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Keratinase Production from Chicken Feather by New Egyptian Local *Streptomyces* Isolates

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THE MAIN objective of the current research was to isolate new *Streptomyces* species that has the ability to degrade chicken feathers through keratinase production. Nineteen Egyptian local *Streptomyces* strains were isolated from soil and tested for their ability to produce keratinase enzyme from chicken feathers. Maximum keratinolytic activity was achieved with a novel *Streptomyces* isolate strain *no 6*. that was identified by 16S rDNA method with a given name of *Streptomyces* sp. NRC FM (Accession no. LC456058). The optimum culture conditions for the highest keratinase production were found to be a pH 9.5, at a temperature of 37˚C and agitation speed of 180rpm. Interestingly, chicken feathers were completely degraded after 9 days of incubation with the novel strain. Keratinase was precipitated using acetone and its molecular weight was estimated to be 30 and 42kDa using SDS-PAGE. The results obtained suggest that the isolated novel *Streptomyces* sp., NRC FM, can be considered as a useful biotechnology tool for valorization of keratin-containing wastes.

Keywords: Feather keratins, Keratinase enzyme, Keratinase-producing microorganisms, *Streptomyces* sp.

Introduction

Feather waste is a big problem for poultry meat industry and millions of tons of feathers are produced every year in several countries worldwide. This waste is mainly keratin in nature as over 91% of the chicken feathers are pure keratin with mainly beta structure. Due to their content of keratin, chicken feathers are resistant to decomposition and proteolysis by wide different agents including proteolytic enzymes, and several physical or chemical agents that affects greatly the process of feather waste disposal (Qiu et al., 2022). A microbial inoculum might convert waste feathers into protein supplements that can be involved in the manufacturing of animal foods (Saarela et al., 2017), in leather and detergent industries (Sastry, 1986; Gessesse et al., 2003; Santha Kalaikumari et al., 2019) and as improving agents of wool fabrics (Gunes et al., 2018). Furthermore, feather decomposition can result in the formation of nitrogenous liquid residues that can be incorporated in hydroponics agriculture and aquaculture (Ichida et al., 2001). Therefore, microbial keratinases possess a vital role for degradation of resistant keratin-containing wastes, including chicken feathers, in a non-polluting process for environmental cleanup (Ichida et al., 2001; Qiu et al., 2022)

Keratinase - producing microorganisms showed previously the ability to degrade feathers from different species of actinomyces, bacteria and fungi, such as *Onygena* sp., *Rhizomucor* sp., *Aspergillus* sp., *Stachybotrys atra* (Friedrich et al., 1999), *Streptomyces albs* (*St. albs*), *St. pactum, St. thermoviolaceus, St. fradiae* (Noval & Nickerson, 1959), *St.* strain BA7 (Korkmaz et al., 2003), *St. glubargensis* (Syed et al., 2009), *St. thermonitrificans* (Mohamedin 1999), *Bacillus* sp. (Suntornsuk & Suntornsuk, 2003), *Bacillus cereus* TS1 *(B. cereus* TS1) (Sivakumar et al., 2013), *Stenotrophomonas* sp. (Yamamura et al., 2002), *B. licheniformis*, *B. pumilus* (Nitisinprasert et al., 1999) and *Vibrio* sp. (Sangali & Brandelli, 2000).

The main goal of this research was to isolate

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new *Streptomyces* isolate that has the ability to degrade chicken feathers through keratinase production. Several techniques were used during this research work including molecular identification of the new strain, optimization for maximum production of keratinize enzyme, isolation of keratinase enzyme and assessment of its activity, and finally confirming the ability of the enzyme to degrade chicken feathers.

Materials and Methods

Organisms and culture conditions

Nineteen Streptomycetes isolated from local soil samples of National Research Centre (NRC) Egypt were revived by inoculating 1mL of spore suspension (7-days old culture) into a basal medium (50 ml) that is composed of: NH_3Cl (1g/L), K_2HPO_4 (1.2g/L), KH_2PO_4 (1.6g/L), $MgCl_2$, (0.96g/L), peptone (1g/L), and 0.5 g chicken feather / flask with a pH 7. After sterilization, incubation period was carried out for 5 days at 28ºC in a rotary shaker (180rpm), thereafter, the cultures were centrifuged under cooling atmosphere, and the supernatants were assayed for keratinase activity (Rashad et al., 2015).

Molecular identification of the selected strain

The strain which produced the highest keratinase activity was selected and its molecular sequence was identified using the 16S rDNA sequence analysis method. Furthermore, we selected this strain for assessing the optimum conditions required for maximum enzyme production. A comparison analysis was carried out using other different *Streptomyces* species (*St.* sp.) sequences found in the Genbank, the National Center for Biotechnology Information (NCBI). Finally, we used the neighbor-joining method to reconstruct the phylogenetic tree (El-Naggar et al., 2019).

Preparation of template DNA

The selected *St.* strain that possessed the highest keratinase activity was propagated in a starch nitrate agar medium for 5 days at 28 ± 2ºC. To obtain a heavy growth, one colony was selected, and a streak was performed by a microbiological loop on a slant of the same medium. Thereafter, we suspended the colonies in a sterilized 0.9% NaCl (saline), centrifuged, and the pellet formed was resuspended in InstaGene Matrix (Bio-Rad, USA). To prepare the template DNA, we heated the suspension at 56ºC for 30 min, followed by another heating period at 100ºC for 10min. Thereafter, we centrifuged the suspension and applied it for PCR analysis, according to manufacturer's procedure (Bio-Rad, USA) (Rashad et al., 2015).

PCR

PCR reaction solution (20µL final volume; Macrogen, Singapore) contains 1µL of template DNA and the following primer sets: forward 16s rRNA primer 27f (5'-AGA GTT TGA TCM TGC CTC AG-3') and reverse 16s rRNA primer 1492r (5'- TAC GGY TAC CTT GTT ACG ACT T-3'). The cycles used for amplification was carried out as 35 cycles at 94ºC for 45sec, followed by 55ºC for 60sec and finally, 72ºC for 60sec. Thereafter, the PCR reaction products (5 to 15µL) were separated using 1% agarose gel electrophoresis. To visualize the DNA bands using UV light, ethidium bromide was added to the gel in a concentration of 10mg/mL water (El-Naggar et al., 2019).

Sequencing

To purify the formed PCR products, we used Montage PCR Cleanup kit (Millipore, Merck, Germany) according to the supplier's instructions. The sequencing procedure was performed using the purified products (1400 bp) and the following primer sets, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3'. A BigDye terminator cycle sequencing kit (Applied Biosystems, USA) was used and the products were sequenced using 3730XL automated DNA sequencing system (Applied Biosystems, USA) (El-Naggar et al., 2019).

Phylogenetic analysis

To identify similarities of the selected active *St.* strain DNA sequence, we had to submit our nucleotide sequence to the GenBank, NCBI, BLASTN programs search nucleotide data bases (https://blast.ncbi.nlm.nih.gov/) (Altschul et al., 1997). Through this program, an accession no. of LC456058 was created and *St.* NRC-FM isolate name was assigned for our partial DNA sequence.

Optimum culture conditions for extracellular keratinase produced by St. NRC FM isolate

Different carbon sources were supplemented individually in the growth medium such as maltose, lactose, dextrose, glucose, xylose,

cellulose, d(-)arabinose, inositol, inulin, fructose, starch, glycerol (1% w/v). In addition, nitrogen sources were achieved by adding solution of equimolar of nitrogen source (0.2% KNO_3). Incubation periods were determined daily for 14 days as well as the suitable pH was examined using acetate buffer with a pH of 5 to 6, phosphate buffer with a pH of 7 to 8 and borate buffer with a pH of 9 to 12.

Preparation of keratin

keratin powder was produced as described before with some modifications (Wawrzkiewicz et al., 1987). Briefly, the feathers were cleaned by washing with soap and water, and dried overnight at 60°C. The dried feathers (10g) were refluxed with DMSO (500mL) at 100°C for 80-120min. To precipitate keratin, 2 volumes of acetone were added and kept in refrigerator at 4°C for 1-2 days. Thereafter, keratin was obtained as a precipitate by centrifugation, washed with water, and finally dried at 4°C.

Isolation of keratinase enzyme and activity assay

After the incubation period, the cultures were centrifuged to remove biomass. The cooled acetone was added to the cooled supernatant at different concentrations and the precipitate was used as a crude enzyme. The enzyme activity was determined in the precipitate.

To measure the keratinolytic activity, feather keratin (10mg) in phosphate buffer (1.5mL, pH 7.4) was added to one ml supernatant that contains keratinase enzyme for 1h at 37°C. To stop the enzymatic reaction, one mL of 10% trichloroacetic acid was added to the reaction mixture for 30 min at 4°C. Thereafter, centrifugation was carried out for 15min at 2500rpm under cooling atmosphere (4°C). A blank tube was prepared by replacing distilled water for the samples. The supernatant was measured using a spectrophotometer at 280 nm, and each 0.1 increase in absorption was considered as one unit of enzyme activity (Friedrich et al., 1999). The amount of protein of culture supernatant was estimated using Lowry method, and bovine albumin was used as a calibration standard (Lowry et al., 1951).

Thermal stability

The influence of temperature on enzyme stability was examined by incubating the reaction buffer (50mM potassium phosphate buffer (pH 7.5)) containing the enzyme over a range of temperatures (30 to 90°C) for 30min, thereafter, the standard enzymatic assay was performed. The main purpose of studying the effect of temperature is to test whether or not the enzyme will maintain its activity over a range of temperature and to determine the best temperature for maximum enzymatic activity. (Letourneau et al., 1998).

Characterization and identification of molecular weight of keratinase

Supernatant was precipitated by cooled acetone and the precipitate (around 50µL) was run on sodium dodecyl sulfate (SDS)-PAGE with standard marker. We used substrate gel electrophoresis for characterization of keratinase. Briefly, SDS-polyacrylamide (12.5%) was allowed to co-polymerize with keratin feather powder, and 0.1% (w/v) gelatin, BSA, casein, or hemoglobin was incorporated as substrate. Thereafter, incubation with phosphate buffer (50mM, pH 5.5) was performed for 48 h at 37°C. In order to detect peptidases, the gels were incubated with Coomassie blue R-250 (0.2%) in methanol-acetic acid-water (50:10:40). The gels got destained using the same solvent, dried and visualized digitally using molecular mass standards (GE Healthcare, Brazil) (Lundy et al., 1995).

Results and Discussion

Screening of local Streptomyces isolates

Data presented in table 1 revealed that the maximum keratinase specific activity was 2.192U/mg followed by 1.248, 0.985, 0.701 and 0.519U/mg with the microorganisms *St*. no. 6, no. 5, no. 9, no. 19 and finally no.16, respectively. Therefore, *St.* no. 6 was chosen for further study (Table 1).

Molecular identification of the selected strain

Our selected active strain (*St.* no. 6) was identified by Microbial Chemistry Lab., NRC, Cairo, Egypt, as *Streptomyces* NRC FM isolate and was included in the collection at GenBank, NCBI, BLASTN programs search nucleotide data bases with accession number LC456058. The 16S rDNA gene sequences of the strain no. 6 were determined and showed the highest similarity to the type strain of *Streptomyces* isolate FM (98% similarity). The phylogenetic tree based on the neighbor-joining method was shown in Fig. 1.

TABLE 1. Screening of local Streptomyces isolates for their ability to produce keratinase

Strain number	Final pH	Reducing sugar µ/mL	Total protein mg/mL	Enzyme activity U/mL	Specific activity U/mg protein
1	8.66	0.592	207.50	62.64	0.302
2	8.38	0.020	103.75	00.00	0.000
3	8.70	0.112	108.25	00.00	0.000
4	8.77	0.020	182.50	00.00	0.000
5	8.50	0.728	187.50	234.00	1.248
6	8.50	0.532	216.25	473.94	2.192
7	8.77	0.232	117.50	0.00	0.000
8	8.52	0.028	69.63	0.00	0.000
9	8.22	0.556	217.50	214.20	0.985
10	8.64	0.820	311.25	83.34	0.268
11	8.53	0.160	62.00	0.00	0.000
12	8.50	0.072	114.13	0.00	0.000
13	8.35	0.076	129.50	0.00	0.000
14	8.74	0.012	138.13	0.00	0.000
15	8.22	0.048	216.75	0.90	0.004
16	8.52	0.532	206.38	107.10	0.519
17	8.50	1.060	344.13	0.00	0.000
18	8.50	0.144	307.38	0.00	0.000
19	8.77	1.568	367.88	257.94	0.701

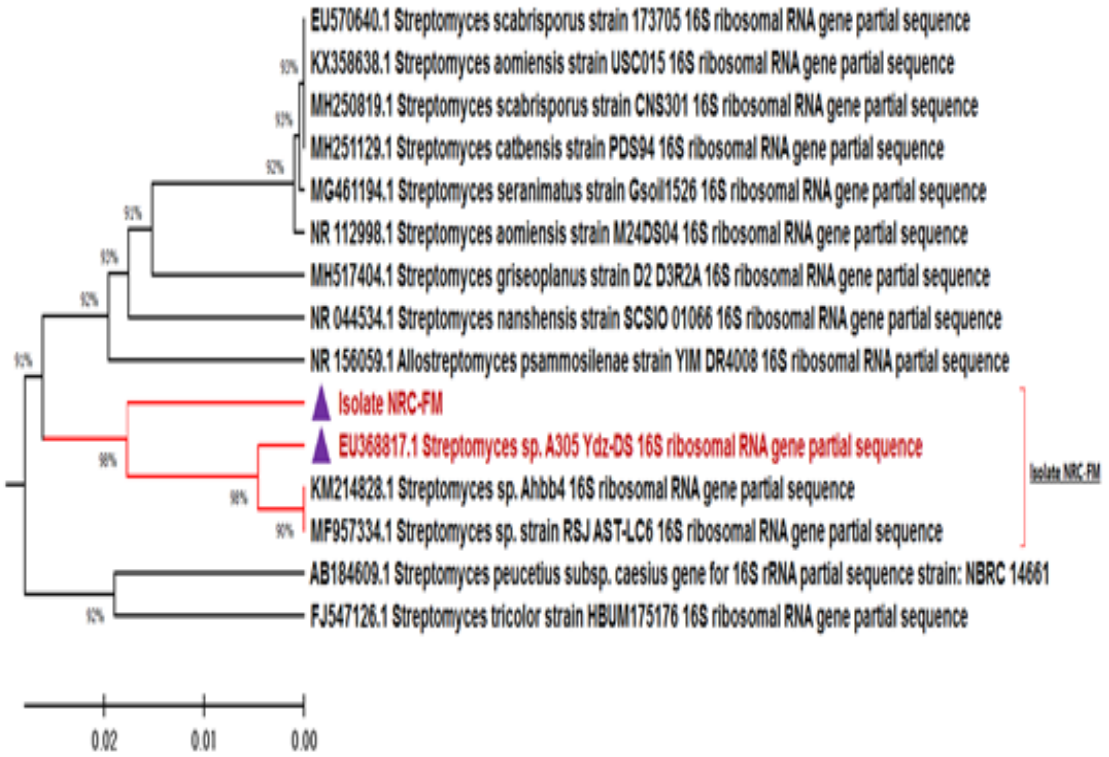

Fig. 1. Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between NRC-FM *Streptomyces* isolate and other species belong to the genus *Streptomyces* [The tree was constructed using the MEGA-X software for carrying out the neighbor-joining method]

Optimum conditions of enzyme production by St. NRC FM isolate

Optimum time course and pH for enzyme production, effect of additional carbon and nitrogen sources were studied.

Optimum time course for enzyme production by St. NRC FM isolate

Figure 2 illustrated that highest enzyme activity was achieved at the 3rd day of incubation and then started to decline sharply. This data was closely related with other research showed that the maximum keratinase production from *B. cereus* (TS1) was recorded at the 3rd day of incubation and the minimum production was recorded at the 5th day of incubation with the microorganism (Sivakumar et al., 2013).

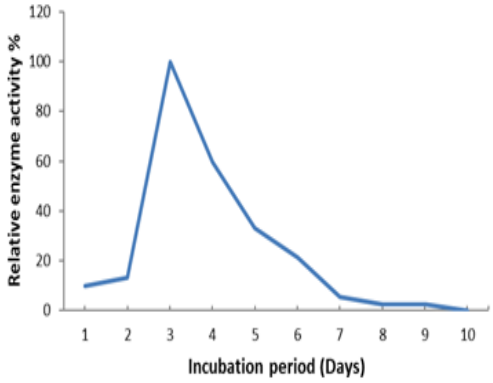

Fig. 2. Effect of incubation period on keratinase activity [Different incubation periods were tested for St. NRC FM isolate maximum keratinase activity. The incubation was carried out on a rotary shaker at pH 7.0, 37ºC and 180rpm]

Optimum pH for enzyme production

As shown in Fig. 3, the optimum enzyme activity was achieved at pH 9.5 and then sharply decreased. Similarly it was shown previously that pH 9.0 was the optimum pH for the maximum keratinolytic activity produced by *St. gulbargensis* (Syed et al., 2009).

Effect of additional carbon and nitrogen sources

The keratinase enzyme activity produced by *St* NRC FM isolate has not been affected by using another source of carbon or nitrogen, to the contrary, it showed an inhibition of the enzyme activity. Therefore, the addition of another carbon or nitrogen sources to the basal medium was not useful. Meanwhile, in another actinomycete strain, it was demonstrated that the addition of other carbon (glucose) or nitrogen (NH_4Cl) sources did not completely repress the keratinase biosynthesis (Ignatova et al., 1999).

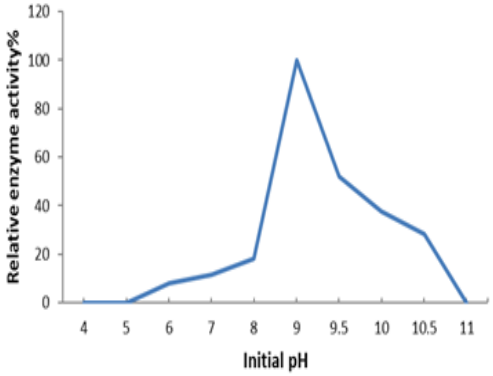

Fig. 3. Effect of initial pH on keratinase activity [Different initial pH was tested for St. NRC FM isolate maximum keratinase activity. The incubation was carried out on rotary shaker for three days at pH 7.0, 37ºC and 180rpm]

Thermal stability

Data illustrated in Fig. 4 revealed that keratinase enzyme retained maximum stable activity between 40-60˚C and after that the activity started to decrease. This data was closely related to other research showed that the favorable temperature for maximum production of keratinase produced by *B. cereus* TS1 was 50˚C (Sivakumar et al., 2013). Other researchers found a temperature range for maximum keratinase activity between 30 and 40˚C when they used *Streptomyces* strain BA to produce a stable keratinase enzyme (Korkmaz et al., 2003).

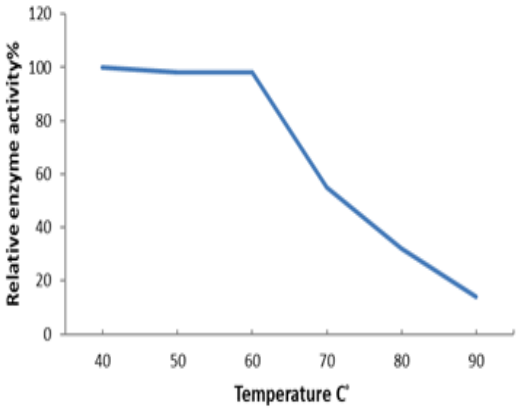

Fig. 4. Thermal stability of keratinase enzyme produced from St. NRC FM isolate at different temperature (40-90˚C) for 30min

Degradation of chicken feathers by St. NRC FM isolate

The degradation of the chicken feathers by *St.* NRC FM isolate was shown in Fig. 5 at different times of incubation period. The percent of chicken feather degradation was obtained in Table 2. The optimum percent of chicken feathers degradation was observed on 9th day of incubation (Table 2 and Fig. 5).

TABLE 2. Percent of chicken feathers degradation by *Streptomyces* isolate NRC FM

Day no.	Degradation %
0	7
1	15
2	15
3	26
4	40
5	55
6	55
7	74
8	74
9	95

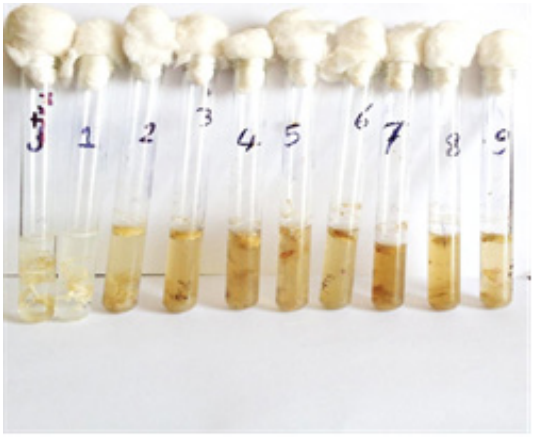

Fig. 5. Degradation of chicken feathers by St. NRC FM isolate [Incubation with chicken feathers was performed for 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 days]

Molecular weight of keratinase

The molecular weight of the enzyme sample produced by *St.* NRC FM isolate was characterized and identified by SDS-PAGE analysis. Two bands were observed at molecular weight of 30 and 42kDa, respectively (Fig. 6). In agreement with our results, several studies revealed that major keratinases produced from different species of *St.* possess a molecular weight range from 20-50kDa (Bockle et al., 1995; Pavani et al., 2017). Furthermore, keratinase produced from *Bacillus* species showed similar molecular weight, as *B. pseudofirmus* FA30-01 has a molecular weight of 27kDa, whereas, *B. licheniformis* PWD-1 has a molecular weight of 30kDa (Lin et al., 1992; Kojima et al., 2006).

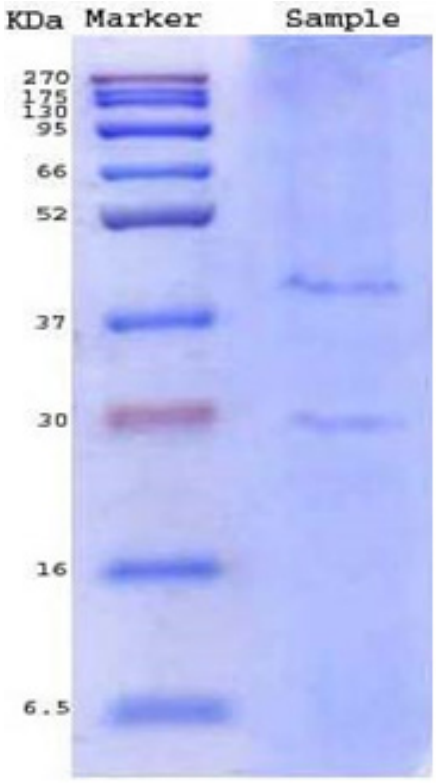

Fig. 6. SDS-PAGE of keratinase enzyme isolated from St. NRC FM isolate [Sample lane: refers to keratinase enzyme, Marker lane: refers to a standard protein molecular weight]

Conclusion

The local novel strain *Streptomyces* NRC FM isolated from NRC, Cairo Egypt, was capable of producing keratinase enzyme. Optimum conditions for maximum production of the enzyme were identified as a pH 9.5, at a temperature of 37˚C and agitation speed of 180 rpm. Moreover, the isolated keratinase enzyme proved its ability to degrade keratin-containing wastes such as chicken feathers in an eco-friendly method. Finally, the produced keratinase enzyme may have several applications including enhancement of cosmetics, detergent and leather industries.

Ethics approval: This research work was

approved by the National Research Centre Medical Research Ethics Committee, Egypt with registration number: 14126072023.

Conflict of Interest: There is no conflict of interest.

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إنتاج إنزيم الكيراتينيز من ريش الدجاج بواسطة نوع جديد من الاستربتوميسيس التى تنمو فى مصر

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الهدف الرئيسي من البحث هو عزل أنواع جديدة من ال *Streptomyces* التى لها القدرة على تحليل ريش الدجاج من خلال إنتاج انزيم الكيراتينيز . تم عزل تسعه عشر سلالة من ال *Streptomyces* المحلية من التربة واختبار قدرتها على إنتاج إنزيم الكيراتينيز من خلال تحليل الكيراتين الموجود في ريش الدجاج. تم تحقيق الحد الأقصى من نشاط الأنزيمى مع العزلة رقم 6 من ال *Streptomyces* و هى عزلة جديدة تم تعريفها على انها *Streptomyces* sp. NRC FM و ذلك باستخدام نتائج التحليل النيوكليوتيدى للحمض النووى الريبوسومى 16 S r RNA و قد تم ايداع هذه السلاله فى بنك الجينات تحت رقم LC45605. تم الحصول على الظروف المثلى لإنتاج انزيم الكيراتينيز عند درجة حموضة 9.5 و عند درجة حرارة 37 درجة مئوية وسرعة التقليب 180 دورة في الدقيقة. كما أثبتت النتائج التحلل التام لريش الدجاج بعد 9 أيام من التحضين باستخدام هذة العزلة الجديدة. تم ترسيب الكيراتينيز باستخدام الأسيتون وقد تم تقدير وزنه الجزيئي بـ 30 و 42 كيلو دالتون باستخدام SDS-PAGE . تشير النتائج التي تم الحصول عليها إلى أن السلاله المعزولة *Streptomyces* sp. NRC FM يمكن اعتبارها بمثابة أداة مفيدة للتكنولوجيا الحيوية لتقيم النفايات المحتوية على الكيراتين.