Production and partial purification of an innovative heat resistant α-keratinase with some remarkable medical and industrial applications

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Received: 23 February 2024
Revised: 11 April 2024
Accepted: 17 April 2024
Published: 14 August 2024

Egyptian Pharmaceutical Journal 2024, 23:670–685

Background and objectives
Keratinase has ultimate practical importance in industry, medicine, food industry and waste management fields. Their applications in wool and silk are as good cleaners and in leather industry as the best ever green dehairing agents providing high leather quality, as well they are used as crucial components of sophisticated detergents. Their medical prospective applications are in prion degradation and human callus removal. They convert keratinaceous wastes to valuable products saving the environment from hard keratin waste pollution. Bacteria, fungi and actinomycetes are effective keratinase producers and they are considered the most suitable sources. This study aims to formulate the production medium and to pinpoint the proper physiological conditions for the potent microorganism producing an efficient α-keratinase enzyme. The partial purification of the crude enzyme was successfully performed. The effect of the reaction temperature on both the crude and the partially purified enzyme (PPE) was duly studied with the thermostability of PPE. Some important applications have been implemented on PPE and these include leather dehairing, cloth stain removal, and topical treatment of human callus.

Materials and Methods
Thirteen recommended microbial strains were screened for effective and applicable α-keratinase productivity. Optimization of the cultural conditions for extracellular enzyme production and also the partial purification of the crude enzyme by ammonium sulphate salting out or by ethanol or acetone precipitation were carried out. The effect of reaction temperature on the enzyme and its thermostability were studied. Finally, the efficiency of the PPE on leather dehairing, stain removal, and human callus treatment was explored.

Results and conclusion
Among the 13 organisms screened, the fungal strain *Trichoderma polysporum HZ-31* was the most potent producer of an influencial α-keratinase. The maximum α-keratinase activity of 58.2 UmL^{-1} was obtained by the previous-mentioned strain after 5-days fermentation medium containing (%, w/v): whole chicken feathers 0.5, glucose 0.2, peptone 0.5, yeast extract 0.5, K_2HPO_4 0.1, KH_2PO_4 0.3, $CaCl_2$ 0.1, $MgSO_4$ 0.1, and pH 7.0. Acetone fractionation of the crude keratinase was the most proper and offered the most promising keratinase fraction PPE at 80–90% acetone. This fraction had high thermostability and was kept at 55°C for more than 98% of its original activity after 60 min heating and this temperature (55°C) was also the optimum for 2 h enzymatic reaction. Conclusively, the present study succeeded in the achievement of a constitutive extracellular alkaline α-keratinase, which successfully proceeded to complete leather unhairing after 12–16 h at 37°C, afforded high performance to cloth blood stain removal with Arial detergent after 2 h at 50°C and complete degradation of the human callus after 4 h at 50°C.

Keywords:
callus biodegradation, leather dehairing, microbial α-keratinase, partial purification, stain removal, thermostability, Trichoderma polysporum HZ-31

Egypt Pharmaceut J 23:670–685

1687-4315

Introduction

Keratinases are proteolytic enzymes predominantly active when keratin substrates are available, that attack disulfide bridges in the keratin to convert them from complex to simplified forms [1–3] by degrading keratins into amino acids and/or peptides [3], then transform the keratinaceous wastes into value-added products [4].

Different properties of microbial keratinases, renders them a green and sustainable material for industrial applications in waste management, textile, leather, and

detergents with advantages over conventional treatments [4], as they are robust enzymes with a wide temperature and pH activity range and are largely serine or metallo-proteases [5].

Their applications in wool and silk are as good cleaners and in the leather industry as the better dehairing agents with the development of greener dehairing technology and leading to high leather quality. Further, their prospective medical application in the challenging field of prion degradation [1–3,5], and human callus removal are reported [6,7], in addition, they find application in the preparation of animal nutrients, protein supplements, and feather meal processing for fertilizers [1].

Keratinases also can serve as important tools to convert keratin-rich wastes, such as feathers into value products applicable to many fields [3,4] and at the same time the environment is protected from with the keratinaceous wastes [4,8].

Accordingly, the increasing demands for these enzymes justified the comprehensive research studies for effective, applicable keratinases production utilizing keratinaceous wastes. This study deals with the formulation of the production medium at the proper physiological conditions applying the potent microorganism producing an efficient α-keratinase. The effect of the reaction temperature on both the crude and partially purified enzyme (PPE) was fully studied with the thermostability of PPE. Moreover, some important industrial and medical applications have been implemented on PPE.

Materials and Methods

Materials

Microorganisms

Thirteen recommended microbial isolates (five bacteria, six fungi, and two actinomycetes) were screened in the present study for the production of extracellular keratinase enzymes. The bacterial isolates tested were *Bacillus licheniformis MSK-103, Bacillus licheniformis N22, Bacillus subtilis NRC-3, Pseudomonas aeruginosa C11,* and *Fervidobacterium pennavorans* and the fungal isolates were *Aspergillus niger, Trichoderma atroviride F6, Trichoderma polysporum HZ-31, A. oryzae NRC-34, Penicillium sp.* and *Doratomyces microspores MZKIB-399*. In addition, the two actinomycetal isolates *Streptomyces pactum DSM40530* and *Streptomyces gulbargensis* were applied in the present study.

The screened microorganisms were collected from the Culture Collection Center, NRC, Egypt, except *Pseudomonas aeruginosa* C11, *Fervidobacterium pennavorans*, *Trichoderma polysporum* HZ-31 and *Penicillium sp.* were provided from the National Center for Agricultural Utilization Research, Peoria, Illinois 61604, USA, also *Aspergillus niger* and *Trichoderma atroviride* F6 were provided from Microbiology Department, Faculty of Science, Ain Shams University, Egypt.

Cultivation, subculturing, and maintenance of the bacterial strains were on the nutrient agar medium, while the fungal strains were on potato-dextrose-agar medium, (PDA), and for the actinomycetal strains were on yeast-extract-malt extract-agar medium. The sub-culturing was performed monthly for all strains.

Media

The following media were used in the present work and had the following composition (g/L):

Bacterial media (Medium 1)

This medium was used for stock culture and maintenance of the bacterial strains and was composed of peptone 5.0, beef extract 3.0, NaCl 8.0, agar 12.0, and pH 7.2 [9,10].

Bacterial growth enhancement medium (tryptone–yeast extract), medium 2

This medium was composed of tryptone 10.0, yeast extract 5.0 and NaCl 10.0, and pH 7 [11].

Fungal and bacterial keratinase production, medium 3

This was composed of whole chicken feathers 5.0, glucose 2.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 1.0, KH_2PO_4 3.0, $CaCl_2$ 1.0 and $MgSO_4$ 1.0, and pH 7.0 [12].

Fungal media

Potato-dextrose-agar (PDA) medium 4

This was applied for stock cultures and culture maintenance of fungal strains and composed of potato slices 500, dextrose 10.0 and agar 20 and pH 7.2± 0.2 [13].

Fungal growth enhancement medium 5

This was composed of peptone 5.0, KH_2PO_4 0.5, yeast extract 1, glucose 10.0 and $MgSO_4.7\ H_2O$ 0.5, and pH 7.2±0.2 [13].

Actinomycetal media

Yeast extract-malt extract, ISP2 medium 7

This was prepared for actinomycetal maintenance, for growth enhancement and composed of malt extract 10.0, yeast extract 4.0, glucose 4.0 and agar 20.0, and pH 7.2± 0.2 [14].

Keratinase production medium 8

This had the same composition as the bacterial keratinase production medium 3.

Keratinaceous wastes

Different keratinaceous wastes (chicken, turkey and duck feathers, animal hairs, and wool) specimens used in this study were freshly obtained from several local market places in Cairo governorate, Egypt.

Buffers

The following three buffers were applied: 0.1 M-Citrate-phosphate buffer pH 4.8–6.2, 0.2 M-phosphate buffer pH 7.0–8.0 and 0.2 M-Carbonate – bicarbonate buffer pH 9.2–10.7

Methods

Maintenance of the tested microorganisms on stock cultures

The bacteria were grown on nutrient agar slants at 37°C for one day, while the fungal strains were maintained on PDA slants, and incubated at 30°C for 7 days. On the other hand, the actinomycetes were maintained on ISP2 slants and incubated for 5 days at 30°C at all the following experiments.

Keratinaceous waste preparation

Different keratinaceous wastes including feathers, hair and wool obtained from chickens, ducks, turkeys, goats, and sheep, were washed under running water to remove proteins, organic and other impurities. Dryness was undergone for 6–8 h at 50°C in an oven, the samples were crushed in a blender and sieved through a 0.1 mm screen. The raw materials are stored at 4°C until use.

Production of the microbial keratinase

To 50 mL of the sterile keratinase production medium 3, 5 mL of the activated fungal inoculum was added and incubated at 30°C on an incubating shaker at 180 rpm for different periods 3, 5, and 7 days.

Separation of extracellular crude keratinase

This was done either by filtration through Whatman filter paper No.1 and centrifugation at 2300 g for 20 min. The keratinase activity, protein content, and the final pH value of the clear supernatant were determined.

Estimation of protein

The protein content was determined colorimetrically by Folin-Ciocalteu phenol reagent (Merck Company, Schuchardt, Germany) by the method of Lowry *et al.*, [15], using bovine serum albumin as the standard.

Keratinolytic enzyme activity was determined by hydrolysis of α-keratin to tyrosine, which was measured by the method of Pridham and Gottlieb [11] with little modification according to Nickerson *et al.*, [16]. This was accomplished by incubating 1 mL of enzyme solution with 1 mL of the following mixture: (0.03 g α-keratin in 2.5 mL carbonate – bicarbonate buffer (0.2 M, pH 8.5), 1.25 mL distilled water, and 1 mM – $MgCl_2$ (0.25 mL), the reaction mixture was incubated in shaking water bath at 40°C, 70 rpm for 2 h, then stopping with 1 mL of 10% (w/v) TCA. The absorbance was measured at 280 nm. The standard curve of tyrosine was plotted applying varied dilutions of pure tyrosine solution.

Keratinase activity unit (Anson unit) was defined as 1 μg of tyrosine released by a certain quantity of enzyme per 2 h reaction at specified conditions.

Optimization of enzyme production

Production of keratinase is affected by various factors and fermentation conditions, such as fermentation period, culture medium, initial pH, inoculum size and age, agitation speed, C and N sources, incubation temperature, and finally different additives. One factor at a time was optimized and then incorporated in the next experiments.

Employment of different keratinaceous wastes for fungal keratinase production

For maximum keratinase production, different keratinaceous wastes (duck feathers, turkey feathers, white chicken feathers, black chicken feathers, goat hair, and sheep wool) were examined. Chicken feathers in the optimized production medium were replaced with equal weights of any of the mentioned above wastes.

Partial purification

Partial purification of the crude keratinase enzyme was performed by salting out with different concentrations of ammonium sulphate or fractional precipitation by ethanol or acetone as described in detail by Ismail *et al.*, [13].

Study of some properties of the crude and partially purified forms

This included the proper reaction temperature and the enzyme thermal stability.

Applications

Some industrial and medical applications of the partially purified enzyme

Assessment of cow leather dehairing

Fresh cow skin was obtained from a market in Cairo, Egypt, cut into medium-sized pieces and kept in a

disposable sterile Petri dish, then 20 mL of keratinase was added to the petri dish containing skin pieces. In the case of control, skin was incubated with only buffer without the enzyme. Then Petri dish was closed and checked after different periods of incubation time (1 and 2 h) at 37°C, by a virtual analysis of skin pieces for dehairing ability [17,18].

Briefly, skin hairs were pulled with a spatula or by hand over the skin to see if the hairs easily detached from the skin. The dehairing impact was distinguished by either No, partially or complete.

Wash performance analysis of keratinase

This was performed using several pieces of cotton cloth stained with harsh stains, as blood or chocolate, which were incubated at different conditions, the first was incubated with keratinase only, while the second was incubated with detergent only, the third was incubated with keratinase and detergent mixture and the last was incubated with distilled water only, after 2 h of incubation period at 50°C, to determine the effect of keratinase washing performance in the presence and absence of detergent [19].

Callus biodegradation

This experiment was carried out by incubating a piece of callus (30 mg) with 1 mL keratinase solution for 2, 4, and 6 h at 50°C and pH 9.2. Before and after the incubation, the keratinolytic activity was measured to determine the quantity of free amino acids liberated from the callus.

Statistics

The data shown in the corresponding Tables and Figures were the mean values of the experiments, the data statistics were often analyzed and the standard error mean (SEM) was calculated [20].

Results and discussions

Screening of some recommended microorganisms for keratinase production

The data recorded (Table 1) showed that, with most cultures of different ages, the protein level was in the usual range and had a consistent relationship with both microbial growth and keratinase activity. It was noticed that the cultures, final pH varied within a very limited range from the neutral to the alkaline value. Also, it was clarified the superiority of some tested fungi followed by bacteria to the actinomycetal organisms in keratinase production. Among all the shaken cultures of the tested microorganisms, 5-days shaken *Trichoderma polysporum* HZ-31 afforded the highest keratinase productivity (56.80 U mL^{-1}), followed by 5-days *Penicillium sp.* (54.80 U mL^{-1}) and 5-day *B. subtilis* NRC-3 (48.32 U mL^{-1}).

In this connection, it was reported that bacteria and fungi have been identified as good decomposers of keratinaceous substrates, which accomplish by extracellular production of keratinolytic enzymes so, most investigations focused on using of bacteria and fungi as they play a key role in keratinase production [21–23]. It is important to refer to the report of the fungus *Trichoderma harzianum* HZN12 (KP235366) as a high potential keratinase producer.

In the case of bacteria as keratinolytic enzyme producers, many reports discussed their efficiency in degrading keratinaceous wastes, such as *Fervidobacterium pennavorans*, which was the first known extreme thermophile capable to degrade native feathers at high temperatures [24].

It was also reported that actinomycetes isn't distributed like bacteria and fungi in the keratinolytic field, although they were efficient in degradation of keratinaceous wastes [25].

The final pH of the culture filtrates of all the investigated microbial strains lied in the alkaline range (7.4–9.3) and changed with the culture age within a very limited range. It was noticed the paramount effect of the alkalinity on both keratinase synthesis and its action, where with any microbial strain investigated, the higher alkaline filtrates had continuously the higher keratinase activity. On the other hand, this indicates the participation of the alkaline protease (alcalase), which acts preferentially at alkaline pHs in the overall protein hydrolysis process and this joint effect is known as the synergistic action of enzymes [26].

In this regard, many reports on keratinase production preferentially at alkaline pHs were published as those from bacteria *Bacillus subtilus* KdN2, [27], *Bacillus licheniformis* ZjuE131410 [28], for fungal *Trichoderma viride* [13,29], *Trichoderma harzianum* HZ N12 [23], and *Fusarium brachygibbasum* AUMC10973 [30], also actinomycetes as *Streptomyces sp.* 594 [31].

Nevertheless, neutral and acidic initial pHs were occasionally reported for some bacterial and actinomycetal isolates [22,32].

Table 1 Screening of some bacteria, fungi and actinomycetes for keratinase activity applying shaken culture technique

Microbial strain	Incubation period (day)	Final pH of CF	PC*	Activity**
Bacillus licheniformis N22	1	7.8	0.597	24.6±0.161
	3	8.1	0.631	25.2±0.024
	5	8.6	0.688	27.8±0.107
Bacillus licheniformisMSK-103	1	7.7	0.44	11.0±0.067
	3	7.9	0.472	11.4±0.129
	5	8.2	0.511	12.0±0.143
Bacillus subtilis NRC-3	1	8.3	1.83	47.3±0.123
	3	8.1	1.73	46.8±0.041
	5	9.0	1.78	48.3±0.024
Fervidobacterium pennavorans	1	7.4	0.325	6.5±0.047
	3	7.5	0.617	6.9±0.145
	5	7.6	0.690	8.6±0.204
Pseudomonas aeruginosa C11	1	8.1	1.11	35.6±0.064
	3	8.9	1.39	39.2±0.146
	5	7.6	1.43	33.2±0.094
Aspergillus niger	3	8.6	1.1	20.36±0.235
	5	8.8	1.4	22.0±0.0128
	7	8.5	1.56	18.6±0.078
A. oryzae NRC-34	3	8.2	1.0	31.4±0.130
	5	8.4	0.81	32.2±0.043
	7	8.7	0.95	36.6±0.246
Doratomyces microspores MZKIB-399	3	7.9	0.58	22.6±0.310
	5	7.9	0.6	25.2±0.225
	7	8.6	0.88	26.4±0.136
Penicillium sp.	3	7.9	0.84	53.9±0.024
	5	8.4	0.99	54.1±0.320
	7	9.1	1.0	54.8±0.104
Trichoderma atroviride F6	3	8.7	1.0	30.5±0.143
	5	8.9	1.7	35.6±0.094
	7	9.3	1.89	38.6±0.007
T. polysporum HZ-31	3	8.4	1.53	52.3±0.147
	5	9.0	1.9	56.8±0.013
	7	8.6	2.1	55.4±0.310
Streptomyces gulbargensis	3	7.7	0.636	14.69±0.233
	5	7.8	0.881	22.41±0.031
	7	8.6	0.952	27.5±0.062
Streptomyces pactum DSM40530	3	8.3	0.710	21.17±0.249
	5	8.2	1.01	25.1±0.036
	7	8.9	1.57	28.01±0.210

In this and the following Tables: U.reaction^{-1}= U/mL enzyme, *PC= protein content of culture filtrate (mg/mL). **Activity=keratinase activity (U.reaction^{-1}).

Factors affecting the extracellular keratinase production by the chosen fungal strain *Trichoderma polysporum* HZ-31

Effect of the fermentation period

Concerning the proper fermentation period for the chosen fungal *Trichoderma polysporum* HZ-31, which was inoculated in the modified medium under the submerged shaken fermentation conditions, and the enzyme activity was followed within 3–9 days and this declared that the optimum productivity was at the 5th day of the incubation at 30°C.

The results also indicated that the keratinase activity increased rapidly during the linear phase of *Trichoderma polysporum* HZ-31 growth. The maximum keratinase activity (57.36 U mL^{-1}) was obtained after 5-days incubation and insignificantly decreased at the extended periods, where only 1.2% keratinase productivity was lost after the seventh day and this loss may be attributed to the depletion of some nutrients. On the other hand, all of the protein content, and the final pH of CF reached their maximum after 5 days of incubation (Table 1). All references in this context reported on the proper microbial keratinase production period to range from 2 to 7 days [33].

Effect of different culture media

Four culture media of varying formulations including the foregoing basal medium 1 (control) were examined to select the most favorable and affording the highest

Table 2 Effect of different culture media *on Trichoderma polysporum* HZ-31 keratinase productivity after 5-days incubation

Production medium No.	Final pH of CF	PC	Activity
1(Basal)	8.9	1.78	58.20±0.172
2	8.5	1.68	56.11±0.137
3	8.3	1.67	55.32±0.200
4	8.0	1.70	55.66±0.071

keratinase productivity. The data (Table 2) showed that, the basal production medium 1 was the most proper and led to the maximal *T. polysporum* HZ-31 keratinase productivity (58.20 U mL^{-1}), although the other medium 2 afforded good record (56.11 U mL^{-1} keratinase productivity). It was noticed that medium 4 enhanced *T. polysporum* HZ-31 growth rather than enzyme production. Accordingly, the culture medium 1 was chosen for keratinase production by *Trichoderma polysporum* HZ-31. In this concern, a similar formulation was designed for considerable keratinase production by *Bacillus licheniformis* RG1 [12].

Effect of the initial pH

Table 3 indicated that the higher keratinase production by *T. polysporum* HZ-31 was continuously exhibited at the alkaline pH values. The productivity at initial pH 9 afforded both the highest keratinase productivity (63.12 U mL^{-1}). On the other hand, the acidic and the slightly acidic and CF protein (1.94 mg mL^{-1}) pH (4.6–6.5) had the most reverse effects on the enzyme production, and more than 53% loss in enzyme productivity was recorded at the initial pH of 4.6. It is worth mentioning that, in all cases the final pH was consistently independent of the applied initial pH and generally lied in the alkaline range (9.3–9.8) and conclusively, it seemed that the final alkalinity was indispensable for the active keratinase production by *T. polysporum* HZ-31.

In addition, both culture growth and protein content of the culture filtrate were in direct relation with the initial pH to record their maximal at pH 9. This accorded to great extent with those reported by many authors, who obtained microbial keratinases on a wide-range of initial pHs including acidic, neutral, and alkaline pH. The alkaline pH 9.0 was reported by many authors [34,35]. Moreover, pH 11.0 was indicated to exhibit the maximum production of keratinase [36]. On the other hand, neutral to slightly acidic pH includes (7.0, 7.5, 7.8, and 8.0) were proper for producing keratinase enzymes [37–39].

Effect of some inorganic and organic nitrogen sources

Table 4 shows the effect of nitrogen source in the production medium on improvement of keratinase production by *T. polysporum* HZ-31 in the presence

Table 3 Effect of the production medium initial pH value on keratinase production by *Trichoderma polysporum HZ-31*

Culture medium Initial pH	Final pH of CF	PC	Activity
4.6	8.3	1.27	29.53±0.164
5.0	8.5	1.55	29.62±0.206
6.5	8.8	1.54	29.93±0.034
7.0 (control)	8.4	1.62	24.43±0.026
8.5	9.3	1.71	55.00±0.097
9.0	9.8	1.94	63.12±0.086

Table 4 Effect of some inorganic and organic nitrogen sources in the culture medium on keratinase production by *Trichoderma polysporum* HZ-31 in the presence (a) and absence (b) of the chicken feathers

N-source (g/l)	Final pH of CF	CP	Activity
Basal medium 1 (control)	**9.8**	**1.94**	**63.12**
a-In the presence of feathers			
Inorganic			
Ammonium molybdate	9.5	1.77	52.44±0.073
Ammonium sulphate	8.1	1.68	51.33±0.012
Sodium nitrate	9.6	1.79	53.45±0.314
Organic			
Urea	8.0	1.57	49.38±0.019
Keratin powder	9.4	1.77	52.22±0.164
Beef extract	9.7	1.94	64.47±0.149
b-In the absence of feathers			
Inorganic			
Ammonium molybdate	8.6	1.78	52.43±0.012
Ammonium sulphate	8.6	1.71	51.25±0.069
Sodium nitrate	8.9	1.76	52.52±0.163
Organic			
Urea	8.1	1.36	45.00±0.101
Keratin	8.4	1.63	50.57±0.130
Beef extract	8.3	1.57	49.02±0.042

of the chicken feathers. This was done by the replacement of both yeast extract and peptone with any of one of two N source groups (on equal N basis) the first group is inorganic and includes ammonium molybdate, ammonium sulphate or sodium nitrate and the second group is organic and includes urea, keratin powder or beef extract. The data declared that among all the sources tested, beef extract was the most promising and afforded the highest keratinase productivity (64.47 UmL^{-1}). In addition, all the other parameters were also the highest.

It was noticed that none of the other tested N sources than beef extract induced keratinase productivity, but led to a clear decrease in the keratinase production. In particular, urea had the most adverse effect on α-keratinase productivity, and more than 21% loss from the control was recorded. All of the inorganic nitrogen sources tested led to very low productivity and caused a loss ranging from 15.3 to 18.6%. The present record on the induction of beef extract on the fungal keratinase productivity accorded to great extent with those reported [40] on beef extract effect for improvement of *B. aerius* NSMK2 keratinase production.

On the other side, many authors applied feathers only as the sole N source for fungal keratinase production from *A. fumigatus* [41]; *T. atroviride* F6 [42], and from *Trichophyton ajelloi* [43], while chicken feather with ammonium mono-phosphate were applied for the optimum keratinase production by *Trichoderma harzianum* MH-20 [13], *Brevibacillus parabrevis* CGMCC 10798 [44]. It is worth noting that, only tryptone was applied to obtain keratinase from the fungal strain *Gibberella intermedia* CA3-1 [45], while only yeast extract was applied to obtain keratinase from *Trichoderma harzianum* HZN12 [23]. In addition, the independence of keratinase production on keratin, was reported as though the keratinous substrates play only indirect roles as inducing compounds and can be replaced by alternative type in nitrogen limited process [46].

On the other hand, concerning the reports of inorganic nitrogen sources effect on keratinase production [39], indicated no effect in the production of keratinase. In addition, chicken feathers was reported as nitrogen source for keratinase production by the fungal strain *T. atroviride* [42].

The data (Table 4) showed the effect of the previously mentioned nitrogen sources, but in the absence of the chicken feathers. The results indicated the compulsory need of feathers for the maximal keratinase production by the fungal isolate *T. harzianum* HZ-31, and the organic or inorganic nitrogen sources in the absence of feathers led to marked loss in productivity, particularly the maximum loss caused with urea and led to 28.7% productivity loss, even use of the beef extract in the absence of feathers led also to high productivity loss (22.33%).

Conclusively, the current data (Table 4) indicated the nature of *T. polysporum* HZ-31 keratinase produced under all the previous conditions as a constitutive keratinase, which has been produced even in the absence of any keratin compound, but it produced in the presence of both white chicken feathers and beef extract. The constitutive nature of the fungal α-keratinases was reported by many authors [13,23,29,45,47–49].

Effect of some carbon sources

The data (Table 5) disclosed that none of the applied saccharides in the optimized medium could improve keratinase production as glucose (control) did, except sucrose, which led to very little improvement (64.59 $U\,mL^{-1}$), and any of lactose, starch and cellulose, led to little lesser values. In this respect glucose was reported as the most preferable carbon source for fungal keratinases production from *T. harzianum MH-20* [13]*;* from *T. harzianum HZN12* [23]; and *Trichophyton ajelloi* [43]. On the other hand, other reports reported that sucrose found of highly significant in the production medium and led to the maximum keratinase yield [38,50].

Effect of the inoculum size and age

The inoculum concentration (15%, v/v) was the most proper and led to the maximum keratinase productivity, and above or below this value caused lower productivity (not shown). Inoculum size from 5 to 12.5% was recommended by many authors [27,36,38,51–53], and these reports were very close to the present study on the proper inoculum size.

Table 5 Effect of different carbon sources in the basal medium 1 on keratinase production by *Trichoderma polysporum HZ-31*

Carbon source	Final pH of CF	CP	Activity
None	8.3	1.29	36.75±0.360
Glucose (control)	9.1	1.93	64.47±0.094
Sucrose	9.5	1.96	64.59±0.086
Lactose	9.3	1.95	62.90±0.046
Cellulose	9.2	1.94	62.82±0.942
Starch	9.0	1.95	62.85±0.136

The inoculum age (ranged from 12 to 72 h) was applied in the production medium at all the optimum conditions previously defined. The data clarified that the inoculum age 48–72 h was the most appropriate and led to the maximal keratinase productivity by *Trichoderma polysporum* HZ-31 accordingly, 48 h-old inoculum was applied. On the other hand, the young inocula yielded less active keratinase, which reached to only 75% that of the optimum. This was completely accorded with those reported elsewhere [13]. However, the young inoculum (16 and 24 h) was recommended by some authors for bacterial keratinases [27,54,55].

Effect of the agitation speed

Varying agitation rates (100–250 rpm) were applied in the bench-top thermostatic shaker, comparing to a stationary culture for *Trichoderma polysporum* HZ-31. The results (not shown) indicated that generally, the shaken technique culture was more suitable for keratinase production than the static technique and a distinct direct relationship existed between agitation speed and keratinase production, final pH, and the filtrate protein till 180 rpm, above which the productivity and the other above parameters considerably decreased. Many authors indicated the speed of 150 rpm was the most suitable for keratinase production from *Trichophyton rubrum* and *Fusarium oxysporum* and *Aspergillus sp*., respectively [56,57]. Moreover, 500 rpm exhibited the highest keratinase yield and activity [19].

Effect of the incubation temperature

Incubation of *T. polysporum* HZ-31 culture was carried out at a wide range of incubation temperatures (20–45°C) and the results (Unrecorded) clarified that, the incubation temperatures (25–30°C) favored keratinase production by *T. polysporum* HZ-31, which achieved the maximal production at 28°C. It was noticed also that the incubation temperature changes (25–40°C) little affected the enzyme productivity and the culture filtrate protein and above 40°C, a marked changes were recorded. On the other hand, the final pH at applied temperatures was consistently alkaline (9–9.7) and changed within a very limited range.

In this respect, 28°C was also reported as the optimum for keratinase production from *Streptomyces sp.* 2M21 [58]. While, 30°C was applied for keratinase production from the fungal strain *T. harzianum* MH-20 and *T. viride*, respectively [13,29]. While, 37°C was applied for keratinase production from *Trichoderma harzianum* HZN12 and *Pseudomonas sp*., respectively [23,59]. The data also clarified that the fungal α-keratinase productivity below or above the optimum range (25–30°C) led to lower productivity and the most reverse effect was recorded at both 20 and 45°C, where more than 75 and 68% loss of α-keratinase productivity were recorded. The big loss of productivity at the moderate temperature 45°C provides clear evidence that the nominated fungal strain *T. polysporum* HZ-31 was a typical mesophilic fungal strain under α-keratinase production conditions and this may not be the same for its produced enzyme and the effect of reaction temperature on the enzyme activity.

Effect of different keratinaceous wastes

The separate effect of some keratinaceous wastes on α-keratinase production by the fungal strain *T. polysporum* HZ-31 compared with the white chicken feathers with the same concentration (0.5%, w/v) as a control was studied. Those wastes were namely; white duck feathers, turkey feathers, white chicken feathers (control), black chicken feathers, goat hair, and sheep wool (pieces and powder) to assist the ability of the fungal strain to utilize any of them instead of the white chicken feathers producing an effective α-keratinase, applying all the optimized fermentation conditions. Any of those wastes were supplemented to the fermentation medium in 0.5% (w/v) concentration after some physical pretreatments. The data (Table 6) declared that both the duck feathers and white chicken feathers (control) were the most favorable and led to the highest keratinase enzyme production, which was very close (66.45 and 66.01 U mL^{-1}), followed by turkey, white feather, chicken black feather, goat hair and sheep wool (64.75, 60.38, 56.63, 49.94 UmL^{-1}, respectively).

It is well known that feathers and animal wool are β-keratin-rich sources. All the tested keratinaceous sources for the α-keratinase production processes except goat hair (mainly consists of α-keratin) are β-keratin-rich. It is worth to note that the fungal strain *T. polysporum* HZ-31 can ably utilize any of those feathers and sheep wool producing α-keratinase. All feathers applied led to considerable α-keratinase

Table 6 Effect of different keratinaceous wastes in the culture medium on keratinase production by *Trichoderma polysporum* HZ –31

Keratinaceous waste	Final pH of CF	CP	Activity
Chicken white feathers (control)	9.8	1.96	66.01±0.432
Duck white feathers	9.8	1.98	66.45±0.017
Turkey white feathers	9.7	1.93	64.75±0.386
Chicken black feathers	9.4	1.89	60.38±0.035
Goat hair	9.1	1.03	56.63±0.021
Sheep wool	9.0	0.89	49.94±0.071

productivity, while sheep wool led to the lowest productivity. Goat hair, even though its rigid structure as α-keratin was utilizable by the fungal strain to produce a considerable adaptive α-keratinase amounts. This was completely accorded with those reported on the utilization of some keratinaceous wastes for the production of eco-friendly skin unhairing agents [48], also partially accorded with the reported data [60]. The comprehensive details of α and β–keratin were early explained [26]. According to the abundance of white chicken feathers as waste in Egypt, it was applied in all the next fermentation processes. In this respect, many investigators used chicken feathers wastes for getting high amounts of microbial keratinase enzymes [12,39,54,61–66].

The proper concentration (0–2%, w/v) of feathers leading to the maximum α-keratinase production was explored. Feather different concentrations in the culture medium under all the specified previously mentioned conditions, were applied. The results (not shown) disclosed that 1%, (w/v) was the most proper concentration, while the higher concentration 1.5% (w/v) afforded slight α-keratinase productivity increase (only 0.5%), so, 1% (w/v) concentration was applied. The absence of feathers from the production medium didn't prevent the fungal strain from synthesizing a constitutive α-keratinase, with less yield (only 26.48% that of the optimum). Many authors reported 1% (w/v) as the proper feather concentration in the microbial α-keratinase production medium [13,29,35,48,52,67]. However, others used higher feather concentrations (2–15%, w/v) for the maximum microbial α-keratinase production [12,38,61,68].

Effect of some additives

Some additives (10 mM) to the optimized production medium for *T. polysporum* HZ-31 keratinase production improvement, were separately tested. Those included any of $CuSO_4$, $CaCl_2$, $KMnO_4$, NaCl, $MgSO_4$, $FeSO4.7H_2O$, $ZnSO_4.H_2O$, and $HgCl_2$. The results clarified that each of $CuSO_4$, $CaCl_2$, $KMnO_4$, NaCl and $MgSO_4$ led to slight productivity increase. On the other hand, other additives as $FeSO_4.7H_2O$, $ZnSO_4.H_2O$ caused productivity decrease to 90.7 and 87.3% that of optimum, respectively. The most adverse effect was with $HgCl_2$, which inhibited *T. polysporum* HZ-31 keratinase productivity (only 63.7% that of optimum). In this concern, addition of $MgSO_4$ was reported to promote microbial α-keratinase production [12,27,39,52]. Also, the addition of $MgSO_4.6H_2O$ with other minerals like NH_4Cl, NaCl, K_2HPO_4, KH_2PO_4, enhanced the keratinase production [69].

Partial purification of T. polysporum HZ-31 keratinase crude enzyme

The partial purification of the crude *T. polysporum* HZ-31 keratinase was performed as described in Materials and Methods and this was done by fractional precipitation with ammonium sulphate, ethanol or acetone, after which the most promising enzyme fraction affording the highest α-keratinase activity was applied for the purpose of some medical and industrial applications.

Table 7 summarized the fractional precipitation with the three agents, which offered a total of seventeen fractions, these included 6 with ammonium sulphate (40–90%), 6 with ethanol (40–90%), and 5 with acetone (50-90%). Generally, the keratinase activity recovery by ammonium sulphate was lower than that of the other two fractional precipitations and amounted to (52.25%), but the recovered protein by it, was the highest amount and amounted to 83.6% of the applied enzyme protein.

The *T. polysporum* HZ-31 keratinase partial purification with ethanol was considerable and afforded 6 fractions. Application of 0 – 40% ethanol concentration afforded the lowest recovered protein (5.3%), however, 60% ethanol fraction afforded the highest total protein quantity (10.0 mg), which represented 13.3% protein recovery of the original, followed by 70, 80 and 50% ethanol fractions. On the other hand, keratinase activities of the fractions were not harmonized in all fractions with their protein level, it was clear that the 60% ethanol fraction was the highest keratinase activity fraction, and recovered 13.5% of the total keratinase activity and possessing 1.02-purification fold. Generally, ethanol precipitation was able to recover 51.1 and 55.0% of the applied enzyme protein and its total keratinase recovery, respectively.

As regards acetone, it was able to recover 33.19 and 53.23% of the applied enzyme protein and keratinase activity, although those values as total were not the highest compared with that of ammonium sulphate or ethanol, all the five acetone fractions efficiently were the highest in their specific activity and purification fold. Acetone precipitation afforded five enzyme fractions. The 50% acetone fraction recovered the lowest protein and activity, although it afforded the highest purification fold, while, the three fractions (50, 60, and 70%) were nearly similar in their protein recovery (3.73, 4.53, and 4.00%, respectively) and recovered activity (9.73, 8.41, and 7.28%, respectively). On the other hand, the two fractions

Table 7 Summary of *Trichoderma polysporum* HZ-31 keratinases partial purification

Saturation or concentration of the precipitating agent (%)	Total protein of fraction (mg)	Recovered protein (%)	Total activity of fraction (U)	Recovered Activity (%)	Specific activity (Umg^{-1}protein)	Purification fold
Culture filtrate	75	100	1076±0.19	100	14.3	1.00
None						
Amm. Sulphate						
40	8.7	11.6	94.50±1.36	8.70	10.86	0.75
50	11.0	14.6	110.00±0.32	10.22	10.0	0.69
60	5.2	6.93	96.60±0.97	8.97	18.57	1.29
70	8.9	11.86	95.00±2.03	8.82	10.6	0.73
80	10.0	13.33	78.37±0.39	7.28	7.83	0.54
90	19.0	25.33	88.90±0.02	8.26	4.67	0.32
100	NP					
Total	62.8	83.6	563.3	52.25	8.97	0.63
Ethanol						
40	4.0	5.3	90.00±0.059	8.30	22.5	1.57
50	6.0	8.0	89.40±0.048	8.30	14.5	1.01
60	10	13.3	146.30±0.368	13.50	14.6	1.02
70	6.8	9.0	101.93±0.512	9.40	14.9	1.04
80	6.1	8.1	84.50±1.070	7.80	13.8	0.96
90	5.6	7.4	83.80±0.236	7.70	14.9	1.04
Total	38.5	51.1	595.90±0.254	55.00	15.48	1.08
Acetone						
40	NP					
50	2.8	3.73	104.8± 0.250	9.73	37.42	2.6
60	3.4	4.53	91.2±0.018	8.41	26.82	1.87
70	3.0	4.0	78.4±0.241	7.28	26.13	1.82
80	8.2	10.93	152.1±0.320	14.13	18.54	1.29
90	7.5	10.0	146.4±0.362	13.62	19.52	1.36
Total	24.9	33.19	572.9±0.021	53.23	23.01	0.61

precipitated at 80 and 90% acetone recovered 14.13 and 13.62% of the initial activity, respectively and 10.93 and10.0% of the initial protein, respectively, according to their similarity and their considerable purity, the two fractions were well mixed and used as the partially purified keratinase enzyme (PPE) and nominated as the 80–90% acetone fraction to be applied in the application experiments.

General properties of *T. polysporum* HZ-31 keratinase (crude and partially purified) forms

Effect of the reaction temperature

The effect of reaction temperature on the fungal keratinase forms (crude and partially purified), was investigated within a wide temperature range (30–75°C). The data (Table 8) declared one optimum reaction temperature (50°C) for the two forms and this provides a preliminary indication of the good thermostability of the two forms. The heat activation of the partially purified form was better than that of the crude one. Moreover, the two forms kept most of their activity until degree 65°C. Generally, the two forms showed a segment of the bell-shapeded curve known for the effect of reaction temperature on the enzymes (not shown).

The partially purified form outperformed the crude form by more than 18% activity. It is well known that temperature increases the reaction velocity and also affects the rate of enzyme destruction, producing a fall in the concentration of active enzyme. At high temperatures, the adverse effect becomes significant as the reaction proceeds. Thus, the remarkable activity detected within a wide range of temperatures (30–65°C) again pointed out the high thermostability of the two enzyme forms. In this

Table 8 Effect of the reaction temperature (°C) on the crude and partially purified *Trichoderma polysporum* HZ–31 keratinase activity

	Keratinase activity* (U/ml)	
Reaction temperature (°C)	Crude enzyme	PPE
30	49.40±0.221	59.51±0.038
35	49.60±0.047	61.80±0.162
40 (control)	54.30±0.132	69.82±0.246
45	57.50±0.159	70.00±0.018
50	57.90±0.627	70.81±0.092
55	57.30±0.146	70.26±0.147
60	56.70±0.327	69.62±0.327
65	55.01±0.024	68.60±0.099
70	45.90±0.302	68.63±0.023
75	45.17±0.149	68.15±0.014

respect, adjusting the temperature at 55°C was the optimum temperature for the keratinolytic activity [70]. As reported elsewhere [70], the enzyme showed its maximum keratinolytic activity at 50°C which accorded with the present result. However, the optimum temperature for other microbial keratinase was at 40°C [46,71–73], also, the optimal keratinase activity by *Bacillus tequilensis* strain Q7 was at 30°C [74]. In addition, the optimum reaction temperatures from 60 to 75°C for microbial keratinases were reported by many authors [75–79].

Thermal stability

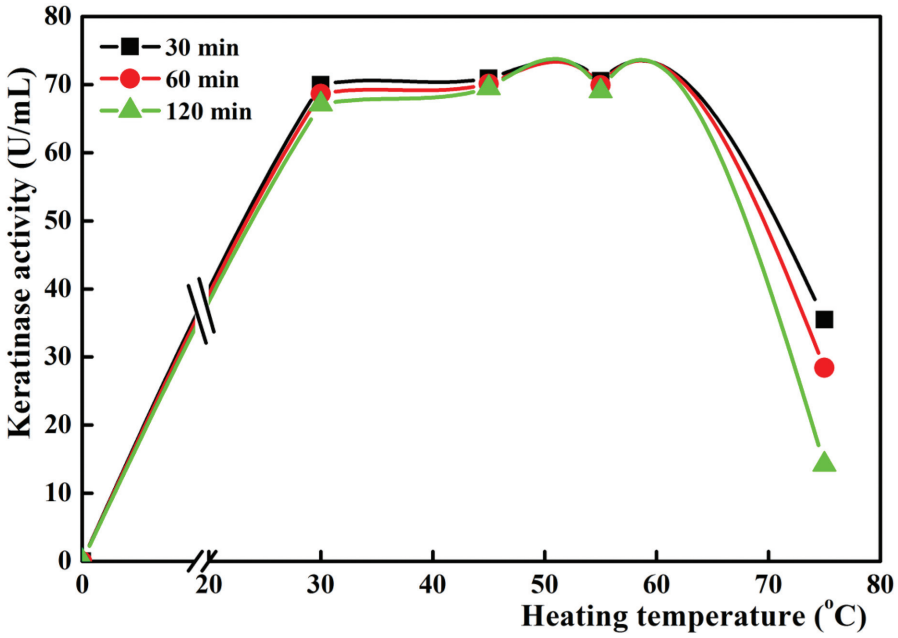
The thermal stability study was carried out only for the partially purified *T. polysporum* HZ-31 keratinase preparation of optimized concentration (1.4 mg mL^{-1}), where it was heated at different temperatures (30, 45, 55, and 75°C) for different time intervals (30, 60, and 120 min) in the absence of its substrate (keratin). After the end of each incubation period, the enzyme was cooled and used in the reaction mixture in the usual manner. The residual activity (%) compared with that in the normal enzymatic reaction with an unheated enzyme solution was calculated. The data (Table 9 and Fig. 1) clarified that, the enzyme exhibited the highest stability under heat treatment of 45°C, where it retained more than 99.90% of the original activity after 30 min heating, furthermore after heating for 60 and 120 min it still kept 98.76 and 97.90% of its original activity, respectively. In addition, it is worthy to mention that the enzyme had a good stability even at elevated temperatures, where it retained 99.33 and 98.43% of its original activity after heating for 30 and 60 min at 55°C, respectively.

Table 9 Thermal stability of *T. polysporum* HZ-31 PPE

Heating temperature (°C)	Time of exposure (min)	Keratinase activity* (U/ml)	Residual activity (%)
30	**30**	69.95±0.024	98.52
	60	68.65±0.661	96.69
	120	67.10±0.034	94.50
45	**30**	70.93±0.015	99.90
	60	70.12±0.097	98.76
	120	69.51±0.166	97.90
55	**30**	70.53±0.007	99.33
	60	69.89±0.214	98.43
	120	69.03±0.148	97.22

Moreover, increasing the temperature to 75°C, it still retained 50, 40, and 20% of its original activity after heating for 30, 60, and 120 min, respectively and this was clear evidence of the excellent thermostability of the partially purified *T. polysporum* keratinase. In this connection, the *P. aeruginosa* 4–3 keratinase showed good thermal stability at 55°C [80]. The suitable incubation period for the reaction mixture to afford the maximum activity of the enzyme was studied. The results (Table 10) clarified the maximum activity of the crude and PPE was afforded after 120 min of reaction and before 120 min, the crude form afforded only 22.55, 13.01, and 1.99% of the optimum after 30, 60 and 90 min, respectively. After 120 min, the crude form activity little decreased and lost 2.64%

Figure 1



Thermal stability of *Trichoderma polysporum* HZ-31 PPE.

from the optimum. Concerning, the PPE form activity after 30, 60 & 90 min reaction, the activity was 22.9, 2.79 & 0.79% lesser than that after 120 min. Extension of the reaction time to 150 min led to only 1.33% decrease than that after 120 min.

Applications

The practical applications of *Trichoderma polysporum HZ-31* partially purified keratinase form in some industrial and medical applications (at lab scale), including leather unhairing, stain removal and topical callus degradation.

Leather unhairing

It is well known that leather industry by the chemical traditional method is the dirtiest industry and produced a toxic pollutants, such as sulfur oxides mixture, hydrogen sulphide, nitrogen oxides and many other gases, as well as the solid remains, the most dangerous of which is the standard carcinogen nitroseamine, which dissolved in the waste water. These pollutants particularly in the unhairing step comprehensively explained all the environmental diseases caused by traditional leather industry applied in Egypt and many developing countries worldwide, and cause the most hard impact on the environment [13]. Therefore, using of an alternative bio-material for dehairing process was necessary and actually participates in environment safe [81]. Based upon this, the partial purified keratinase (PPE) was used in this experiment and showed an excellent result of goat skin as shown in Fig. 2, after 1 h the hair was partially removed and after extended time (12–16 h) of incubation with keratinase, it showed quietly complete hair removing.

On the other hand, application of chemicals in dehairing process required more than 20 hours of incubation, and even then, some hair fragments could still be visible on the goat's skin. Additionally, the skin's quality had many bad flaws, which had completely overcome by the safe natural keratinase preparation [73]. In this respect, the use of crude keratinase in the tanning industry for both dehairing skins and hydrolyzing hair into amino acids for environmental processing was comprehensively reported by [82].

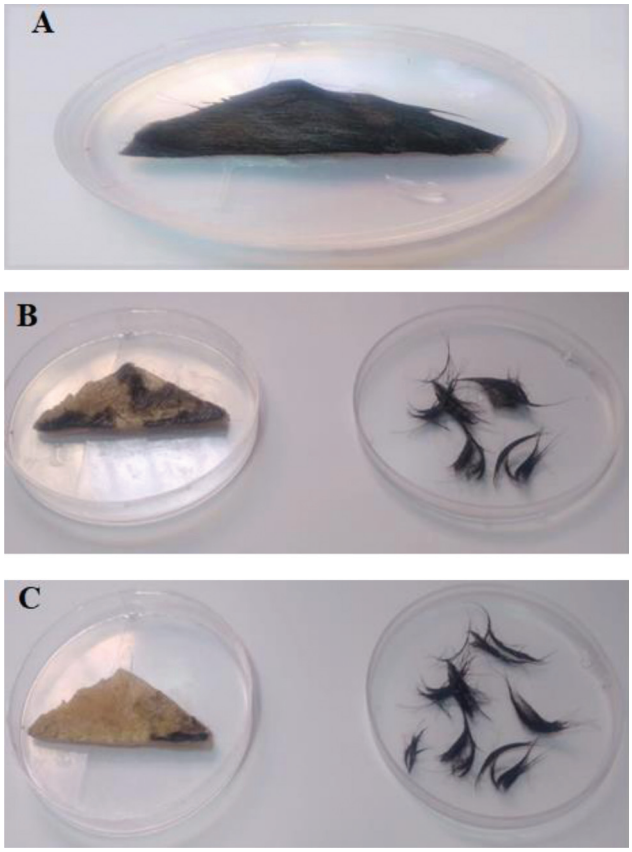
Table 10 Effect of the enzymatic reaction time on the crude and partially purified *T. polysporum* HZ –31 keratinase activity

	Keratinase activity (U/ml)	
Reaction time(min)	Crude	PPE
30	48.30±0.289	54.90±0.097*
60	54.25±0.076	69.22±0.183
90	61.12±0.058	70.64±0.082
120 (control)	62.49±0.077	71.98±0.247
150	60.71±0.247	70.26±0.088

Washing performance

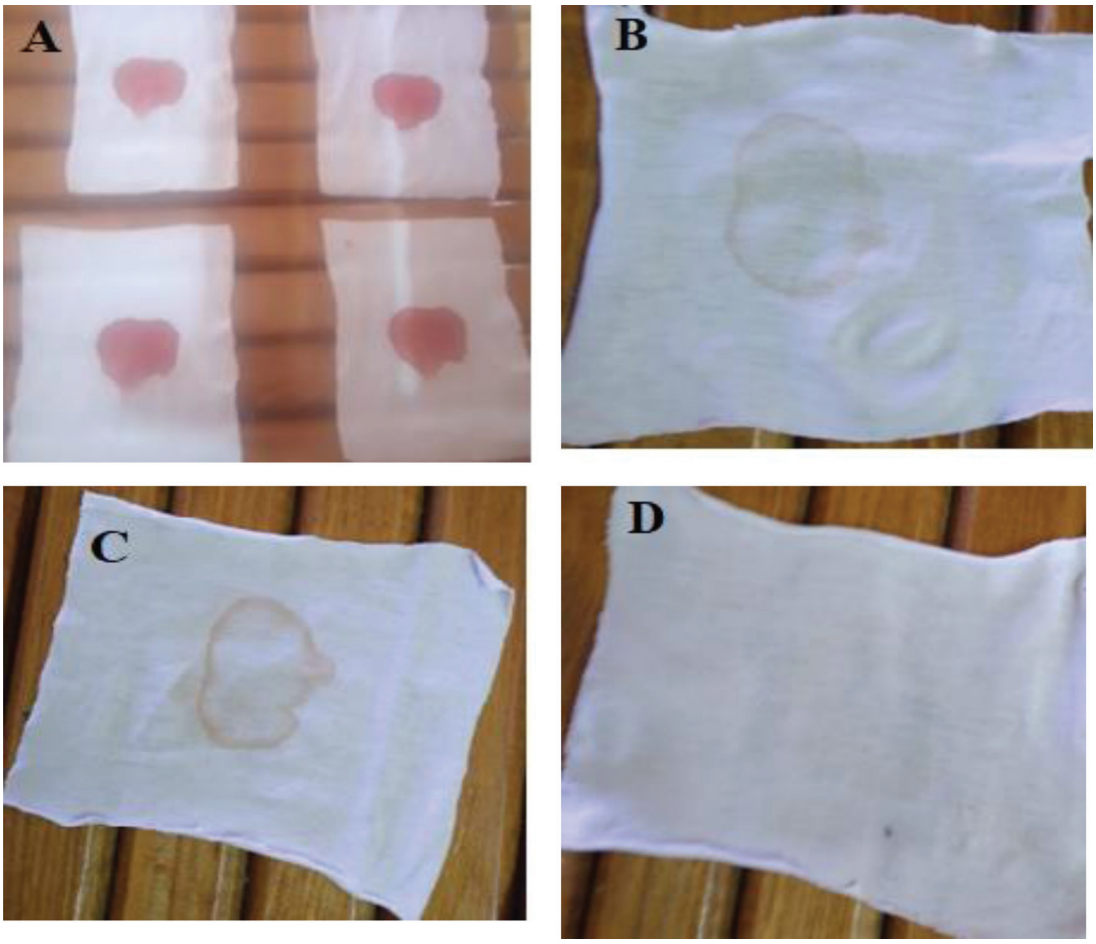
PPE exhibited good efficiency for blood stain removal and had this property as haemoglobinase enzyme. This strongly candidates for using as a bio-additive in detergents industry for cleaning purpose of blood stains on clothes, where it showed its high efficiency in this experiment. As shown in Fig. 3, A- Control, B- partially removal of the blood stain using the enzyme only. C-partially removal of blood stain applying the detergent only and, D- complete removal of blood stain with the enzyme-detergent mixture and this indicated the PPE good stability and compatibility when mixed with the laundry detergent (Ariel) and this was also reported [7]. In addition, keratinase called pedobacter was prepared and exhibited high stability with laundry detergents when were tested even as solid or liquid where, it retained 37% of its activity in the presence of 5 mg/mL of Ariel detergent [83]. Added to that many authors supported using of proteases in industry as a new type of a cleaning bio-additive in detergent formulation [84–87].

Figure 2



The effect of *T. polysporum* HZ-31 keratinase (PPE) on cow's skin: (A) Native skin specimen (control), (B) skin after 1 h of treating with the enzyme, (C) Skin after 2 h of treating with the enzyme.

Figure 3



Wash performance of keratinase (PPE): A) blood-stained cloth treated only with water (control), B) blood-stained cloth treated PPE only; C) blood-stained cloth treated with detergent (Arial) only; D) blood-stained cloth treated with mixture of PPE and Arial detergent.

Callus biodegradation

The medical application of the tested partially purified *Trichoderma polysporum HZ-31* keratinase (PPE) was topically applied on the human callus instead of the chemical preparations as collomak. Collomak is a medical topical solution applied for the treatment of warts, verrucas, corns and calluses and composed of the following active ingredients (%, w/v): salicylic acid 20, lactic acid 5, polyclocanol 600, 2 and other inactive ingredients, namely: diethyl phthalate, ethyl cellulose and acetone. This preparation is manufactured in Egypt by October Pharma. Beside the corrosive effect of lactic acid, some other components have many bad effects on human skin. Certainly, in no way can a comparison be made between the harmful unspecified chemical drug (collomak) and the other safe and specified *T. polysporum* HZ-31 α-keratinase preparation.

Callus was easy, safe degradable by the partially purified *T. polysporum* HZ-31 keratinase after gentle repeat (Table 11). In this respect, many previous studies indicated the ability of keratinase to hydrolyze callus into soluble peptides and free amino acids [6]. Callus degradation processes were reported to be under certain experiments [7].

Conclusively, it could be decided here that the present study succeeded in achievement a constitutive production of an extracellular α-keratinase related to the alkaline keratinases group. The product was pale greenish powder, instantly soluble in water and buffer solutions. This product successfully proceeded a number of safe applications at the laboratory level as the above mentioned. Production process of the above-mentioned product is of a double-benefit value, the

Table 11 Effect of keratinase enzyme (PPE) on callus biodegradation at room temperature

Incubation time of enzyme with callus (h)	Keratinase activity (U/mL)
Without callus (control)	18
2	31
4	43
6	43

first is the utilization of keratinaceous wastes saving the environment and the second is the achievement of an enzyme preparation of diverse important industrial and medical applications. Also, it could be decided here that the partially purified keratinase, which is proper for most industrial applications, must be purified to be acceptable in medicinal (rather than the topical uses) and food industries [88].

Conclusion

Keratinase have an ultimate practical importance in industry, medicine, food industry and waste management fields. The present study aimed the production of an innovative purified heat resistant α-keratinase with promising industrial and medical applications. The fungal strain *Trichoderma polysporum HZ-31* was reported as the most potent α-keratinase producer with a maximum enzymatic activity (58.2 UmL^{-1}) after 5-days incubation in a production medium containing (%, w/v): whole chicken feathers as recommended substrate 0.5, glucose 0.2, peptone 0.5, yeast extract 0.5, K_2HPO_4 0.1, KH_2PO_4 0.3, $CaCl_2$ 0.1, $MgSO_4$ 0.1, and pH 7.0. Partial purification studies recommended acetone fractionation of the crude keratinase and at 80–90%, concentration it offered the most promising keratinase fraction. This fraction had high thermostability and kept at 55°C more than 98% of the original after 60 min heating and this temperature (55°C) was also the optimum for 2 h enzymatic reaction. Conclusively, the present study succeeded in the achievement of a constitutive extracellular alkaline α-keratinase, which successfully proceeded complete leather unhairing after 2 h at 37°C, afforded high performance to cloth blood stain removal with Arial detergent after 2 h at 50°C and complete degradation of the human callus after 4 h at 50°C.

Acknowledgments

Authors express their full appreciation for the authorities of the National Research Centre, Egypt and the Faculty of Science-Ain Shams University for any facilities provided to complete this submitted article.

Authors' contributions

S.S.E.: She conducted all laboratory experiments and collected the related references. A.A.H.: She followed the experiments and participated in preparing the submitted article as well the carefully editing and revision. S.M.E.: She followed-up all administrative procedures required to complete the research and reviewed the article after its preparation. A.-M.S.I.: He proposed the research topic, developed the work plan, designed laboratory experiments as well the enzymatic preparation applications. He also contributed to writing the article and its carefully revision to be at the required level.

Financial support and sponsorship

Nil.

Conflicts of interest

The authors declare there are no conflicts of interest.

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