ISOLATION AND MOLECULAR IDENTIFICATION OF *BACILLUS* STRAINS PRODUCING KERATINASE ENZYME

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ABSTRACT:

Feather waste, generated in large quantities as a byproduct of commercial poultry processing, is almost pure keratin. Keratin is insoluble fibrous protein and resistant to degrade by most proteolytic enzymes. Keratinase enzyme belongs to the class hydrolase which are able to hydrolyze insoluble keratins more efficient than other proteases. Microbial keratinolytic hydrolysis represents an attractive alternative to improve the nutritional value of feather wastes. In the last decade, as a result of the widespread use of PCR and DNA sequencing, 16S rRNA sequencing has played a pivotal role in the accurate identification of microorganisms and the discovery of novel isolates in microbiology laboratories. The aim of this study was the genotypic identification of keratinolytic bacteria isolated from soil and feather samples of chicken feather dump site. Thirty-two isolates were obtained and characterized according to morphological characteristics and biochemical tests. All isolates were tested for their keratinase enzyme activity. The best five isolates for keratinase enzyme activity were selected and identified by 16S rRNA gene sequence analysis. DNA was extracted from each of the five isolates. The target regions of the rRNA genes were amplified by PCR and sequenced using suitable universal primers 27F and 1492R. The sequence data were analyzed and aligned in the order of increasing genetic distance to relevant sequences against a library database to achieve an identity match. The DNA sequences of the phylogenetic tree results confirmed the identity of the five isolates as *Bacillus thuringiensis*, *Bacillus velezensis*, *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus licheniformis*.

Key words: Characterization, keratin, degradation, 16S rRNA, identification, *Bacillus*, Phylogenetic tree.

INTRODUCTION

Feathers are composed of over 90% protein and produced in large amounts as a waste by poultry processing worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage **(Onifade *et al.*, 1998 and Gousterova *et al.*, 2005).** Considering high protein content of keratin wastes, they could have a great potential as a source of protein and amino acids for animal feed and for many other applications. However, keratin in its native state is not easily degradable by common proteolytic enzymes such as trypsin, pepsin and papain, because of its insolubility and multiple disulfide bonds. The current processes of hydrothermal treatment used to make keratin wastes more digestible are expensive and destroy certain amino acids, yielding a product with poor digestibility and variable nutrient quality **(Wang and Parsons, 1997).** Development of enzymatic and/ or microbiological methods to improve the nutritional value of keratin wastes

is an attractive alternative, as it offers cheap and mild reaction conditions **(Onifade *et al*., 1998)**. Degradation of keratin by micro-organisms is performed by specific proteases (keratinases) **(Onifade *et al*., 1998; Wang and Shih, 1999 and Singh, 2003)**. Almost all keratinases are inducible and different keratin-containing materials such as feathers, hair and wool can be used as substrates for keratinase enzyme production **(Gupta and Ramnani, 2006).** Use of microbial keratinase for keratin degradation is the innovative solution for recycling feather waste and reducing pollution. Conversion of feathers into feather meal, dietary protein for animal feed by using physical and chemical treatment is significant. These methods can destroy certain amino acids and decrease protein quality and digestibility. The utilization of agro-industrial residues may increase energy conservation and recycling **(Veenayohini and Sangeetha, 2016).** To overcome the loss of amino acids due to keratin hydrolysis microbial keratinases are used. Among numerous microbial groups many keratin degraders derive from the bacterial genus *Bacillus*. The present study was undertaken with the following objectives of isolation and identification of keratinolytic bacteria from soil sample collected from poultry waste dumping yard and assessment of keratinase enzyme activity. Nowadays, 16S rRNA gene sequencing is the widely accepted modern molecular approach employed for identification of bacteria as the genotypic methods are more accurate than morpho-physiological or phenotypic identification **(Contreras *et al*., 2013).** Moreover, 16S rDNA is the significant point for molecular identification and taxonomical classification of bacteria due to the mosaic composition of phylogenetically conserved and variable regions within the gene **(Hisham *et al*., 2019; Mitra and Roy, 2010; and Janda and Abbott, 2007).** The prime objective of the present study was to identify keratinase enzyme producing bacteria isolated from the soil and feather at Fayoum region, using both morpho-physiological and 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Sample collection, serial dilution, and isolation of bacteria:

Isolation of bacteria was carried out according to **(Iruolaje *et al*., 2016)**, with some modifications as the following: the soil and feather samples were aseptically collected in sterile bags from the poultry farm at Fayoum region. The collected samples were immediately transferred into laboratory. Serial dilutions for each sample was prepared by adding 1 g of the soil sample or feather to 9 ml of sterile water. Then serial dilution up to 10^{-8} was done using sterile water and from appropriate dilutions (10^{-7} and 10^{-8}) 1 ml of sample was poured into the nutrient Luria-Bertani broth (LB) agar plates respectively (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 L of distilled water, and pH to 7.0 with 1 N NaOH. The widely used rich medium called Luria-Bertani broth is popular with bacteriologists because it permits fast growth and good growth yields for many species as the method of **(Sezonov *et al*., 2007).** All inoculated and control plates were incubated at 37℃ for 24h. After the incubation, different bacterial colonies were picked up

by inoculation loop and it's were streaked on respective plates containing nutrient agar. Then the colonies were continuously streaked on nutrient agar palates by different streaking plate methods until get purified colonies of isolates. Isolated different purified colonies were sub cultured on nutrient agar slants.

Morphological and biochemical characterization of keratinolytic isolates:

Thirty-two purified isolates were characterized by biochemical analysis using catalase production and lactose fermentation of carbohydrates tests as the methods of **(Hemraj *et al*., 2013).** Gram's Method of staining and observed under microscope for Gram, and motility tests as the methods of **(Tuhin-Al-Ferdous *et al*., 2013)** were performed under morphological tests.

Screening for keratinolytic activity, and preparation of inoculums and feather meal:

Screening for keratinolytic activity was carried out according to **(Veenayohini and Sangeetha, 2016)** with some modifications as the following: one loop full of thirty-two different bacterial isolates was taken from nutrient agar slants and inoculated into the conical flasks containing sterilized nutrient broth. Inoculated broths containing conical flasks were incubated at 37°C for 24h and it was used as inoculums for screening of keratinase enzyme activity of isolates. The plates containing Basic medium used for keratinase screening and activity (g L^{-1}, pH 7.5) NaCl, 0.5; $MgCl_2.6H_2O$, 0.1; $CaCl_2$, 0.06; KH_2PO_4, 0.7; K_2HPO_4, 1.4 as the method of **(Lin *et al*., 1995; Hossain *et al*., 2007)** and 1% feather keratin substrate. The plates were prepared and wells were formed on agar plates by removing agar using 0.9 cm diameter cork borer. The wells were filled with 0.1 ml of isolated supernatant broth respectively and the plates were incubated at 37°C for 48h. Around the well, clear zone forming five isolates were selected for the further work. The five isolates, which degraded keratin effectively and it was further identified by physical and biochemical tests and then taken for growth and identification study by 16S rDNA gene sequence.

Molecular identification of the keratinase highly-producing isolates, and PCR amplification of 16S rDNA gene:

16S rDNA sequence coding region was amplified by PCR. The sequences of the PCR products were determined directly with the prokaryotic 16S ribosomal DNA universal primers 27F (5´-AGA GTT TGA TCC TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´) as (**Cai *et al*., 1999).** PCR amplifications were performed in 25µl final reaction volumes containing 12.5µl Master Mix, 3µl of genomic DNA as the template, 2µl Forward primer, 2µl Reverse primer, 5.5µl deionized water. The PCR reaction was distributed into each of the PCR tubes. Mineral oil was layered on top of the reaction mixture. Amplification was performed in PCR Thermal Cycler with PCR conditions as follows: Pre-heating at 94°C for 1.5 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at a 72°C for 1.5 min, this cycle was repeated at 30 cycles and finally performed at 72°C for 5 min. PCR products were

electrophoresed in 1% agarose gel. The resulting bands were then visualized with UV Trans illuminator. PCR products were sent to the BASE for sequencing 16S rDNA gene. The sequencing results were subjected into National Center for Biotechnology Information (NCBI) GenBank (www.ncbi.nlm.nih.gov) and Basic Local Alignment Search Tool (BLAST) was adopted to search GenBank database for sequence homology analysis. *Bacillus* species with similarity more than 97% was considered as the same. Phylogenetic tree of *Bacillus* species strains was constructed with Clustalw software as the method of **(Wirawati *et al*., 2019).**

RESULTS AND DISCUSSION

Isolation of keratinolytic bacteria:

The bacterial isolates that used in this study were collected from feathers waste samples (Feather and soil) from different sites at Fayoum region. The results showed that thirty-two isolates were obtained and characterized by morphological, biochemical, and then were tested for their keratinase enzyme activity.

Morphological and biochemical characterization of keratinolytic bacteria:

Thirty-two isolates (Isolate Code Number from FAK 1 to FAK 32) were subjected to phenotypic characterization gram staining, catalase, lactose fermentation, motility tests, and keratinase enzyme activity test. Thirty-two isolates were characterized using morphological characters. The results showed that all thirty-two isolates were gram-positive staining except for (FAK 2, FAK 19, FAK 20, FAK 21, FAK 22, FAK 29 and FAK 30) that were negative gram staining. All thirty-two isolates were long rods shape except for (FAK 2, FAK 5, FAK 9, FAK 12, FAK 19, FAK 20, FAK 21, FAK 22, FAK 23, FAK 24, FAK 29, FAK 30 and FAK 31) that were short rods shape. Isolates (FAK 13, FAK 16, FAK 18, FAK 25 and FAK 27) were positive of gram staining (Fig. 1). The results showed that all thirty-two isolates were positive for catalase production. All isolates untested for lactose fermentation test except for isolates (FAK 9, FAK 20, FAK 21, FAK 22, FAK 29 and FAK 30) were negative lactose fermentation. All thirty-two isolates were positive motile for motility test.

Screening for keratinase enzyme production:

All the thirty-two isolates were screened for keratinase enzyme production by agar well plating method on feather meal agar plates (NaCl - $MgCl_2.6H_2O$ - $CaCl_2$ - KH_2PO_4 - K_2HPO_4- keratin). The results showed that among thirty-two bacterial isolates, twenty-three isolates showed clear zone around the well after the incubation. As it indicates the ability of each isolate from the twenty-three to degrade the keratin. That means each isolate from the twenty-three was capable of producing keratinase enzyme. The best of twenty-three isolates were five for their ability to degrade the keratin (FAK 13, FAK 16, FAK 18, FAK 25 and FAK 27) (Fig. 2). The results showed that the other nine isolates did not produced zone around the well (FAK 2, FAK 5, FAK 8, FAK 9, FAK 20, FAK 21, FAK 22, FAK 29 and FAK 32). The degrading rate of keratin for each isolate from the nine

isolates was 0. That means each isolate from the nine did not produce keratinase enzyme.

Molecular identification of the keratinase highly-producing isolates, and nucleotide sequence accession numbers:

The results indicated that the best five isolates for keratinase enzyme production were previously identified by the 16S rRNA sequence and identified as five species of the genus *Bacillus* (*Bacillus thuringiensis, Bacillus velezensis, Bacillus megaterium, Bacillus subtilis* and *Bacillus licheniformis*). They were registered at GenBank with accession number |MK788291|, |MK788292|, |MK788293|, |MK788294| and |MK788295|. PCR amplification of 16S rRNA gene of the five species strains of *Bacillus* produced a single fragment approximately of (1500) bp by gel electrophoresis as shown in (Fig. 3). Biochemical identification of the bacterial strains was confirmed via PCR ampilification and partial sequencing of the 16S rRNA genes using the primer pairs 27F/1492R by the same method for reliable identification by (**Ha *et al*., 2019).** The bacterial strains confirmed as *Bacillus cereus*, *B. thuringiensis* or *B. megaterium* by 16S rRNA gene sequencing. Also, **(Barus *et al*., 2017)** studied the diversity of protease-producing *Bacillus* spp. from fresh Indonesian tempeh based on 16S rRNA gene sequence. The aim of their investigation was to study the genetic diversity of *Bacillus* spp. in tempeh production, and all isolates were identified to be similar to *B. pumilus*, *B. subtilis*, *B. megaterium*, *B. licheniformis*, *B. cereus*, *B. thuringiensis*, *B. amyloliquefaciens*, *Brevibacillus brevis*, and *Bacillus* sp. Bacterial strains were identified also by using genomic DNA isolation and 16S rRNA gene sequencing to investigate the diversity of genus *Bacillus* at species level using eubacterial universal primers 27F and 1492R **(Nair and Raja, 2018).** The *Bacillus megaterium* exopolysaccharide producer was also isolated from the Egyptian soil and identified by molecular biology technique, 16S rRNA **(Abu Shady *et al*., 2011).** Also, molecular studies of *Bacillus* diversity have been reported and investigated by using the same molecular method as **(Freitas *et al*., 2008).** Their results indicated that 16S rDNA partial sequencing assigned all the isolates to the *Bacillus* genus, with close genetic relatedness to the *Bacillus subtilis* and *Bacillus cereus* groups, and to the species *Bacillus sphaericus*. Many previous reports also used 16S rRNA sequencing technique as in the present study. Many previous reports demonstrated that 16S rRNA sequencing technique has played a pivotal role in the accurate identification of known and the discovery of novel *Bacillus* species strains isolates in many different laboratories **(Hisham *et al*., 2019; Senthilraj *et al*., 2016; Hakovirta *et al*., 2016; Khusro, 2015; Sabir *et al*., 2013; Mitra and Roy, 2010; Gomaa and Momtaz, 2006, and Wisotzkey *et al*., 1992).**

Phylogenetic analysis of the 16S rDNA sequences:

The phylogenetic tree obtained by sequence analysis of 16S rDNA of the five species strains of *Bacillus* and the other sequence of *Bacillus* species strains which obtained from sequence data GenBank was represented in (Fig. 4). It showed

that isolate *Bacillus licheniforms* |MK788295| is similar to isolate *B. licheniformis* |MH628666| and the homology between them is (98.06%), *Bacillus subtilis* |MK788294| is similar to isolate *B. subtilis* |MH045986| and the homology between them is (99.57%), *Bacillus velezensis* |MK788292| is similar to isolate *B. velezensis* |MN093795| and the homology between them is (99.57%), *Bacillus megaterium* |MK788293| is similar to isolate *B. megaterium* |KM191301| and the homology between them is (100%) and *Bacillus thuringiensis* |MK788291| is similar to isolate *B. thuringiensis* |MH921668| and the homology between them is (99.00%).

The results obtained from the present study, keratinolytic bacteria were presented in chicken feather and feather dumping sire soil along with different verity of non keratinolytic bacterial species. The isolated keratinolytic bacterial *Bacillus* species strains were partially identified as *Bacillus thuringiensis*; *Bacillus velezensis*; *Bacillus megaterium*; *Bacillus subtilis* and *Bacillus licheniformis*. Similarly, **(Lin *et al*., 1995)** reported that keratinolytic bacterial strains were isolated from soil and other natural sources and that were identified as *Bacillus* sp. as a potential keratinolytic organism and its possible use in field studies for biodegradation of feather were studied. However, the induction of keratinolytic enzyme produced by the species of *Bacillus* with feather powder, human hair and nails, guinea pig hair and cow horn and hooves was also, reported **(Cheng *et al*., 1995).** In the present investigation, the optimum temperature for the growth and production of keratinolytic protease enzyme by *Bacillus* species strains was 37°C. This temperature optimum for maximum keratinolytic enzyme production by this organism is almost similar with that of by other *Bacillus* sp. **(Cheng *et al*., 1995; Wang and Shih, 1999)**, but dissimilar with keratinolytic Vibrio sp. kr2 **(Sangali and Brandelli, 2000)**, *Lysobacter sp*. **(Allpress *et al*., 2002)** and *Streptomyces sp*.

Conclusion:

According to the results obtained from this present study, the results obtained from the 16S rDNA gene sequence analysis the isolates were conformed and identified as *Bacillus thuringiensis*; *Bacillus velezensis*; *Bacillus megaterium*; *Bacillus subtilis* and *Bacillus licheniformis*. The five species strains of bacterial genus *Bacillus* are widely associated with chicken feathers and soil of feather dumping site. The five species strains effectively degrade the keratin protein substrates. This character of the five species of bacterial genus *Bacillus* is leads to wide range of keratinase activity, among these characteristics of isolates is well adaptive for the large scale bio-conversion of keratin waste. The industrial application of these strains for large scale bio conversion of keratin waste into poultry feed stuff creates significant importance.

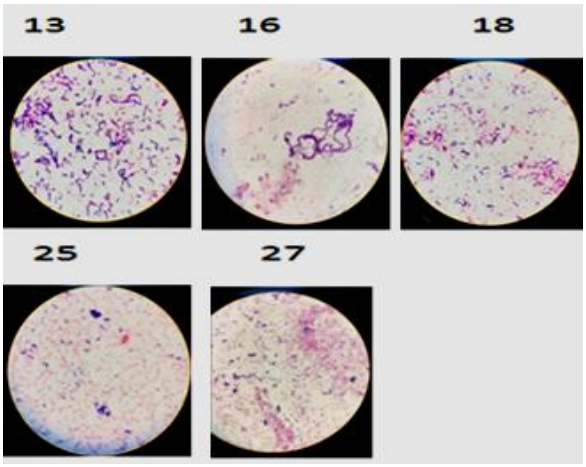

Fig. 1: Gram staining of FAK 13, FAK 16, FAK 18, FAK 25 and FAK 27 isolates showing gram staining positive.

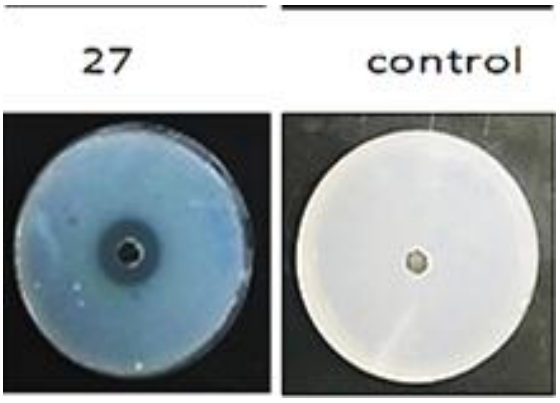

Fig. 2: Keratinase activity of FAK 27 and control isolates as indicated by clear zone surrounding wells on keratin plates after incubation at 37°C for 48 h.

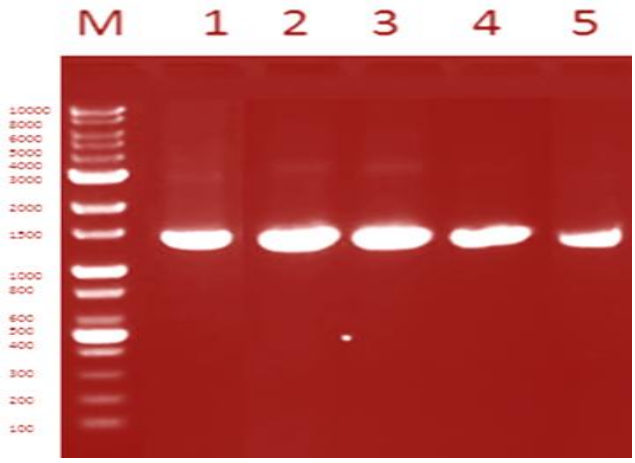

Fig. 3: Agarose gel analysis of PCR products from amplification of 16S rDNA of the five selective *Bacillus* species strains. Lane M: 1Kb plus DNA ladder; Lane 1: *B. thuringiensis*; Lane 2: *B. velezensis*; Lane 3: *B. megaterium*; Lane 4: *B. subtilis*; Lane 5: *B. licheniformis*

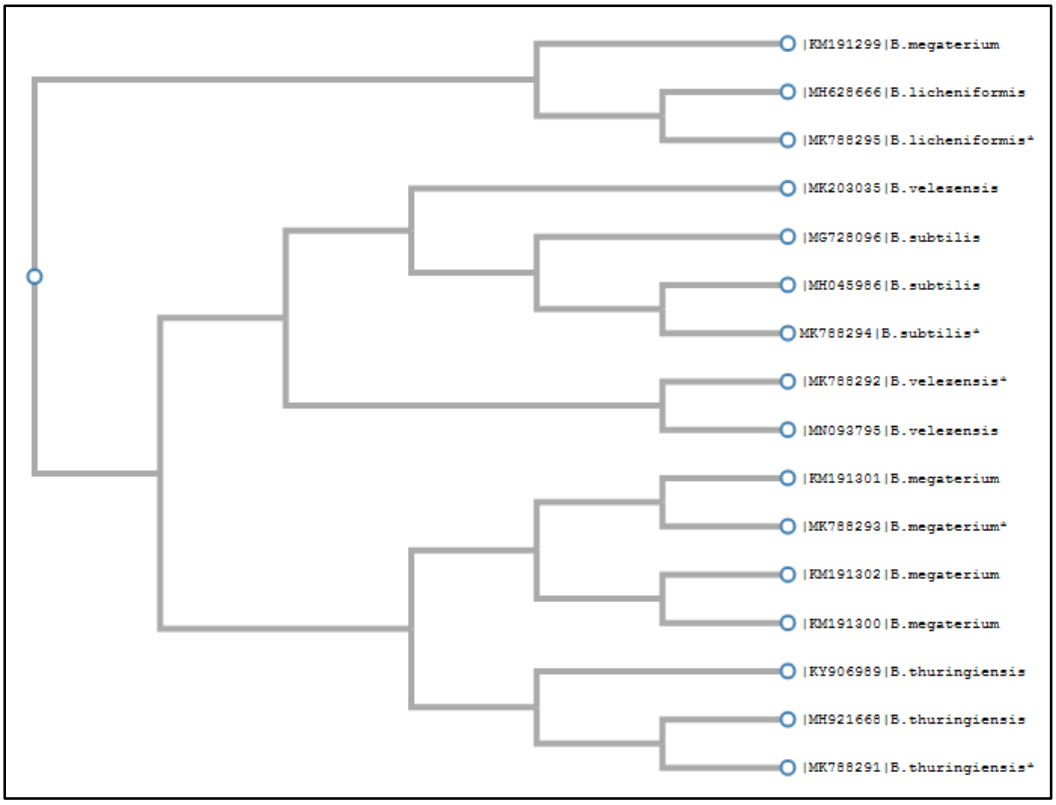

Fig. 4: Phylogenetic tree showing the relationship between the five selective *Bacillus* species strains and the closely related members of *Bacillus* species obtained from NCBI database according to 16S rDNA gene sequence.

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عزل وتعريف جزيئي لسلالات بكتيريا من جنس ال *Bacillus* منتجة لإنزيم الكيراتينيز

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الملخص العربي

تعتبر مخلفات الريش الناتجة بكميات كبيرة من مزارع الدواجن ومحلات ذبح وبيع الدواجن مصدر أساسي لتلوث البيئة. ونسبة كبيرة من تلك المخلفات هي بروتين الكيراتين. ويعتبر الكيراتين بروتين غير قابل للذوبان وغير قابل للتحلل بواسطة الإنزيمات المحللة للبروتين. وإنزيم الكيراتينيز ينتمي إلى فئة الإنزيمات المحللة للكيراتين حيث يكون قادر على تحليل الكيراتين الغير قابل للذوبان بكفاءة أكبر من الإنزيمات الأخرى المحللة للبروتين. ويعتبر التحليل المائي بواسطة البكتيريا المحللة للكيراتين بديلا هاما لتحسين القيمة الغذائية لمخلفات الريش والدواجن. وفي العشرة سنوات الماضية، نتيجة للإستخدام الواسع النطاق لتسلسل ال PCR وال DNA، فقد لعب تكنيك التسلسل الجينى ال 16S rRNA دورا محوريا في التعريف الدقيق للبكتيريا وإكتشاف سلالات جديدة منها في مختبرات الأحياء الدقيقة. وكان الهدف من هذه الدراسة هو العزل والتعريف الوراثي الجزيئى للبكتيريا المنتجة لإنزيم الكيراتينيز المعزولة من عينات التربة والريش في مواقع التخلص من ريش الدواجن. وقد تم الحصول على 32 عزلة نقية وقد تم توصيف العزلات على أساس الخصائص المورفولوجية، الإختبارات البيوكيميائية وقد تم إختبار نشاط كل عزلة في إنتاج إنزيم الكيراتينيز. وقد بينت النتائج أن 23 عزلة منها كانت منتجة لإنزيم الكيراتينيز. وقد تم إختيار أفضل 5 عزلات منها فى إنتاج إنزيم الكيراتينيز وقد تم تعريف كل عزلة من العزلات ال 5 المنتجة لإنزيم الكيراتينيز بواسطة تحليل تتابع ال 16S rRNA. وقد تم إستخلاص الحمض النووي ال (DNA) من كل عزلة من العزلات ال 5. وتم إكثار الجينات المستهدفة من جينات 16S rRNA بواسطة تفاعل ال PCR وقد تم تحديد تتابعاتها الجينية بإستخدام بادئين معروفين عامين مناسبين. وتم تحليل بيانات تتابعات الجينات ومحاذاتها من أجل معرفة القرابة الوراثية إلى التتابعات المقابلة لها فى قاعدة بيانات بنك الجينات وذلك لتحقيق التطابق ودرجة القرابة الوراثية وتعريف العزلات. وقد أكدت نتائج تتابع الجينات لنتائج شجرة القرابة الوراثية تعريف العزلات البكتيرية ال 5 وقد تم تعريفها. وقد كانت ال 5 عزلات البكتيرية المنتجة لإنزيم الكيراتينيز هى 5 سلالات كل سلالة منها تابعة لنوع واحد من 5 أنواع تابعة للجنس باسيلس وهى:- سلالة باسيلس سيورنجينسيس - وسلالة باسيلس فيليزينسيس - وسلالة باسيلس ميجاتيريم - وسلالة باسيلس سبتيلز- وسلالة باسيلس ليشينيفورمس.