Statistical analyses

As said above, all mentioned experiments were performed in triplicates and mean of the results were reported. To show the significant difference between groups, one-way ANOVA followed by Holm-Sidak multiple comparison test and two-way ANOVA using SPSS software (version 18, SPSS Inc., Chicago) were applied. A probability level of $p < 0.05$ was considered statistically significant.

Results and Discussion

Keratinase production and concentration

Figure 1 shows the growth and keratinase production curves of the bacterial strain. Maximal keratinase activity of about 20 U/mL was reached after 48 h cultivation. Under the culture conditions, the keratinase production started from the early logarithmic growth phase, and the maximal enzyme production was achieved after 48 h cultivation when cell growth reached in the middle of logarithmic phase, and then decreased with increasing the incubation time (Figure 1). These finding suggested that the extracellular keratinase of *S. marasensis* was produced as a primary metabolite. Similar pattern of the early-exponential secretion of an extracellular protease was reported by Massaoud

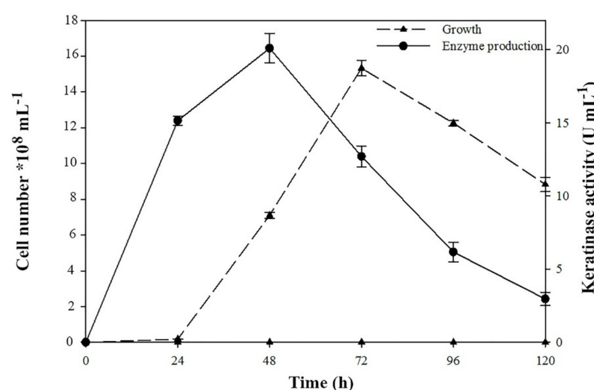

Figure 1. Growth and keratinase production profile of *S. marasensis* over the time.

Table 1. Concentration summary of keratinase produced by *S. marasensis.*

Purification steps	Volume (mL)	Total activity (kU)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	500	10.2	472.8	21.6	100.0	1.0
Dialysed precipitate	20.0	7.6	129.1	58.9	74.5	2.7

et al. where the considerable proteolytic activity of *Xenorhabdus kozodoii* was obtained after 4 h incubation at 30 °C (22). However, the reported results of Khardenavis *et al.* were in contrast to the obtained results of the present study (18). They observed the maximal keratinolytic activity of *Serratia* sp. HPC 1383 after 24 h cultivation while the growth curve reached to its maximum point (1340 mg/L biomass) after 4 day cultivation (18). Abdel-Fattah *et al.* described keratinase production of *Bacillus licheniformis (B. licheniformis*) ALW1 which reached maximally to 25.2 U/mL under static condition (23).

After concentrating the clear culture supernatant of *S. marasensis*, a 2.7-fold purification of the cultural supernatant was achieved with some loss in keratinolytic activity (Table 1). More than 70% of the initial enzyme activity of the supernatant was detected in the concentrate. Similar results were previously reported by Martínez *et al.* who applied acetone precipitation protocol for concentrating the keratinase produced by *Paecilomyces lilacinus* (24). They found that the enzyme was completely precipitated in the presence of acetone 75% without any loss of keratinase activity while the recovery of the enzyme decreased to 60% when acetone concentration reached to 95% (24). In the study of Zhang *et al.* concentrating the keratinase containing supernatant (produced by *Brevibacillus parabrevis* CGMCC 10798) using ammonium sulphate (70%, w/v) led to purification fold of 1.08 and recovery percent of 65.04% (19).

In general, sequestering the essential water molecules around the enzyme structure either by organic solvents (like acetone) or inorganic salts (such as ammonium sulphate) at high concentration might rigidify the structure of protein and negatively affect the catalytic activity of the enzymes (14).

The feather degrading activity of the concentrated keratinolytic content of S. marasensis

Owing to the broad applications of keratinases and since they are usually from metallo- and serine-proteases families, the keratinolytic potential of the concentrated supernatant of *S. marasensis* toward pretreated feathers was studied (20). The obtained results of the Bradford method showed 26% yield for keratin extraction from 100 mg of pretreated feathers by the keratinolytic activity after 12 h incubation. The attained results of the applied reaction mixtures over the time can be seen in Figure 2. The obtained results from this primary study indicated that the *S. marasensis* keratinolytic enzyme may be suitable for enzymatic treatment of feathers bio-waste.

In the study conducted by Abdel-Fattah *et al.* the capability of keratinase produced by *B. licheniformis* ALW1 for degradation of feather waste was determined (23). They observed 63% native feather degradation in redox free system (23). In another study performed by Yusuf *et al.* the ability of *Alcaligenes* sp. AQ05-001 for secretion of a multi heavy metal tolerant keratinase was evaluated where 83% of the feathers contaminated by a mixture of highly toxic metals were eliminated after 36 h incubation (25).

Zhang *et al.* evaluated the keratinolytic activity of *Brevibacillus parabrevis* (*B. parabrevis)* towards different native sources including human hair, wool, azocasein, feather powder, gelatin, and casein among which azocasein and feather powder was degraded by 100% and 90%, respectively (19).

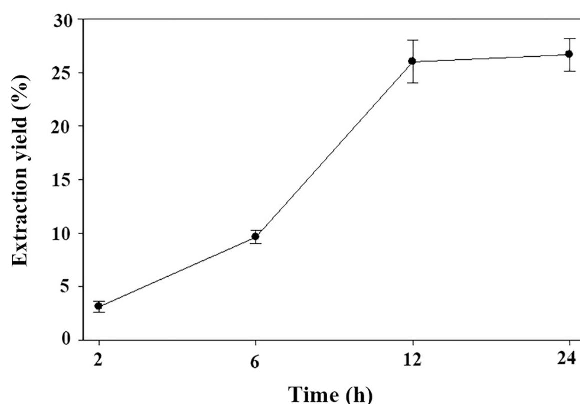

Figure 2. The rate of keratin degradation (measured as extraction yield%) over the time in the presence of keratinase of *S. marasensis* as determined by the protein released assay.

Effect of pH, temperature, and different additives on the keratin hydrolysis activity of the enzyme

The results of pH-temperature influences on the keratinolysis yield are shown in Figure 3. Keratinolytic activity of the crude enzyme was maximally found at 40 °C and pH 9.0. It has been reported that most of the bacterial derived kertinases optimally work at pH range 5−9. For example, the purified keratinases of *B. parabrevis* CGMCC 10798 displayed its maximum activity at pH 8. In the case of temperature, the purified keratinolytic proteases of bacterial origin reported to have different optimum temperature. The purified alkaline keratinase of *B. subtilis* DP1 exhibited the optimum temperature of 37 °C while the keratinase of *Brevibacillus parabrevis* CGMCC 10798 showed its maximum activity at 60 °C.

Enhancement of keratinolytic activity of the crude enzyme in the presence of [BMIM][PF_6] and several organic solvents, surfactants, and reductants were also examined. Maximal yield, *i.e.* 80%, was obtained when keratinolytic reaction of the enzyme extract was performed in 20% [BMIM][PF_6] for 12 h (Figure 4).

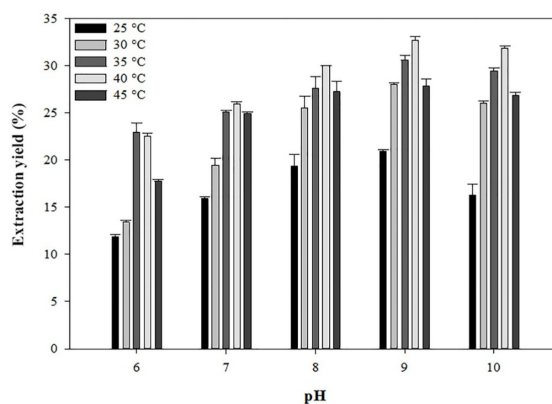

Figure 3. Effect of pH-temperature on the keratinolytic activity (measured as extraction yield%) of the concentrated enzyme.

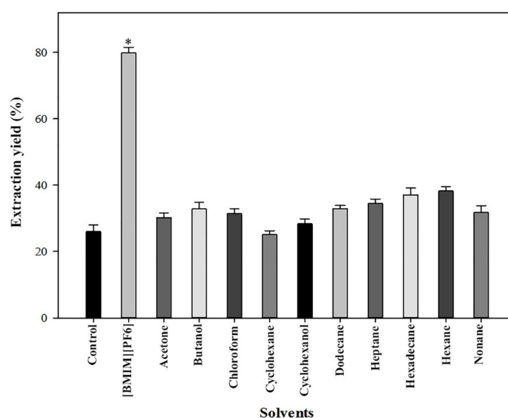

Figure 4. Changes in the keratinolytic activity (measured as extraction yield%) of the concentrated culture broth in the presence of different solvents.

Reusability of the recovered IL was checked and the results showed the recovery of approximately 91% after three cycles. Łaba and Szczekala found that keratinases from *Bacillus cereus (B. cereus)* was produced about 0.96 mg/mL (~10%) protein from 100 mg of the pretreated feathers in the presence of 10 mM Na_2SO_3 (20). Wang *et al.* studied the degradation of intact chicken feathers by simultaneous use of a keratinolytic protease from *Thermoactinomyces* sp. CDF and the chemical reducers (26). They observed complete degradation of the chicken feathers in the presence of reducers after 1 h of incubation, while no significant disintegration of chicken feathers was obtained in the absence of reducing agents (26). Some ILs and chemical reducers have been already used to extract the keratin from feathers (16, 26 and 27). In the study of Wang and Cao, the maximal yield of keratin was about 21% with the mass ratio of feathers to IL 1:40 after 4 h (16). To the best of our knowledge, this should be the first report concerning the effective extraction of keratin from feathers using a stable crude extract with keratinolytic activity in a hydrophobic IL. As shown in Figure 5, among the other additives, most remarkable effect on the amount of keratinolysis was obtained when the reaction was carried out in

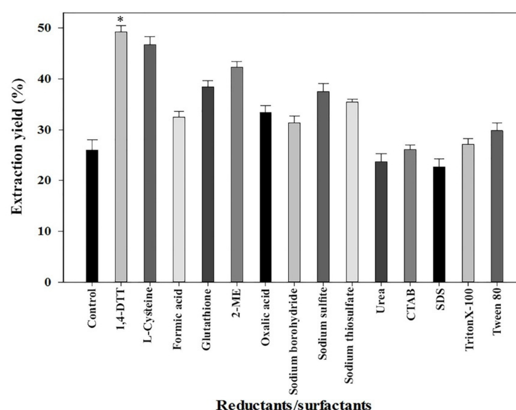

Figure 5. Keratin degradation yield (measured as extraction yield%) of the concentrated keratinolytic extract in the presence of reducing agents and surfactants.

the presence of 1,4-DTT, L-cysteine, 2-ME, and hexane, respectively (Figures 4 and 5). Except for SDS, urea, and cyclohexane, other applied test compounds enhanced the efficiency of the keratinolysis reaction (Figure 5). The tested chemicals and solvents might raise the rate of keratinolysis reaction through different mechanisms. Indeed, some parameters increased the efficiency of keratinolytic protease for biodegradation of keratin such as (i) cooperative action of a reductant with reducing potential of the disulfide bonds of keratin, (ii) an IL with the power of dissolution of cuticle and cortex of feathers and (iii) a surfactant or a hydrophobic solvent with increasing the accessibility of the enzyme to the hydrophobic substrate (28). In general, the solvents are usually incompatible with the enzymatic stability and decrease the catalytic activity of the enzyme (5). It has been shown that the solvents and ILs separate critical bound water from the surface of the mesophilic enzymes leading to unfolding the enzyme and cause the enzyme to be less stable (14, 15). However, in the case of halophilic enzymes, the solvent stability is mainly evidenced as a generic trait because of high critical water concentration at their active site (29).

Conclusion

In this study, a concentrated culture broth extract possessing interesting stability and high keratinolytic proficiency from *S. marasensis* was described. The potential of the keratinolytic extract for highly degrading of keratin in a hydrophilic IL revealed that it might be a useful eco-friendly route for the application in the recycling and environmental pollution control of the keratinous wastes. With the activity in the both hydrophobic and hydrophilic organic solvents, the stability in the presence of the salts, surfactants, metals, and chemicals, as well as high temperatures and over a versatile pH range, was studied. Further studies are merited to find out the related catalytic abilities of the enzyme in various harsh industrial conditions.

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References

(1) Elleuche S, Schröder C, Sahm K and Antranikian G. Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. *Curr. Opin. Biotechnol.* (2014) 29: 116-23.

(2) Promchai R, Boonchalearn A, Visessanguan W and Luxananil P. Rapid production of extracellular thermostable alkaline halophilic protease originating from an extreme haloarchaeon, *Halobacterium salinarum* by recombinant *Bacillus subtilis*. *Biocatal. Agric. Biotechnol.* (2018) 15: 192-8.

(3) Karan R, Capes MD and DasSarma S. Function and biotechnology of extremophilic enzymes in low water activity. *Aquat. Biosyst.* (2012) 8: 4.

(4) Samaei-Nouroozi A, Rezaei S, Khoshnevis N, Doosti M, Hajihoseini R, Khoshayand MR and Faramarzi MA. Medium-based optimization of an organic solvent-tolerant extracellular lipase from the isolated halophilic *Alkalibacillus salilacus*. *Extremophiles* (2015) 19: 933-47.

(5) Marhuenda-Egea FC and Bonete MaJ. Extreme halophilic enzymes in organic solvents. *Curr. Opin. Biotechnol.* (2002) 13: 385-9.

(6) Moshfegh M, Shahverdi AR, Zarrini G and Faramarzi MA. Biochemical characterization of an extracellular polyextremophilic α-amylase from the halophilic archaeon *Halorubrum xinjiangense*. *Extremophiles* (2013) 17: 677-87.

(7) Khardenavis AA, Kapley A and Purohit HJ. Processing of poultry feathers by alkaline keratin hydrolyzing enzyme from *Serratia* sp. HPC 1383. *Waste Manage.* (2009) 29: 1409-15.

(8) Paul T, Jana A, Mandal AK, Mandal A, Mohpatra PKD and Mondal KC. Bacterial keratinolytic protease, imminent starter for NextGen leather and detergent industries. *Sust. Chem. Pharm.* (2016) 3: 8-22.

(9) Rahayu S, Syah D and Suhartono MT. Degradation of keratin by keratinase and disulfide reductase from *Bacillus* sp. MTS of Indonesian origin. *Biocatal. Agric. Biotechnol.* (2012) 1: 152-8.

(10) Gupta R, Rajput R, Sharma R and Gupta N. Biotechnological applications and prospective market of microbial keratinases. *Appl. Microbiol. Biotechnol.* (2013) 97: 9931-40.

(11) Vidyasagar M, Prakash S and Sreeramulu K. Optimization of culture conditions for the production of haloalkaliphilic thermostable protease from an extremely halophilic archaeon *Halogeometricum* sp. TSS101. *Lett. Appl. Microbiol.* (2006) 43: 385-91.

(12) Sinha R and Khare S. Isolation of a halophilic *Virgibacillus* sp. EMB13: Characterization of its protease for detergent application. *Indian J. Biotechnol*. (2012) 11: 416–26.

(13) Vijayaraghavan P, Jebamalar TRJ and Vincent SGP. Biosynthesis optimization and purification of a solvent stable alkaline serine protease from *Halobacterium* sp. *Ann. Microbiol.* (2012) 62: 403-10.

(14) Gao WW, Zhang FX, Zhang GX and Zhou CH. Key factors affecting the activity and stability of enzymes in ionic liquids and novel applications in biocatalysis. *Biochem. Eng. J.* (2015) 99: 67-84.

(15) Naushad M, ALOthman ZA, Khan AB and Ali M. Effect of ionic liquid on activity, stability, and structure of enzymes: a review. *Int. J. Biol. Macromol.* (2012) 51: 555-60.

(16) Wang YX and Cao XJ. Extracting keratin from chicken feathers by using a hydrophobic ionic liquid. *Process Biochem.* (2012) 47: 896-9.

(17) Rezaei S, Shahverdi AR and Faramarzi MA. Isolation, one-step affinity purification, and characterization of a polyextremotolerant laccase from the halophilic bacterium *Aquisalibacillus elongatus* and its application in the delignification of sugar beet pulp. *Bioresour. Technol.* (2017) 230: 67-75.

(18) Khoshnevis N, Rezaei S, Samaei-Nouroozi A, Amin M, Moshfegh M, Khoshayand MR and Faramarzi MA. Enhanced production and characterization of a highly stable extracellular protease from an extreme halophilic isolate *Salicola marasensis*. *Iran. J. Pharm. Res.* (2018) 17: 1392-412.

(19) Zhang RX, Gong JS, Su C, Zhang DD, Tian H, Dou WF, Li H, Shi JS and Xu ZH. Biochemical characterization of a novel surfactant-stable serine keratinase with no collagenase activity from *Brevibacillus parabrevis* CGMCC 10798. *Int. J. Biol. Macromol.* (2016) 93: 843-51.

(20) Łaba W and Szczekala KB. Keratinolytic proteases in biodegradation of pretreated feathers. *Pol. J. Environ. Stud.* (2013) 22: 1101-9.

(21) Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* (1976) 72: 248-54.

(22) Massaoud MK, Marokhazi J, Fodor A and Venekei I. Proteolytic enzyme production by strains of the insect pathogen *Xenorhabdus* and characterization of an early-log-phase-secreted protease as a potential virulence factor. *Appl Environ. Microbiol*. (2010) 76: 6901-9.

(23) Abdel-Fattah AM, El-Gamal MS, Ismail SA, Emran MA and Hashem AM. Biodegradation of feather waste by keratinase produced from newly isolated *Bacillus licheniformis* ALW1. *J. Genet. Eng. Biotechnol.* (2018) 16: 311-8.

(24) Martínez YN, Cavello I, Cavalitto S, Illanes A and Castro GR. Studies on PVA pectin cryogels containing crosslinked enzyme aggregates of keratinase. *Colloids Surf. B Biointerfaces*. (2014) 117: 284-9.

(25) Yusuf I, Ahmad SA, Phang LY, Syed MA, Shamaan NA, Khalil KA, Dahalan FA and Shukor MY. Keratinase production and biodegradation of polluted secondary chicken feather wastes by a newly isolated multi heavy metal tolerant bacterium-*Alcaligenes* sp. AQ05-001. *J. Environ. Manage.* (2016) 183: 182-95.

(26) Wang L, Cheng G, Ren Y, Dai Z, Zhao ZS, Liu F, Li S, Wei Y, Xiong J and Tang XF. Degradation of intact chicken feathers by *Thermoactinomyces* sp. CDF and characterization of its keratinolytic protease. *Appl. Microbiol. Biotechnol.* (2015) 99: 3949-59.

(27) Ji Y, Chen J, Lv J, Li Z, Xing L and Ding S. Extraction of keratin with ionic liquids from poultry feather. *Sep. Purif. Technol.* (2014) 132: 577-83.

(28) Chen J, Vongsanga K, Wang X and Byrne N. What happens during natural protein fibre dissolution in ionic liquids. *Materials* (2014) 7: 6158-68.

(29) Zaks A and Klibanov AM. Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci.* (1985) 82: 3192-6.

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