Journal of Innovations in Pharmaceuticals and Biological Sciences
www.jipbs.com
JIPBS
e-ISSN: 2349-2759 p-ISSN: 2395- 1095

Research article

Production of keratinolytic enzymes from industrial wastes for employing in leather industry

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Abstract

The use of chicken feather waste as a substrate for the production of keratinolytic enzyme blend has a potential to generate value-added products. In this study, Six fungal isolates were screened for this purpose. *Trichoderma viride* showed the highest keratinase activity (69 U ml^{-1}) at 5-day incubation period. Three other proteolytic enzymes along with α-keratinase were produced in the medium, alkaline, neutral and acidic proteases enzymes. The alkaline protease showed the highest activity (2453 Anison unit) with weak collagenase activity. Partial purification of crude enzyme showed a purification fold of keratinase enzyme reached 5.99 times with 37% protein recovery at 80% ammonium sulphate saturation using bulk precipitation technique. By applying this fraction on hide specimen it was cleared that it was the most proper for unhairing process.

Key words: Leather, dehairing, Keratinolytic enzymes, *Trichoderma viride*.

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1. Introduction

Leather tanning industry considered as one of the highly polluting industries accompanied with bad effect on the environment. The worldwide production of leather was about 24 billion m^2 by 2005 using a huge amount of hazardous chemicals in their processing (about 3.5 million tons). Most of these pollutants are discharged into the effluent. The tannery processes of leather are producing not only high amount of toxic materials but also an offensive odor emanating from the decomposition of proteinous waste material and the presence of sulphide, ammonia and other volatile organic compounds [1].

Lately, the enzyme blend was used in the tannery processes of leather. Microbial proteases are today used in soaking, un-hairing, bating of hides [2]. In this respect, many enzyme preparations were developed for these purposes in the market as lederzim RV, lederzim CA, and Erhavit MC [2,3].

In fact, enzyme blends accomplished many weighty advantages, specially on quality

of the final leather product and overcome all the environmental pollution by chemicals and other wastes [2,4].

2. Materials and Methods

Microorganisms and culture conditions
The microbial isolates (five fungi and one bacterium) were screened for the production of protease blends with special reference to keratinase enzyme. The microbial isolates screened were *Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum, Trichoderma viride, Trichoderma harzianum* and *Bacillus subtilis*. These fungal and bacterial species were isolated from different soil sources of Cairo governorate and identified in Taxonomy Department, Ain-Shams University, Cairo city, Egypt.
The fungal isolates were maintained as single spore on potato-dextrose-agar (PDA) medium, subcultured on PDA slopes and incubated at 30 ^{o}C for 7 days. The bacterial isolate *B. subtilis* was maintained on nutrient agar (NA) medium and incubated at 37 ^{o}C for 3 days.

Media
The following media were used and had the following composition (g L^{-1}) [5].

Fungal isolates maintenance and sub-culturing (PDA) medium
Potato slices, 500; dextrose, 10; agar, 20 and distilled H_2O.

Fungal inoculum (growth enhancement) medium
Peptone, 5; Yeast extract, 1; glucose, 10; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 0.5 and distilled H_2O.

Protease blend preparation medium by fungi or bacteria [6]
Milled white feather, 20; $(NH_4)H_2PO_4$, 1; $MgSO_4.7H2O$, 2; KCl, 0.2 and pH was adjusted to 7.5.

Raw cow-hide specimens
These were provided as dried rawhide by Radio Tannary-Misr-El Kadeema, Cairo, Egypt. This was pretreated by 25% (w/v) NaCl as preservative, then wetted just before unhairing by 3% (w/v) wetting agent, Egyptol.

Wetting agent, Egyptol
This was purchased from the Chemicals Marketing Corp., Cairo, Egypt.

Bovine keratin powder
This was purchased from ICN Bio-chemicals Inc. Ohio, USA.

Fungal inoculum preparation
The fungal inoculum was prepared by cultivation of the fungal strain in growth enhancement medium and the culture was incubated in bench-top shaker at 30^{o} C for 48 h. 5 ml of inoculums pellets were used for fungal culture inoculation.

Fungal enzyme preparation
Five ml fungal pellets of each fungal culture were transferred to 250 ml Erlenmeyer flask containing 50 ml enzyme blend production medium. The fungal culture was incubated in bench-top shaker at 30 ^{o}C, and the culture was lasted for 4, 7 and 9 days. Thereafter, the culture was centrifuged at 6000 rpm to separate the mycelium from the culture filtrate. The clear supernatant was taken for measuring keratinase, protease (acidic, neutral and alkaline) and collagenase activities.

Protein content measurement
The protein content of the enzyme preparations was determined by the method of Lowry [7].

Assay for different protease activities (acidic, neutral or alkaline)

The different protease (acidic, neutral or alkaline) activities were assayed in each culture filtrate as follows; one ml of clear enzyme was mixed with 1 ml of 1.5% (w/v) casein solution in 0.03 M phosphate buffer for acidic (pH 5.7) and neutral protease and of 0.1 M carbonate-bicarbonate buffer for alkaline protease (pH 10). The mixture was incubated at 37 ^{o}C for 60 min then the reaction was stopped by adding 3 ml of 15% TCA and left for 30 min. at room temperature. The mixture was centrifuged at 4000 rpm for 5 min and the supernatant was used for measurement of released soluble proteins and amino acids released according to Lowry [7]. Control was prepared by adding TCA solution at zero time. One unit of any protease was defined as the amount of enzyme required to liberate 1 microgram of tyrosine during one minute of reaction (Anson unit, AU).

Assay for α-keratinase activity

Keratinase activity was determined according to the method of Nickerson *et al.* [8] as follows, one ml enzyme solution was added to 5 ml borate buffer (pH 8.5), 50 mg α-keratin, 2.5 ml distilled water and 0.5 ml of 1 mM-$MgCl_2$. The mixture was incubated for 2 h at 37^{o} C, then stopped by 1 ml of 10% (w/v) trichloroacetic acid and left for 30 min at room temperature, then centrifuged and the released amino acids in the clear supernatant were measured as tyrosine [7]. One unit of keratinase activity was defined as the amount of enzyme required to liberate one microgram tyrosine under the specified conditions.

Assay for collagenase activity

This was detected by Gover and Ramanthan [9] method using Scanning electron microscope for detection. The surface holes and the opening up of fiber bundles of grain surface which composed of collagen indicates the activity of collagenase enzyme.

Scanning electron microscope (SEM) analysis

The skin specimen was examined using SEM (JEOL/ 1230, Services Central Lab, National Research Centre, Cairo, Egypt) to notice the quality of the skin grain and estimate the effect of collagenase enzyme.

Preparation of hides for enzyme treatment (British Leather Confederation, BLC method)

A fresh fleshed cow-hide skin was soaked in sodium chloride solution for one hour then washed twice with tab water. The specimen was treated with a commercial detergent solution (Egyptol) and cut into 5 cm^2 pieces then washed twice with sterile water. The hide pieces was sterile with alcohol and left to dry at sterile conditions. A set of conical flasks with different pHs (7, 8.5, 8.9, 10) buffers (50 ml) was prepared and inoculated with 2 ml of crude enzyme. The hide pieces were transferred to these flasks and shaked for two hours at 37^{o} C in incubated-shaker. The skin pieces were gently scraped with fingers to remove loose hairs. Lime and Na_2S were used instead of the protease blend for comparison. At the end of the process, the skin fragments were fixed as described by Lopes *et al.*[10] and the unhaired leather was evaluated by scanning electron microscope (SEM) analysis to detect the quality of enzyme treated skin.

Ammonium sulphate precipitation

The enzyme was first precipitated from the cell free supernatant by the addition of ammonium sulphate at different saturation levels ranging from 20-80% (v/v) then stirred for 1 h at 4°C. The mixture was stored overnight at 4^{o}C and the precipitated protein was collected by

centrifugation at 12,000 X*g* for 20 min and 4°C then re-suspended in a minimal volume of 0.1M phosphate buffer, pH 8.0 to obtain the concentrated enzyme suspension. The enzyme suspension was finally dialyzed overnight in the same buffer, then dried and stored at -20°C.

3. Result and Discussion

The main goal of this study is to produce a potent enzyme blend used in dehairing process of leather to overcome the pollution problems accompanied with the traditional chemical methods without deformation of the grain layer of leather.

Six fungal isolates were screened for study of their abilities to produce keratinase enzyme using the production medium recommended by Cantera [6] using dry ground feather as a sole carbon source and incubation periods 5,10,17 days at 28^{o}C by static culture method.

Table 1 showed that all fungal strains had the ability to produce keratinase enzyme with different activities. *Trichoderma viride* showed the highest keratinase activity (69 Uml^{-1}) at 5-day incubation period.

The final pHs of all culture was in alkaline range (7.5-8.6) during all incubation periods and were not a significant parameter for keratinase enzyme production.

The protein content of the culture filtrate was increased exponentially with the age of the culture and reached the highest values at 17 days incubation period. There was no clear relation between the protein content and keratinase activity.

The effect of different feather concentrations on keratinase production is displayed in Figure 1. The fungal growth and keratinase production increased with the increase of feather concentration. The optimal concentration was 2.0% at which the highest keratinase activity (69 U ml^{-1}) was reached.

Table 1. Screening of six fungal isolates for keratinolytic enzymes production in different incubation periods

Fungal isolate	Incubation period (day)	pH of CF	Protein content of CF (mg/ml)	Keratinase activity (U ml^{-1})
1	5	7.50	0.92	2.44
	10	8.03	1.10	1.95
	17	8.50	1.34	1.68
2	5	8.0	1.60	7.64
	10	8.5	1.84	9.12
	17	8.5	2.07	9.12
3	5	8.2	1.70	12.08
	10	8.3	1.93	5.42
	17	8.6	2.26	2.96
4	5	7.64	0.52	16.26
	10	8.00	0.66	26.37
	17	8.30	0.99	54.21
5	5	7.6	0.19	2.46
	10	7.6	0.23	5.91
	17	7.7	0.26	7.88
Trichoderma viride	5	8.34	1.35	69.00
	10	8.50	2.03	50.00
	17	8.60	2.70	30.00

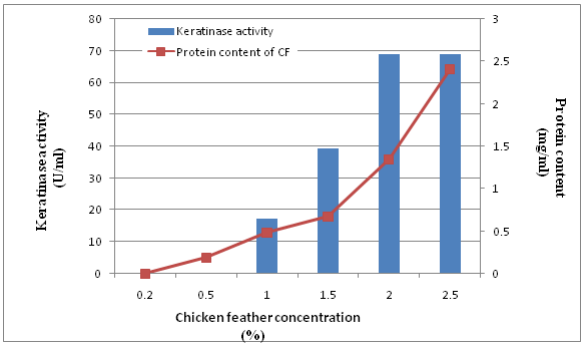

Figure 1. Effect of waste (chicken feather) concentration in culture medium on *T. viride* Keratinase productivity

The effect of different feather concentrations on keratinase production is displayed in Figure 1. The fungal growth and keratinase production increased with the increase of feather concentration. The optimal concentration was 2.0% at which the highest keratinase activity (69 Uml^{-1}) was reached.

These results are matching with those reported with Hamdy & Mohamed *et al.*[11, 12]. Suntornsuk and Suntornsuk reported that higher concentrations (3 and 5%) might cause substrate inhibition or repression of keratinase production [13].

The effect of different percentages of keratinolytic wastes (feather and cow hair) on the productivity of keratinolytic enzymes is presented in Table 2.

The effect of different percentages of keratinolytic wastes (feather and cow hair) on the productivity of keratinolytic enzymes showed that the cow hair is not suitable for keratinolytic enzymes production and this may be related to the highly crystalline, α-helical structure of the α-keratin of hair containing a high percent of disulphide bonds. In comparison, the chicken feather has β-pleated sheet structure or β-keratin with low percent of disulphide bonds and weak crystalline structure. This vision is matched with Williams & Ferdinand [14, 15].

There were three other proteolytic enzymes along with α-keratinase were produced in the medium, alkaline, neutral and acidic proteases enzymes. The alkaline protease showed the highest activity (2453 Anison unit). Cantera & Feigel pointed to the synergistic action of the alkaline and neutral proteases with α-keratinase in leather unhairing process [16, 17].

To optimize the enzyme blend properties ammonium sulphate precipitation technique was applied for partially purification of the crude enzyme. From the results summarized in Table 3, an extracellular keratinolytic enzymes were precipitated from the culture filtrate of *T. viride* using different saturation levels of ammonium sulphate ranging from 25-100% saturation. The results showed that the fractional precipitation by 60% ammonium sulfate saturation was the best, showing the highest activity (615.14 U/ml) and specific activity 95.67 U/mg with recovered protein reached 22.97%.

While, the highest activity was achieved at 80% ammonium sulphate saturation using bulk precipitation technique (Table 4); where, the purification fold of keratinase enzyme reached 5.99 times with 37% protein recovery.

Figures 2&3 showed the effect of partial purification by ammonium sulphate on the activity of the other proteases in culture filtrate of *T. viride.* The highest activities for alkaline, neutral and acidic proteases were found in the precipitate resulted from 80% ammonium sulfate saturation using bulk precipitation technique. On the other hand, the collagenase enzyme showed weak activity (+1) in 60% and 80% saturations, while this activity was slightly increased (+2) in 100% saturation. These results assured that the 80% ammonium sulphate saturation part was the most proper for unhairing process. The purification fold of keratinase enzyme reached 5.99 times more than crude one at 80% amm. Sulphate saturation.

Table 2. Effect of different keratinolytic wastes (chicken feather and cow hair) combination in culture medium on *T. viride* Keratinase productivity Keratinase productivity

Feather concentration (%, w/v)		pH of CF	Protein content of CF (mg/ml)	Keratinase activity (U ml^{-1})
Feather	Hair			
0.0	2.0	7.04	0.192	NA
0.5	1.5	7.67	0.241	19.71
1.0	1.0	7.24	0.443	32.04
1.5	0.5	8.0	0.886	46.82
2.0	0.0	8.34	1.350	69.0

Table 3. Fractional precipitation of the crude keratinase enzyme of *T. viride* by salting out with different ammonium sulphate saturation

Ammonium sulphate satu. (%)	Protein content of the fraction (mg/ml)	Total protein (mg/ Fraction)	Protein recovery (%)	Keratinase activity (U ml^{-1})	Keratinase activity (Umg^{-1} protein)	Total keratinase activity of fraction (U)	Keratinase activity recovery (%)	Purification fold
None (CF, 1400ml)	0.51	714	--	47.5	93.15	66509.1	--	1
25	NP*	NP	--	NP	NP	NP	--	--
40	NP	NP	--	NP	NP	NP	--	--
50	NP	NP	--	NP	NP	NP	--	--
60	6.43	164	22.97	615.14	95.67	15689.42	102.7	1.03
70	NP	NP	--	NP	NP	NP	--	--
80	5.6	98	13.73	502.3	89.7	8790.25	96.24	0.96
90	NP	NP	--	NP	NP	NP	--	--
100	5.51	55.07	7.71	475.19	86.24	4749.31	92.58	0.93
Total		317.07	--	1592.63	--	29228.98	--	--
Recovery (%)		44.4	--	--	--	44	--	--

NP = no precipitation

Table 4. Bulk precipitation of the crude keratinase enzyme of *T. viride* by salting out with different ammonium sulphate saturation

Ammonium sulphate satu. (%)	Protein content of CF (mg/ml)	Total protein (mg/Fraction)	Keratinase activity (U ml^{-1})	Keratinase activity (U mg^{-1} protein)	Total keratinase activity of fraction (U)	Purification fold
None (CF, 1400ml)	0.51	714	47.5	93.15	66509.1	1
80	4.15	261.8	2315.04	557.84	146042.5	5.99
Recovery (%)	---	37	---	---	581.06	---
100	0.876	55.2	103.24	117.85	6505.32	1.27
Recovery (%)	---	7.7	---	---	9.7	---

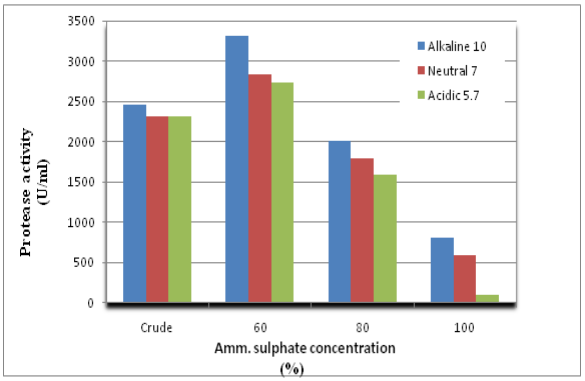

Figure 2. Detection of other proteolytic enzymes (alkaline, neutral and acidic) in the ammonium sulphate fractional precipitate of the crude *T. viride* enzyme blend

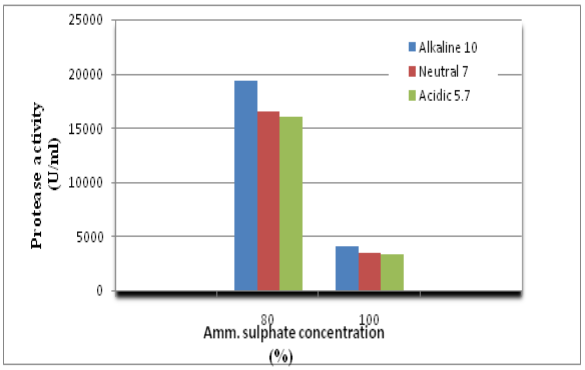

Figure 3. Detection of other proteolytic enzymes (alkaline, neutral and acidic) in the ammonium sulphate bulk precipitate of the crude *T. viride* enzyme blend

These results are matched with Abdallah *et al.*[18] who found that the highest recovered activity was achieved with 50% saturation ammonium sulphate which exhibited 13.18 fold purification of extracellular alkaline protease produced by *S. rochei* NRC 24. Also, proteases from *S. albidoflavus* and *S. alboniger* are precipitated by 45% and 40% ammonium sulphate saturation, respectively [19, 20]. On the other hand, Al-Askar *et al.* reported that the maximum precipitation of protease content from the culture filtrate of *S. griseorubens* E44G was reached at saturation of 70% of ammonium sulphate, giving the highest proteolytic activity and specific activities [21]. Salting out with ammonium sulphate was also reported by many investigators [22, 23].

By applying the crude enzyme produced from *T. viride* strain on cow leather the unhairing process was completely accomplished in only 24 hour at pH 10 without any deformations in the grain layer of leather using BLC technique (Figure 4) and this assured that there was no collagenase activity. The visual observation showed that the leather specimen kept with its natural features without any deformations in the grain layer and this was assured with SEM examination (Figure 5). These results refer to the success of this enzyme blend in leather industry with good

leather quality. In comparison with the crude enzyme the total time of unhairing was lowered by about five hours in 80% ammonium sulphate saturation using bulk precipitation technique (Table 5).

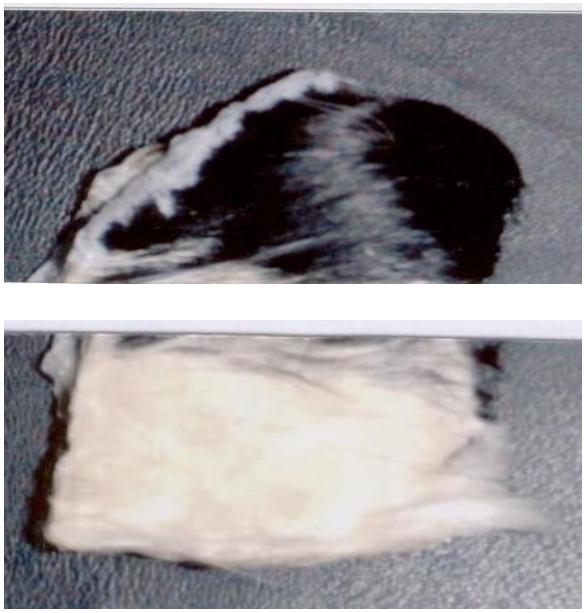

Figure 4. Cow skin specimen before and after enzymatic treatment

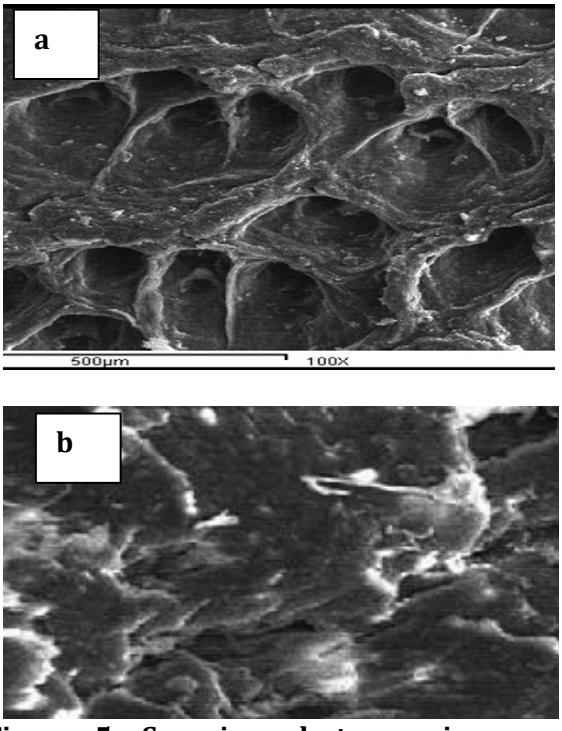

Figure 5. Scanning electron microscope (SEM) of (a) chemical and (b) enzymatic treated cow skin.

Table 5. Effect of different *T. viride* ammonium sulphate enzyme fractions on BLC unhairing efficiency

Ammonium sulphate satu. (%)	Total time of unhairing (h)	
	Static	Shaking
60	18	1.5
80	24	2
100	26	2

Finally, we can conclude that the partially purified enzyme blend produced from the fungal strain *T. viride* can be employed in leather industry with low-cost production and high leather quality.

This article does not contain any studies with human participants or animals performed by any of the authors

Funding: This study was not funded by any organization.

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