Iraqi Journal of Science, 2023, Vol. 64, No. 10, pp: 5051-5063
DOI: 10.24996/ijs.2023.64.10.14

ISSN: 0067-2904

Optimize and Purification of Keratinase Produced from Local *Aspergillus Terreus* A13 Isolate Using A Feather as Substrate

Ali J. R. Al-Sa'ady
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 10/9/2022 Accepted: 6/11/2022 Published: 30/10/2023

Abstract

Keratin is a fibrous, insoluble structural protein that is highly cross-linked with hydrophobic, hydrogen, and disulfide bonds. Keratinases are enzymes that belong to the category of serine hydrolases that are capable of breaking down keratin. The results of the determination of the better fermentation system showed that the production of keratinase from local *A.terreus* A13 isolate by submerged fermentation (SmF) system was the best system to give the highest specific activity (113.4 U/mg) of keratinase compared with solid-state fermentation (SSF). The optimum conditions for keratinase production by SmF, were determined via cultivation conditions, including carbon source, nitrogen source, temperature, pH of the medium, and time of incubation were optimized to enhance the production of total keratinase production in a culture of *A.terreus A13* with incubator shaker. The highest product of total keratinase was achieved in feather broth with 2 % sucrose, and 0.5 % soya bean, with a pH of 5.5 at 28 °C for 8 days. Separation and purification of keratinase from a local isolate of *A.terreus* A13 was done by precipitating with 0-75 % saturated ammonium sulfate, then by ion-exchange chromatography on DEAE-Cellulose column and sephadex G-150 gel. Partially purified keratinase gave an activity of 5.1 U/ml, protein concentration of 0.004 mg/ml, and specific activity of 1275 U/mg with purification fold of 4.96 and 49 % as yield. The aim of the present study was to optimize the production of keratinase from *A. terreus* A13, cultivated using optimum conditions, and its use for the biodegradation of feathers.

Keywords: Optimum conditions, Solid state fermentation, keratinase, Purification.

تحديد الظروف المثلى وتنقية الكيراتينيز المنتج من العزلة المحلية لفطر *Aspergillus terreus* A13 باستخدام الريش كمادة اساس

علي جبار رشك الساعدي
[1]قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

الكيراتين هو بروتين هيكلي ليفي غير قابل للذوبان ومرتبط بشكل كبير مع اواصر كارهة للماء ، وهيدروجينية ، وكذلك اواصر ثنائية الكبريتيد. الكيراتينازات هي إنزيمات تنتمي إلى فئة السيرين المحللة القادرة على تكسير الكيراتين. أظهرت نتائج تحديد نظام التخمير الأفضل أن الكيراتيناز المنتج من العزلة المحلية لفطر *A.terreus* A13 عن طريق نظام التخمير المغمور (SmF) كان أفضل نظام ليعطي أعلى فعالية نوعية (113.4 وحدة /

*Email: ali.jabbar15@yahoo.com

مجم) لانزيم الكيراتينيز بالمقارنة مع تخمرات الحالة الصلبة (SSF). تم تحديد الظروف المثلى لإنتاج الكيراتينيز بواسطة نظام التخمير المغمور، من خلال ظروف الزراعة ، والتي تتضمن: المصدر الكربون ، والمصدر النيتروجين ، ودرجة الحرارة ، ودرجة الاس الهيدروجيني للوسط ، وكذلك وقت الحضانة لتحسين إنتاج الكيراتينيز الكلي في وسط زراعي لفطر *A.terreus* A13 وفي حاضنة هزازة. تم تحقيق أعلى انتاج كلي من انزيم الكيراتينيز في وسط الريش المحتوي على 2٪ سكروز ، 0.5٪ فول الصويا ، مع اس هيدروجيني 5.5 عند درجة حضن في 28 درجة مئوية لمدة 8 أيام. تم فصل وتنقية الكيراتينيز من العزلة المحلية لفطر *A.terreus* A13 بالترسيب بكبريتات الأمونيوم وبنسبة تشبع 0–75 ٪, بعدها تم تنقيته باستخدام كروماتوكرافيا التبادل الأيوني على عمود DEAE–Cellulose و وعمود الجيل sephadex G–150. أعطى الكيراتينيز المنقى جزئياً فعالية 5.1 U / ml وتركيز البروتين 0.004 مجم / مل وفعالية نوعية بحدود 1275 U / mg مع عدد مرات تنقية 4.96 و وحاصل يقدر 49٪. الهدف من هذه الدراسة هو تحسين إنتاج الكيراتينيز من فطر *A. terreus* A13 ، المزروع باستخدام الظروف المثلى للانتاج ، واستخدامه للتحلل الحيوي للريش.

Introduction:

Keratin is a fibrous, type of insoluble structural protein that is highly cross-linked with di-sulfide, hydrogen, and hydrophobic bonds. Keratinases are enzymes that belong to the category of serine hydrolases that can break down keratin [1]. The main protein in chicken feathers is keratin, which is one of the hardest animal proteins to degrade. Keratin is distinguished by its densely packed shape in -helixes and -sheets with a high degree of disulfide linkages [2]. So they aim to hydrolyze highly rigid, tightly cross-linked structural polypeptide keratin that is resistant to routinely used proteolytic enzymes like trypsin, pepsin, and papain, microbial keratinases have become crucial in biotechnology. When these enzymes are degraded, keratinous substrates such as hair, feathers, wool, hooves, nails, and horn are present in high amounts [3].

The extracellular enzyme keratinase is employed in the biodegradation of keratin. It can only be produced when a keratin substrate is present [6]. In the poultry business or industry, keratinase is a possible enzyme for hair and feather removal [4]. *Bacillus* species and fungi, including the species *Aspergillus, onygena, Absidia,* and *Rhizomucor*, have both been used to manufacture this enzyme. *Trichophyton mentagrophytes* is one of the darmatophytes, as well as other genus includes: *Microsporum canis, T. rubrus, T. gallinae, M. gypseum,* and *M. canis* [3]. It is likely the only genus being commercialized for the production of alkaline keratinases, and the genus Bacillus is a significant source of these enzymes [5, 7].

Aspergillus terreus is a member of a family of filamentous ascomycete fungi that is crucial in the environment because it breaks down organic materials that are found in nature. About 180 species make up this genus, and some of them cause diseases for people, animals, and plants. Several other species are crucial to the biotechnological sector's ability to produce chemicals, enzymes, and medications [8]. Undoubtedly, these various *Aspergillus* species are essential for serving as microbial cell factories for a variety of industries, including the food, beverage, and pharmaceutical industry [9]. The present study aimed ~~was~~ to optimize the production of keratinase from *A.terreus* A13, cultivated using optimum conditions, and its use for the biodegradation of feathers.

Materials and methods:

Potato Dextrose Agar (PDA) from Himedia, India. Sodium acetate (CH3COONa), Sodium hydroxide (NaOH), Ethanol 95%, and other materials from BDH, England.

Isolates collections:

Collection of seventy-three fungal isolates from different areas. Forty-one isolates from the Biotechnology Department\Collage of Science\Baghdad University, three isolates from Collage of Biotechnology\Al-Nahrain University, seventeen isolates from Biology Department\College of Science\Baghdad University, four isolates as standard isolates, and eight isolates from Tropical Biological Research Unit- Collage of Science\Baghdad University. These fungal isolates were isolated from the soil and diagnosed in the laboratories of these centers. In the laboratory, all the plates of isolates were transferred directly. Under serial conditions, the isolates were subcultured on potato dextrose agar, then incubated at 30°C, after that the isolates were screened for the production of keratinase as indicated by the strategy portrayed by Mazotta, *et al*. [10].

Screening the fungal isolates for Keratinase production:

Using the single spore isolation approach, spores from 73 fungal isolates were obtained using 2% Tween-20 solution, diluted to $8x10^6$ spores/ml, and then collected. A hemocytometer was used to count the spores [9]. The inoculum was grown in a 250 ml flask containing 50 mL of feather broth (containing (gm/L) (pH 6): 20gm feathers, 5gm yeast extract, 1 gm MgSo4, and 1 gm K2HPO4) at 28±1°C on a rotary shaker at 120 rpm for 8 days [8]. Screening experiments of isolates on this media for keratinase production were performed. The biomass of the fungi was separated by filtration using Whatman No. 1 filter paper and discarded after the fermentation process had been going on for 8 days. Centrifugation at 10,000 rpm for 10 min was used to recover the superannuation. One local *A.terreus* A13 isolate was chosen based on the screening results and recultivated on the same production media (pH 6.0) [10].

Enzyme assay:

Enzyme activity was estimated by a protocol of Chitte [11] with some modifications. A mixture of 1.8 ml of Tris-HCl (0.02 M, pH 8) containing 0.1% keratin with 0.2 ml of the crude enzyme solution was added in a test tube, then incubated for 30 min at 45 °C. five ml of 15% trichloroacetic acid (TCA) was added to the reaction after incubation to stop its (TCA).

The mixture was centrifuged at 10,000 rpm for 15 minutes. A blank was made by combining 3 ml of TCA with the same substrate as before, followed by the addition of 0.2 ml of extracted enzyme. The identical procedures as with the samples under investigation were applied to the blank. A cuvette for the spectrophotometer was filled with 3 ml of the supernatant. To determine the proteolysis activity of the keratinase, the absorbance at 280 nm was measured. The amount of enzyme needed to release 354 micrograms of an amino acid under specific circumstances was used to define one unit of keratinolytic activity.

Assay for Protein Concentration:

The protein concentration in samples was assessed and determined using the Bradford technique [12].

Types of fermentation system:

Two paradigms of fermentation system were used for keratinase production from the local *A.terreus* A13 isolate, these systems were submerged (SmF) and solid state fermentation (SSF). The medium in submerged fermentation was described above, while in solid-state fermentation the medium was composed of:

A 250 mL flask containing Feathers 5 gm, 0.5 gm yeast extract, 0.1 gm MgSo4, and 0.1 gm K2HPO4, then moisture 60 % of distilled water, the pH was adjusted to 6.0, then sterilized at 121°C for 15 minutes. This medium was used for keratinase production by the *A.terreus* A13 isolate. Each flask containing production media was incubated with 2 ml of spore suspension

8×10^6 spores/ml., the flasks of SmF group at 28±1 ºC were incubated with the rotary shaking incubator (140 rpm) for 8 days. Whereas the flasks of SSF were incubated at 28±1 °C for 8 days. The keratinase activity and protein concentration were estimated.

Extraction of keratinase produced in solid-state fermentation:

After the finish of SSF fermentation, the activity and keratinase concentration were estimated. At first, the culture was extracted by using 25 ml of sodium acetate buffer (0.2 M pH 6). The mixture was incubated in a rotating shaker for 2 h at 28±1°C at 140 rpm. Then the extract was filtrated by Whitman filter paper No. 1 for the biomass removed from the filtrate.

Optimization of cultural conditions of keratinase production:

Optimum conditions for keratinase production by the selected isolate were performed using SmF. These parameters were carbon sources, nitrogen sources, incubation temperature, pH, and incubation period.

Carbon sources:

Seven types of carbon sources were used to determine the optimal carbon source of keratinase production from local *A.terreus* A13 isolate. These carbon sources including (20 gm/l): lactose, starch, fructose, sucrose, cellulose, maltose, and glucose, were added separately in different flasks containing feather broth. Then inoculated with 8×10^6 spores/ml, and the inoculation was incubated for 8 days at 28±1°C; after that, the keratinase activity and protein concentration were estimated [13].

Nitrogen sources:

Eight types of nitrogen sources were used to determine the optimal nitrogen source of keratinase production from local *A.terreus* A13 isolate. These nitrogen sources including: Soya bean, Yeast extract, Urea, Ca(No3)2, Casein, NH4Cl, NaNo2, and (NH3)2So4, were added separately in different flasks containing feather broth plus sucrose. Then inoculated with inoculum size 8×10^6 spores/ml, the inoculation was incubated for 8 days at 28±1°C. The keratinase activity and protein concentration were estimated [9].

Temperature:

To select the optimum temperature for enzyme production from *A.terreus* A13 isolate, flasks containing medium that was selected as an efficient medium for keratinase production were inoculated with inoculum size 8×10^6 spores/ml and incubated at different temperatures 25, 28, 30, 35, 37, 40, and 45 °C for 8 days. Then the keratinase activity and protein concentration were estimated [9].

pH:

The selective broth medium was prepared at different pHs 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8. All flasks were inoculated with 1 % (8×10^6 spores/ml) of *A.terreus* A13 isolate and incubated for 8 days at 28±1°C, the keratinase activity and protein concentration were estimated after that [13].

Incubation period:

The selective broth medium was inoculated with 8×10^6 spores/ml of *A.terreus* A13 isolate and incubated at 28±1°C for different incubation periods (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 days). And then the keratinase activity and protein concentration were estimated [8].

Purification of keratinase:

Separation of keratinase:

After 8 days of the fermentation process, the biomass of *A.terreus* A13 isolate was separated utilizing filtration employing Whatman No. 1 filter paper. The supernatant was recovered employing centrifugation at 10,000 rpm for 10 min. The keratinase activity and protein concentration were estimated.

Purification of keratinase by ion exchange chromatography:

The precipitation step was done by adding solid ammonium sulfate to the crude extract with (0-75) saturation rates. The exchanger DEAE-cellulose was prepared as described by Whitaker, [14]. The DEAE-cellulose was packed into the column (28×1.7 cm), and then the column was equilibrated with the same buffer overnight. Using a pasture pipette, 13 ml of dialyzed keratinase was carefully passed through a DEAE-cellulose column, washed with 0.005 M Tris-HCl buffer pH 8.0, and then attached proteins were eluted with Tris-HCl buffer pH 8.0 and a NaCl gradient of 0.1–1 M. This process was carried out at a flow rate of 30 ml/hour through the exchanger. Protein fractions weighing 3 ml were collected. Protein contents were traced in accordance with absorbency at 280 nm and activity estimated. The fractions that ~~were~~ showed activity were collected and concentrated with sucrose; the peaks were estimated by plotting the absorbance at 280 nm versus the fraction number.

Purification of keratinase by gel filtration chromatography:

The Sephadex G-150 column (65 1.5 cm) was designed and packaged in accordance with the manufacturer's instructions (Pharmacia- Sweden). The flow rate of 30 ml/hour and 0.02 M Tris-HCl buffer at a pH of 8.0 was used to equilibrate the column overnight. The column was then filled with14 ml of keratinase enzyme and the column was washed with 225 ml of Tris-HCl buffer pH 8.0. Three milliliter fractions were gathered. Each fraction's absorbency at 280 nm was tested, and the activity was estimated.

Results and discussions:

Screening the fungal isolates for keratinase production:

After the collection of seventy-three diagnosed soil fungal isolates. One local *A.terreus* A13 isolate was chosen based on the screening findings. Selection of the active isolate was done depending on the keratinase activity that was measured with feathers as substrate. The findings indicated that one isolate ~~has the capacity to~~ can produce high keratinase yield when using selective culture production media, accordingly isolate A13 which is identified as *Aspergillus terreus* and is selected as the most active isolate for keratinase production compared with other isolates ~~which~~ gives a lower yield of keratinase.

Type of fermentation system:

Two fermentation systems were used for keratinase production from *A.terreus* A13 employing feathers as substrate, these systems were SmF and SSF. The results of this experiment exposed that the production of keratinase using *A.terreus A13* isolate employing SSF gave the lowest yield compared with SmF system which gave the highest concentration. The specific activity of keratinase produced by SmF was 113.4 U/mg, while the other system (SSF) showed a lower concentration of enzyme produced (Figure 1).

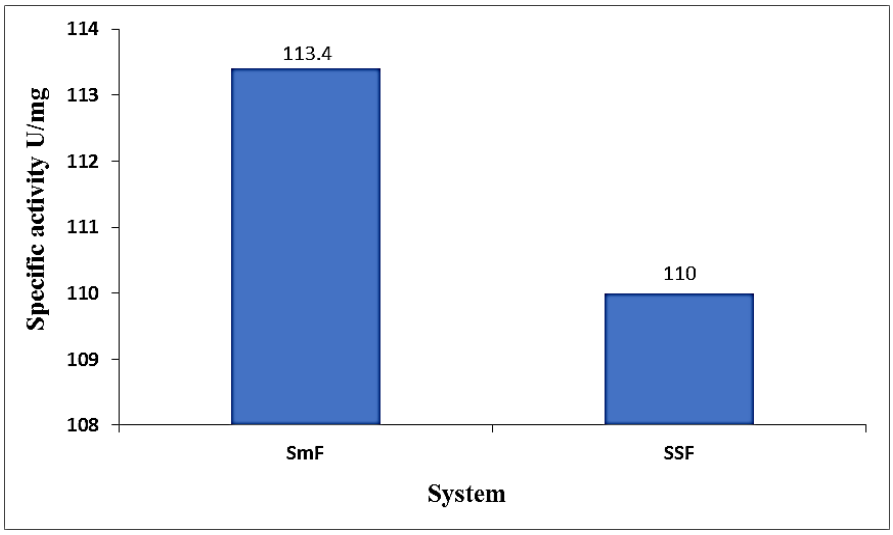

Figure 1: Production of keratinase from local *A.terreus* A13 isolate employs various systems.

The outcomes agreed with the results of El-Ayouty, *et.al.* [15], who demonstrated that submerged fermentation was a better system for the production of keratinase by *Aspergillus flavipes*. While Shata and Farid [16] found that solid-state fermentation was best system for the production of keratinase from *Streptomyces* sp. Previously published reports provided supporting evidence that different paradigms of solid substrates, including orange peel, wheat bran, barley, rice straw, sugarcane bagasse, etc., can increase the yield of primary metabolisms in *A.terreus* [9, 13].

Effect of carbon source:

As shown in Figure (2), the result indicated that *A.terreus A13* potentially used various carbon sources for keratinase production. Among the 7 kinds of carbon, sources ~~were~~ examined, the maximum keratinase production was obtained in sucrose plus feathers medium (167.8 U/mg) while keratinase is rarely produced in other carbon. Glucose, often is a great source of carbon for microbial growth, it interfered with the formation of various compounds of primary and secondary metabolites. (13).

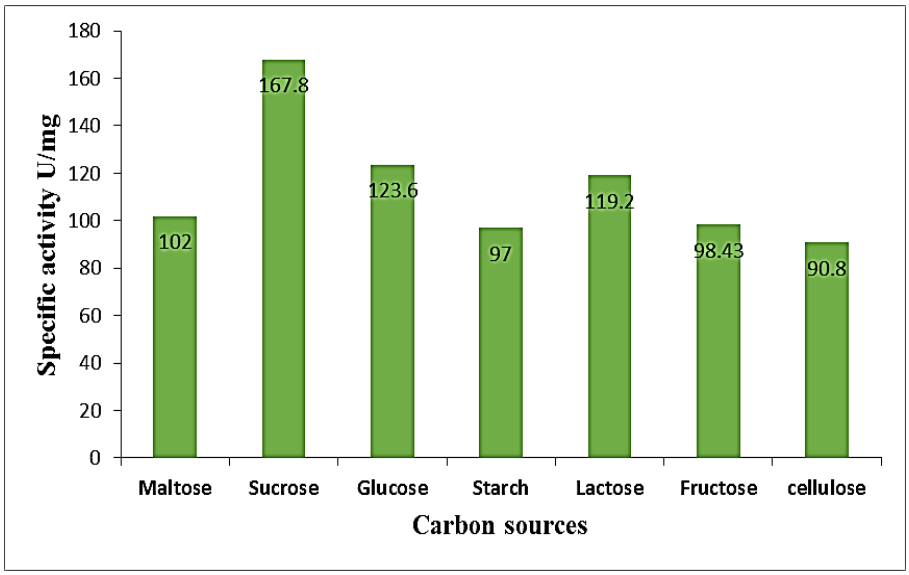

Figure 2: Impact of carbon source on keratinase production from local *A.terreus* A13 isolate using SmF, pH 6 incubation at 28 °C for 8 days.

These results disagree with the results of [16], which found that the best carbon source for maximum keratinase production from *Streptomyces* sp. under solid-state fermentation was glucose. Mazotto, *et.al.*, [17], showed that the better nitrogen and carbon source for keratinase production from *Aspergillus niger* was keratin. Other studies that employed other sugars like; starch, sorbitol, lactose, maltose, and sucrose also found increased alkaline keratinase production [9, 13, 16], suggesting that the optimum carbon source for enzyme production varies depending on the kind of microbe.

Effect of nitrogen source:

Various nitrogen sources such as Soya bean, Yeast extract, Urea, Ca(No3)2, Casien, NH4Cl, NaNo2, and (NH3)2So4 were supplemented separately for keratinase production from *A.terreus A13.* Different nitrogen sources used in fermentation are reported to have an impact on the growth of microorganisms and the synthesis of keratinase. The results showed that Soya bean was the best nitrogen source for keratinase production. As shown in Figure (3), yeast extract as a nitrogen source yielded higher keratinase production with a specific activity of 207 U/mg compared with the other nitrogen sources. According to previous studies, main metabolite production like keratinases depends on a variety of nutrients found in organic nitrogen sources. In actuality, *A. terreus* A13 can produce keratinase when various nitrogen sources are present.

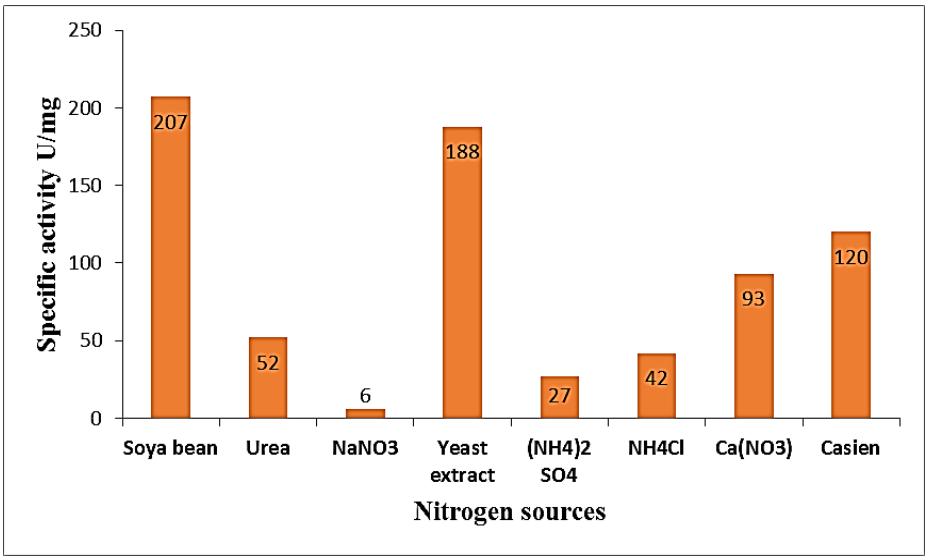

Figure 3: Effect of nitrogen source on keratinase production from local *A.terreus* A13 isolate using SmF, pH 6, incubation at 28 °C for 8 days.

Mohamad, *et.al.* [13], found that the maximum production of the keratinase enzyme from *Pseudomonas* sp. was peptone as a nitrogen source when it was added to the feather medium. While Nnolim, *et.al.*, [18], found that added nitrogen sources in the production media of the keratinase production from *Bacillus sp.* lead to a decrease in enzyme production. However, the presence of nitrogen in all forms is regarded as essential for the production of enzymes. In addition, the existence of nitrogen in both kinds (NO3 and NH4 +) as well as salts usually contains zinc ions is considered to be necessary for the production of keratinase [13].

Effect of temperature:

The findings displayed that the temperature had a tremendous impact on the growth of the fungi. Temperature, on the other hand, affected keratinase production, with an increase in the

yield of the enzyme at 28 °C. The highest yield (208 U/mg) of keratinase was detected at 28 °C (Figure 4). This outcome is similar to the result of the keratinase production from *A.terreus* [8].

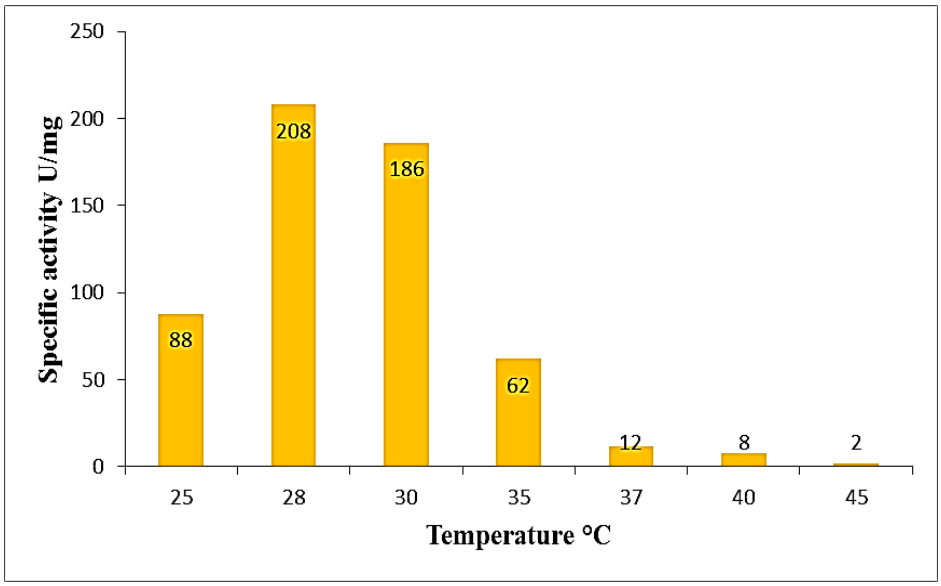

Figure 4: Effect of temperature on keratinase production from local *A.terreus* A13 isolate using SmF, pH 6, and incubation for 8 days.

The better temperature for keratinase production from this fungus was 28 °C. These correspond with primary metabolite production in other microorganisms. Temperature is a significant parameter that influences the success of an SmF system. Some of the results indicated that the enzyme production corresponded closely to the growth of the fungi, and the optimum temperature for keratinase production by *A.terreus* A13 is similar to the optimum temperature of the growth of the fungi [9, 10]. This observation was in agreement with those reported by [17], which showed that the highest keratinase was obtained at a temperature of 28 °C which was optimum for the growth of the fungi.

Influence of pH:

Local *A.terreus* A13 isolate was grown in feather medium with 20 gm/l glucose and 5 gm/l soya bean over an 8-days period to investigate the initial pH effect on fungi growth and keratinase production. At pH 5.5, keratinase had the highest specific activity (302 U/mg). Figure 5 demonstrated the relationship between keratinase production and the initial pH of *A.terreus* A13 growth. The better pH was between pH 3 and 8. This finding suggested that pH plays a role in keratinase formation. In a related experiment, it was discovered that the keratinase synthesis during fungal growth and keratinase production was correlated with a suitable concentration of hydrogen ions in the medium (pH 5.5). On the other hand, when the pH of the culture media rose or fell below pH 5.5, the fungal growth was inhibited.

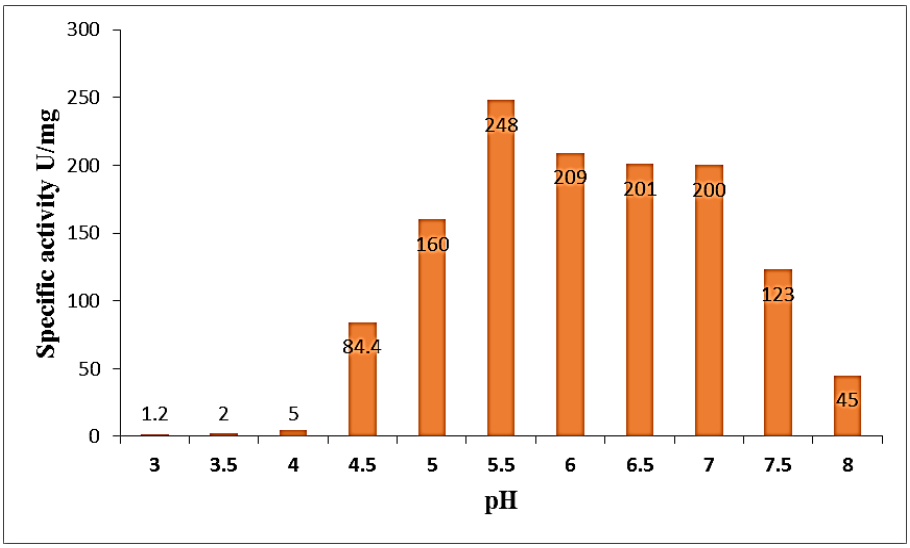

Figure 5: Effect of pH on keratinase production from local *A.terreus* A13 isolate using SmF, incubation at 28 °C for 8 days.

The crucial element in biomass increase and keratinase formation was the variable shape of fungi under a different initial pH value [19]. The solubility of salts, the ionic state of substrates, the absorption of different nutrients, and product biosynthesis can all be impacted by the pH of the medium. In general, cells can only grow within a limited pH range, and pH frequently affects metabolite production [17, 20]. Koutb, *et.al.*, [8], found that the best pH for ~~the~~ feather biodegradation and keratinase production from *Aspergillus terreus* was 7.5. While Akhter, *et.al.*, [21], found that the best pH for the feather biodegradation and keratinase production from *Bacillus subtilis* and *Pseudomonas* sp. were 7.0 and 10.0, respectively.

Effect of incubation time:

By incubating the selected isolates at various time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 days), the keratinase production parameter was first optimized. Conical flasks were incubated for various time durations of spaced one day apart in order to find the optimal incubation time for keratinase synthesis. The largest amount of keratinase (252 U/mg) was produced after 8 days of incubation (Figure 6).

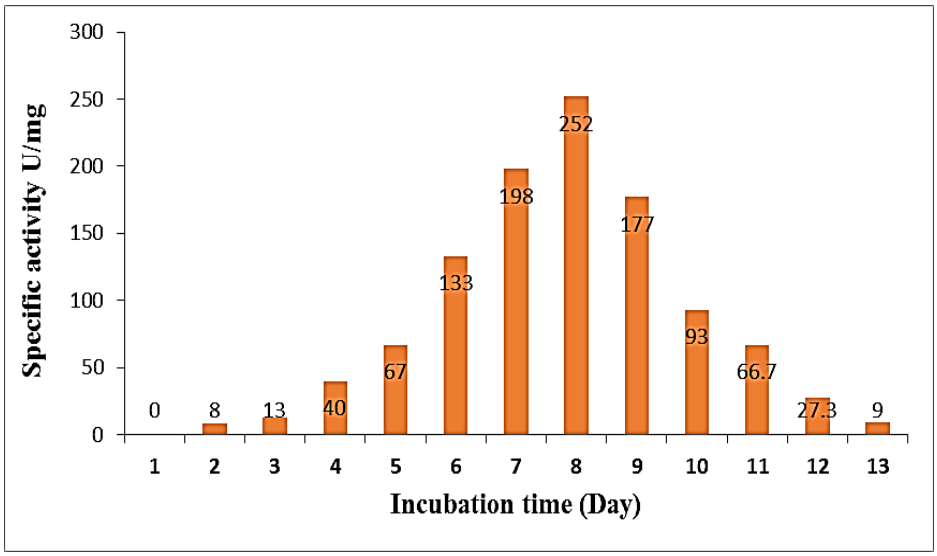

Figure 6 : Effect of incubation time on keratinase production by local *A.terreus* A13 isolate using SmF, pH 5.5.0, incubation at 28 °C.

The keratinase production was decreased after 9 days. This decrease in keratinase production occurred as a result of reducing nutrients in the medium. Koutb, *et.al*. [8], found that the better incubation time for feather biodegradation and amino acid production from feather using *Aspergillus terreus* was 25 days. Singh, *et.al*. [22], found ~~the~~ that better incubation time for the keratinase production by *Bacillus subtilis* was 72 hours, using a feather medium.

Purification of keratinase by ion exchange chromatography :

Purification of keratinase from local *A.terreus* A13 isolate was done using ion exchange chromatography after keratinase precipitation via ammonium sulphate powder (0-75 % saturation). During the experiment, it was found that washing with Tris-HCl (0.005 M, pH 8.0), allows the manifestation of three peaks ~~which~~ are represented via fractions 18-35, 37-45, and 47-48 as shown in Figure (7). Addition of 225 ml of Tris-HCl with NaCl gradients (elution step) allows three peaks to be obtained and represented by fractions 64-82, 83-89, and 91-97. Each fraction was tested for keratinase activity and concentration. In elution step, the fractions of 64-82 ~~were~~ showed keratinase activity. In addition, the outcomes indicate that keratinase produced by *A.terreus* A13 carried negative charges which are attracted with the positive charge of DEAE-cellulose therefore in the elution step the keratinase was eluted. As described in Table (1) protein concentration of 0.005 mg/ml, an activity of 5.9 U/ml, and specific activity of 1180 U/mg, with a purification fold of 4.6 and a yield of 62 % were obtained.

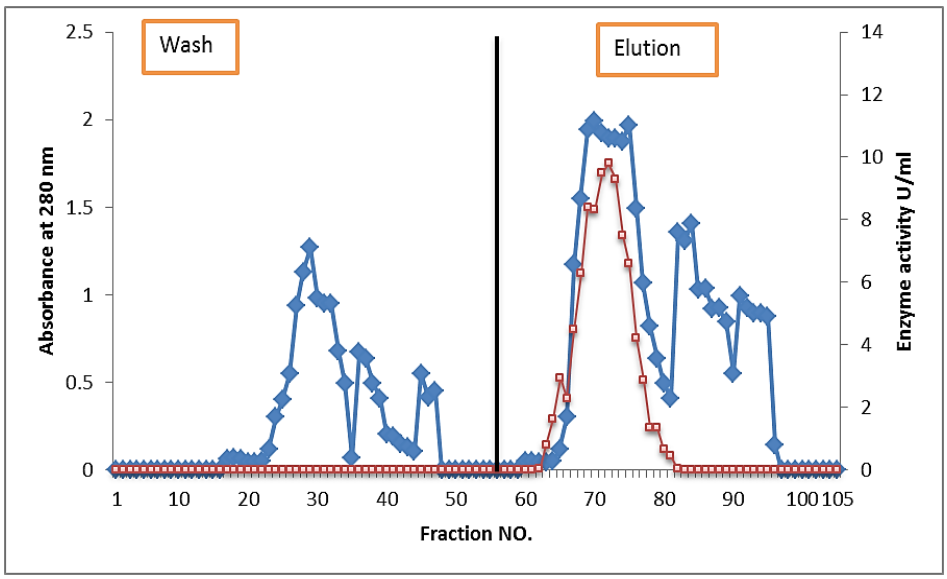

Figure 7: Ion exchange chromatography for purification of keratinase from local *A.terreus* A13 isolate by using DEAE-Cellulose column (28-1.7 cm) equilibrated with Tris-HCl (0.005 M, pH 8.0), 3 ml for each fraction and in flow rate 30 ml/ hr, eluted with Tris-HCl with NaCl gradient (0.1-1 M).

Gel filtration chromatography:

After ion exchange, the gel filtration step was followed, this step was done using Sephadex G-150 gel. The results of the keratinase purification by gel filtration chromatography showed five peaks of separated protein. Only one peak exhibits activity in fractions 30-42 after enzymatic activity estimation; these fractions are gathered and combined and the volume is concentrated by sucrose to 13 ml. The gel filtration process results in an enzymatic yield of 49%, a purification fold of 4.96, and a specific activity of 1275 U mg (Table 1).

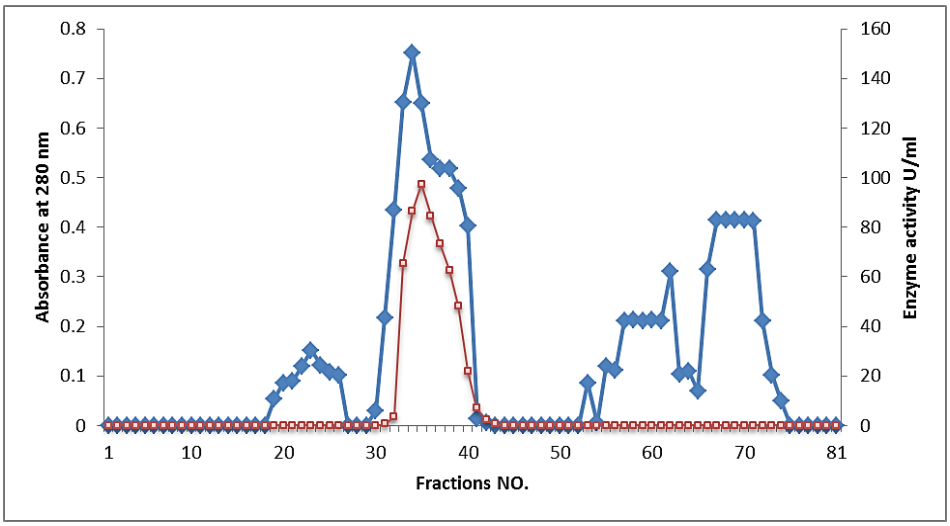

Figure 8 : Purification of keratinase by using Sephadex G-150 column (65×1.5 cm), 3 ml for each fraction with a flow rate of 30 ml/hour, eluted with Tris-HCl (0.005 M, pH 8.0).

Table 1: Purification steps of keratinase from local *A.terreus* A13 isolate.

Step	Volume ml	Activity U/ml	Protein concen. mg/ml	Specific activity U/mg	Total activity	Fold	Yield %
Crude enzyme	75	1.8	0.007	275	135	1	100
Precipitation by ammonium sulphate (0-75 % saturated) after concentration by sucrose	15	9.8	0.015	653.3	127.4	2.5	94.4
Purification by Ion-exchange chromatography (after concentration by sucrose)	14	5.9	0.005	1180	83	4.6	62
Purification by Gel-filtration chromatography (after concentration by sucrose)	13	5.1	0.004	1275	66.15	4.96	49

The purification of the keratinase enzyme from *Aspergillus flavus* by Kim [23] revealed that the fraction obtained at 0-80% ammonium sulfate saturation showed 2.39-fold purification, and that the active protein peak showed 11.53-fold purification after being purified by gel filtration in Sephadex G-100 and then by ion exchange chromatography on DEAE-Sephadex A-50. According to zymograms and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the purified keratinase is a monomeric enzyme with a molecular weight of 31 kDa.

Acknowledgements:

This study is supported by the College of Science, ~~to~~ the presidency of Biotechnology Department, and University of Baghdad.

Conflict of interest:

The author declares no conflicts of interest.

References:

[1] J.D. Allpress, G. Mountain, and P.C. Gowland. Production, purification and characterization of an extracellular keratinase from *Lysobacter* NCIMB 9497. *Lett Appl Microbiol*, vol. 34, pp. 337-342, 2002.

[2] P. Anbu, A. Hilda, H. Sur, B. Hur, and S. Jayanthi. Extracellular keratinase from *Trichophyton* sp. HA-2 isolated from feather dumping soil. *Int Biodeterior Biodegrad*, vol. 62, pp. 287-292. 2008.

[3] V.T. Arasu, T. Sivakumar, V. Ramasubramanian, K. Nalini, and R. Kiruthiga. The potential application of keratinase from *Bacillus* sp as plant growth promoters. *J Pure Appl Mcirobiol*, vol. 3, pp. 583-590, 2009.

[4] C. Bernal, J. Cairo, and N. Coello. Purification and characterization of a novel exocellular keratinase from *Kocuris rosea*. *Enz Microb Technol*, vol. 38, pp. 49-54, 2006.

[5] S.C.B. Gopinath, P.Anbu, T.Lakshmipriya, T.H.Tang, Y. Chen, U.Hashim, A.R. Ruslinda, and M.K.M. Arshad. Biotechnological Aspects and Perspective of Microbial Keratinase Production. *BioMed Research International*, vol. 1, pp. 1-10, 2015.

[6] D. Voet, and J.G. Voet. Biochemistary. Fourth edition. Printed in the United States of America. 2011.

[7] A. Hasani, A. Kariminik, and K. Issazadeh. *Streptomycetes*: Characteristics and Their Antimicrobial Activities. *International journal of Advanced Biological and Biomedical Research*, vol. 2, no. 1, pp. 63-75, 2014.

[8] M. Koutb, F.M. Morsy, M.M.K. Bagy, and E.A. Hassan. Optimization of Extracellular Keratinase Production by *Aspergillus terreus* Isolated from Chicken's Litter. *Journal of Advanced Laboratory Research in Biology*, vol. 3, no. 3, pp.210-216, 2012.

[9] A.J.R. Al-Sa'ady, and G.M. Aziz. Optimization of Lovastatin Production from A Local Isolate of *Aspergillus terreus* A50 in Solid State Fermentation by Classical and Statistical Methods. *Iraqi Journal of Science*, vol. 61, no. 10, pp. 2525-2539, 2020.

[10] A.M. Mazotto, R.R.R. Coelho, S.M.L. Cedrola, M.F. Lima, S. Couri, E.P. Souza, and A.B. Vermelho. Keratinase Production by Three *Bacillus* spp. Using Feather Meal and Whole Feather as Substrate in a Submerged Fermentation. *Enzyme Research*, pp. 1-7, 2011.

[11] R.R. ChitteV.K. , Nalawade, and S. Dey. Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8. *Letters in Applied Microbiology*, vol. 28, pp. 131–136, 1999.

[12] M. Bradford. A rapid and sensitive method for the quantization of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem*, vol.72, pp. 248- 254, 1976.

[13] N. Mohamad, L. Phang, and S. Abd-Aziz. Optimization of metallo-keratinase production by *Pseudomonas* sp. LM19 as a potential enzyme for feather waste conversion. *Biocatalysis and Biotransformation*, pp. 1-10, 2017.

[14] J. R. Whitaker. Principle of enzymology for the food science. Marcel Dekker Inc. New York. vol. 5, pp. 153-158, 1972.

[15] Y.M. El-Ayouty, A. EL-said, and A.M. Salama. Purification and characterization of a keratinase from the feather-degrading cultures of *Aspergillus flavies. African Journal of Biotechnology*, vol. 11, no. 9, pp. 2313-2319, 2012.

[16] H.M.A.H. Shata, and M.A.F. Farid. Medium optimization for keratinase production by a local *Streptomyces* sp. NRC 13S under solid state fermentation. *J Appl Biol Chem*, vol. 56, no. 2, pp. 119−129, 2013.

[17] A.M. Mazotto, S. Couri, M.C.T. Damaso, and A.B. Vermelho. Degradation of feather waste by *Aspergillus niger* keratinases: Comparison of submerged and solid-state fermentation. *International Biodeterioration & Biodegradation*, vol. 85, pp. 189-195, 2013.

[18] N.E. Nnolim, L. Mpaka, A.I. Okoh,and U.U. Nwodo. Biochemical and Molecular Characterization of a Thermostable Alkaline Metallo-Keratinase from *Bacillus* sp. Nnolim-K1. *Microorganisms*. Vol. 8, pp. 2-24, 2020.

[19] C. Bernal, I. Diaz, N. Coello. Response surface methodology for the optimization of keratiase production in culture medium containing feathers produced by *Kocuria rosea*. *Can J Microbiol*, vol. 52, pp. 445-450, 2006.

[20] A. J. R. AL-Sa'ady, and M. H. Hilal. Determination of the Optimum Conditions for Extracting Polyphenol Oxidase and Laccase Enzymes From *Malva Parviflora* and Their Role in The Decolorization of Some Dyes. *Iraqi Journal of Science*, vol. 61, No. 2, pp: 306-313, 2020.

[21] M. Akhter, L.W. Marzan, Y. Akter, and K. Shimizu. Microbial Bioremediation of Feather Waste for Keratinase Production: An Outstanding Solution for Leather Dehairing in Tanneries. *Microbiology Insights*, vol. 13, pp. 1–12, 2020.

[22] S. Singh, H. Masih, G.E. Jeyakumar, R. Lawrence, and P.W. Ramteke. Optimization of Fermentative Production of Keratinase by *Bacillus subtilis* Strain S1 in Submerged State Fermentation Using Feather Waste. *Int.J.Curr.Microbiol.App.Sci*, vol. 6, no. 12, pp. 1499-1510, 2017.

[23] J.D. Kim. Purification and Characterization of a Keratinase from a Feather-Degrading Fungus, *Aspergillus flavus* Strain K-03. *Mycobiology*, vol. 35, no. 4, pp. 219-225, 2007.