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Characterization of *Streptomyces Smyrnaeus* Ati-92 Isolated from Soil Samples in Basrah City

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Abstract

The present study aimed to isolate and identify local *Streptomyces* from different sites of Basrah city, Iraq. A total of 150 samples of (soil, cow feces, buffalo feces) were collected. However, thirty-one local isolate of promising *Streptomyces* spp. were obtained from soil, cow, and buffalo feces after cultivated on the Inorganic Salt Starch Agar ISP4, Depend on the morphological, microscopic, biochemical characteristics, and antimicrobial activity, the isolate S13 was found to be a member of the genus *Streptomyces*. Furthermore, genetic diagnoses were performed on the *Streptomyces* 13. The analysis of 16S rRNA gene sequence similarity and the phylogenetic tree showed that *Streptomyces* S13 isolate was closely related to *Streptomyces smyrnaeus* (99.51%), was deposited in the NCBI gene bank (*Streptomyces*) Ati-92 smyrnaeus with accession number (LC495904).

Keywords: *Streptomyces*, Isolation, Morphology characterization, Antimicrobial activity, 16S rRNA

Introduction

Streptomyces are members of the family Streptomycetaceae (order Actinomycetales), which is the largest genus of Actinobacteria. Waksman and Henrici have discovered the species of *Streptomyces* in 1943 (Hasani et al.,2014). In general, they are recognized as a Filamentous form bacteria, gram-positive, aerobic, and non-motile that form the extensively branched substrate and aerial mycelium produce, and pigmentation were observed. More than 660 species of *Streptomyces* have been discovered and used for the production of important secondary metabolites in the past several decades. Indeed, most species prefer temperatures in the range 25 - 35 °C., the optimum pH value is between 6.5 - 8. Saturated fatty acids are synthesized by bacteria (Kim et al., 2003). high Guanine-Cytosine (57-75%) genome content (Castaneda-Cisneros et al., 2020). These bacteria were isolated from different sites of soil, plant roots, lake sediments, marine sources, and rivers (Tatar et al., 2020), Antibacterial, antitumor agents, antiviral, and anthelmintic are many of the large bioactive compounds produced by *Streptomyces* (Li et al., 2019). More recently, Pratiwi et al, (2020) found that *Streptomyces* sp was the best source of a bioactive compound *Streptomyces* is a significant producer of pharmacologically active secondary metabolites, especially antibiotics, according to studies. Researchers are interested in studying *Streptomycetes* from mangrove habitats because of their capability to produce a wide range of bioactive metabolites (Law et l, 2020). Moreover, *Streptomyces* secrete a wide range of enzymes, such as protease, amylase, lipase, pectinase, cellulose, xylanase,

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asparaginase, and transglutaminase, and these are an important application for agricultural purpose, pharmaceuticals, and food processing. Therefore, it is widely recognized as an industrial microorganism (Al- Dhabi et al., 2020). *Streptomyces* sp. that known for their ability to produce Transglutaminase which has significant higher yield than other microorganism this enzyme has been applied for modifying and improving protein characterization (Nur'amaliyah et al., 2016).

Therefore, it is generally recognized as an industrial microorganism, Microbial transglutaminase is widely used in the food industries to improve food qualities. So, we need to find more and more *Streptomyces* are the major sources of the enzyme from different habitats for transglutaminase activity in the hope of getting a new *Streptomyces* isolate that produces an enzyme.

Materials and Methods

Collection of Samples

A total of 150 samples (soil, cow feces, buffalo feces) were collected from different sites of Basrah city in the depth vary from 15 to 20 cm and were transferred into sterile polyethylene bags, labeled, transported aseptically to the laboratory, and stored at 4 °C. (Abdulhameed, 2013). Cow and buffalo feces, fruits (figs and apples), legumes (lentils, chickpeas, and beans), meat (beef), and fish (carp) were also taken from local markets and different Basrah sites.

Isolation of *Streptomyces*

Ten grams of each soil and feces were dried in an air oven at 45°C for 3 hr. then, was pretreated with 1 gram of calcium carbonate and incubated at 37 °C for 4 days, Adequate serial dilution was prepared for all samples by suspended 1 g of each (soil, cow and buffalo feces, figs, apples, lentils, chickpeas, beans, beef, and fish Carp) in 9 ml of sterile peptone water. The contents were diluted (10^{-1} to 10^{-4}) In addition, 1 ml of each dilution was spread onto the International Streptomyces Project (ISP). Plates were incubated at 28 for 7 days, the *Streptomyces* colonies were purified and maintained in ISP4 Inorganic Salt Starch Agar (Himedia) (Abdulhameed, 2013).

Diagnostic tests:

Morphological tests:

The bacteria were activated by growing on ISP4 Agar medium for 7 days at 28 °C. The colonies were examined for shape, appearance, edge, and color on the growth medium. The cultural characteristics such as the spore chain morphology, color of substrate and aerial mycelium of the local isolate on ISP4 medium were recorded using a light microscope (Ernstieitz Wetzlirgm , Germany) with some modification according to the method of Rosana et al., (2014).

Biochemical tests :

Diagnostic characters of the local isolate was determined according to the Bergey's Manual of Systematic Bacteriology (Kampfer, 2012), biochemical characteristics generally used are gram stain, starch hydrolysis, citrate utilization test, oxidase test, catalase test, gelatin hydrolysis, casein degradation, nitrate reduction, anaerobic growth, urea degradation, motility test,

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Physiological characterization, such as NaCl tolerance (5,7 and 10%), the effect of pH (4-12), and temperatures(4-55) were also tested.

Antimicrobial activity:

Streptomyces isolate was evaluated for their activity against five pathogenic microbes: *Bacillus subtilis*, E. *coli*, *Staphylococcus aureus* *Aspergillus niger*, and *Candida albicans* were obtained from the Biotechnology laboratory of Agriculture college, University of Basrah. The antimicrobial activity of the isolate was determined by the cross-streak method on Mueller Hinton Agar (Himedia) depending on the agar well diffusion method, The spores, and bacterial suspension were added and spread on a medium using a sterile glass L-shape. and Then, 50 µl of the pure isolate was poured into wells (5 mm). The plates were incubated at 30°C for 1 to 7 days, the inhibition zones were observed and measured (Jabur, 2004).

Genetic analysis of the most common MTGase isolate:

The DNA was extracted using a mini Kit provided by the Korean company i-genomic BYF according to the manufacturer's instructions, Electrophoresis was used to detect DNA on a 1 % agarose gel, according to the method of Sambrook et al., (1989).

Amplification and sequencing of the 16S rRNA gene:

PCR amplification of the 16 S rRNA genes of the local *Streptomyces* isolate was identified using the primers, F; 5'-AGAGTTTGATCCTGGCTCAG- 3' and R; 5'-GGTTACCTTGTTACGACTT- 3', by the method described by Lamilla et al.,(2018)

Preparation of agarose gel and Electrophoresis of PCR products:

PCR products were loaded onto 1.5% agarose slabs according to the method of Sambrook et al. (1989). The amplified gene products were sent for sequencing to the Macrogen company, Korea, , Then, the sequence was compared for similarity of *Streptomyces* with their reference species was approved using the NCBI BLAST program available at the(www.ncbi.nlm.nih.gov), Also, the construction of phylogenetic tree was displayed using the MEGA7 program.

Statistical analysis

Data was analyzed using Special Program for Statistical System (SPSS).

Results and Discussion:

Isolation of *Streptomyces*:

A total of 31 bacterial isolates were obtained from different sites including soil, cow feces, and buffalo feces, they were divided into three groups based on the sources and isolation sites. The soil were mixed with calcium carbonate (1:10) and incubated at 37 °C for four days. The drying and addition of calcium carbonate to the soil led to reducing the number of vegetative bacteria other than *Streptomyces*., also raising the pH value, which inhibits the growth of other microorganisms such as fungi and increases in growth of *Streptomyces* (Kampfer, 2012). A 100 fold increased in *Streptomyces* colonies may be achieved by the addition of calcium carbonate to soil samples (Bagyoko et al., 2018; Maiti et al., 2020). ISP4 agar is considered a selective and sufficient medium for their growth. As demonstrated by various scientific studies that indicate the importance of using this medium, all of these factors contributed to the bacteria ability to

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evolve independently of others (Kampfer, 2012). Other sources of isolation, such as beef, carp fish, figs, apples, lentils, chickpeas, and beans, failed to obtain bacteria because the soil is a rich source of *Streptomyces*., which is the primary source.

After that, they were grown on ISP culture medium, which is a selective medium for the growth of this bacteria and has been used in many research articles. (Korn- Wendisch and Kutzner, 1992).

Identification of Bacterial Isolates:

Morphological tests:

Morphological features of 31 locally isolated colonies were identified and named as S1 to S31 S12, S13, S20, S25, S26, S29, S30, and S31 had single, circular, white, cottony colonies, whereas isolates S15, S16, S17, S18, and S19 had a creamy color and diffused in medium, while other isolates S1, S2, S3, S21, S22, S27and S28 as yellow. S4, S5, S6, S7, S8, S9, and S14 as brown, Isolates S10, S11, S23, and S24 were gray pigmentation colonies with thick growth, was observed as shown in Figure 1 (A, B, C, D, E,). All of the thirty-one isolates produced a wide range of colored colonies, which are one of the major diagnostic features of the genus *Streptomyces* and are influenced by several factors such as the medium composition, temperature and pH, as well as the age of the culture (Kampfer, 2012). Furthermore, The bacterial isolates were identified based on the production of moist earthy odor and chalky appearance of colonies, These results are similar to other previous studies (Rahman, 2008; Thakur et al., 2007; Lapaz et al., 2017).

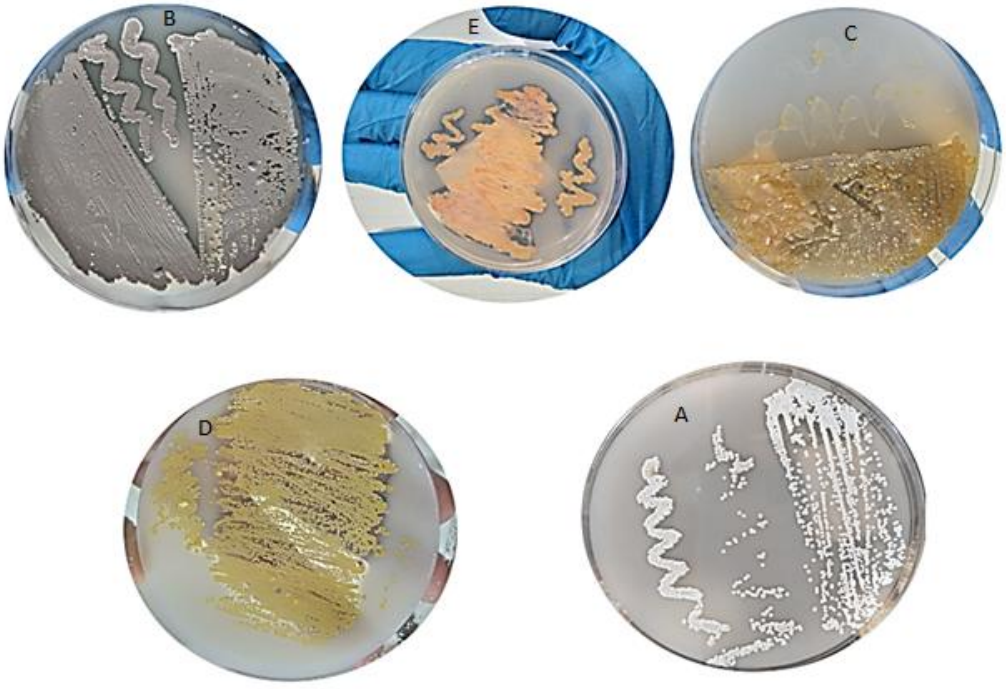

Figure 1: Colony morphology of *Streptomyces* spp. isolated from soils in Basrah using ISP4 media.

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Figure (2) Shows the microscopic characteristics of S13 isolate.. The isolate was found that a gram-positive filamentous bacterium., some isolates are thin and long, like hair, while others are thick, wavy, filament branches. Then confirmed by using the slide culture method, which is one of the best technique for characterizing filamentous bacteria isolates at the genus level due to its role in showing the substrate and aerial mycelium that distinguishes these bacteria from one another (Kampfer, 2012). It was known as the substrate mycelium because it was heavily branched, smaller, and non-dividing, and it lacked spores and was white. on the other hand Aerial mycelium was less branched and thicker, forming a short chain of spores that all of which were white as shown in Figure (3), *Streptomyces* bacterium is positive for aerobic pigment-forming spores and non-motile (Kampfer ,2012),also, Thirumurgan et al. (2018) found that *Streptomyces albogriseolus* isolated from the marine environment of the Bay of Bengal was positive for Gram stain,. Kusuma et al., (2020) confirmed the *Streptomyces harenosi* isolated from soil is gram-stain positive and non-motile.

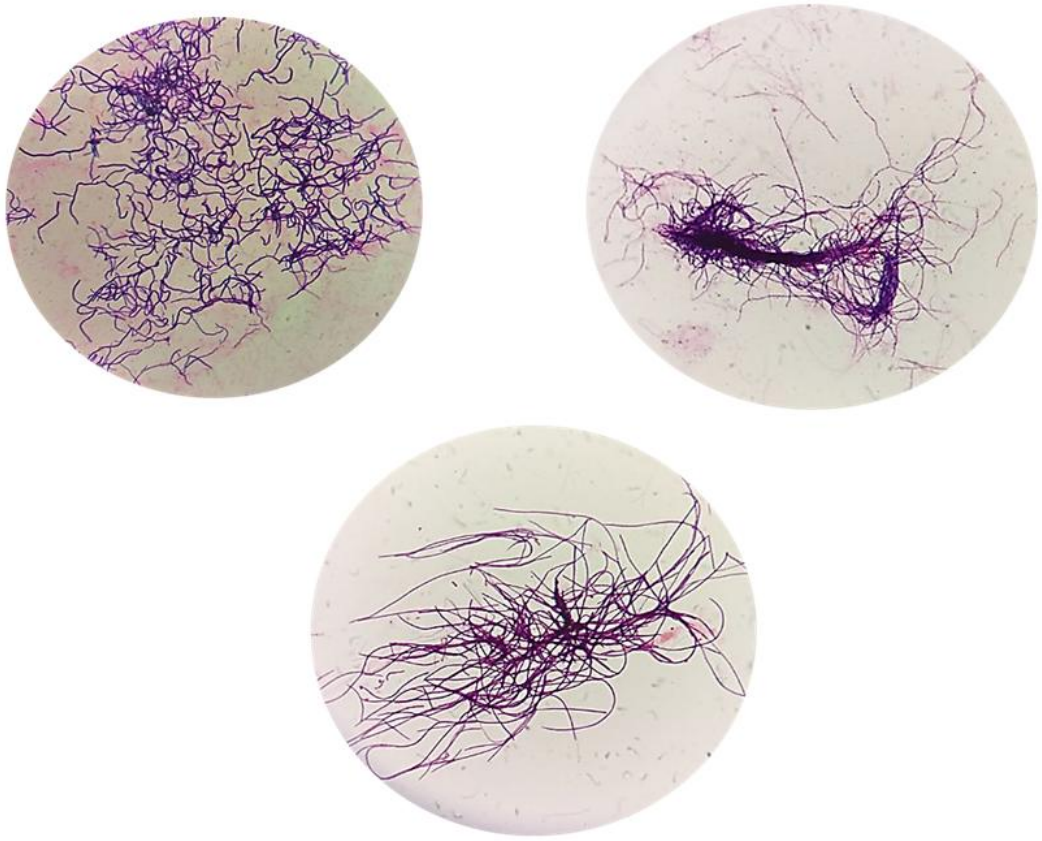

Figure 2: Microscope image of locally isolated bacteria

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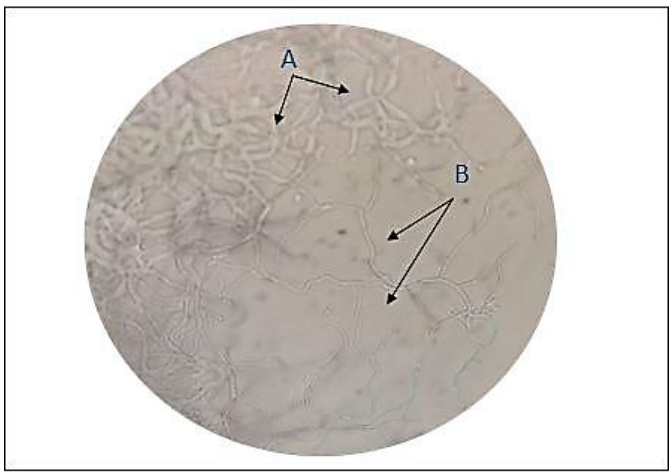

Figure 3: Aerial mycelium and substrate mycelium of locally isolated bacteria, A: aerial mycelium, B: substrate mycelium.

Biochemical tests:

The isolate *Streptomyces* S13, was selected from primary screening which was based on the highest transglutaminase activity (unpublished data) which was referred to as biochemical analysis.

Table 3 shows that this isolate could hydrolyze starch, utilization of citrate, catalase, and degradation of gelatin are positive, whereas oxidase, nitrate reduction, casein degradation, and decomposition of urea are negative, No growth under the anaerobic condition, On the other hand, the isolated growth at different concentration of NaCl (5-10%) and pH values (4-12). Moreover, local isolate can grow at (26, 28, 30, 35, 40, 45) °C, but not at (4, 10, 50, 55) °C. Depend on the morphological, microscopic, and biochemical characteristics, the isolate S13 was found to be a member of the genus *Streptomyces*., our findings were consistent with previous studies' (Tatra et al., 2014, Islam et al., 2014; Thirumurugan et al., 2018 ; Zhao et al., 2019).

Table 3. The physiological and biochemical characteristics of Streptomyces S13 isolate

Characteristic	*Streptomyces* S13
Starch hydrolysis	+
Citrate utilization	+
Catalase	+
Oxidase	–
Gelatin hydrolysis	+
Casein hydrolysis	–
Nitrate reduction	–
Urease	–
Mobility	–
Anaerobic growth	–
NaCl (%) for growth5%	+
7%	+

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10%	+
pH tolerance 4	+
6	+
8	+
10	+
12	+
Temperature for growth26 C	+
28	+
30	+
35	+
40	+
45	+
4	–
10	–
50	–
55	–

+ : positive test result – : negative test result

Antimicrobial activity:

Table (4) and Fig. 4 show that the *Streptomyces* S13 isolate which can produce antimicrobial agent against some pathogenic microorganisms (Gram-negative, gram-positive bacteria, and fungi), The results showed that isolate having antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and also indicated antifungal activity against *Aspergillus niger* and *Candida albicans* with inhibition zone about 15-30 mm, The inhibitory activity is attributable to their ability to produce many types of secondary metabolites such as antibiotics (Kampfer, 2012), Our results agreed with findings of (Abdulhameed, 2013; Tatar et al., 2014; Bagayoko et al., 2018) in which growth of pathogenic microorganisms was inhibited by a substance produced from *Streptomyces* S13, The inhibitory function of MTGase-producing bacterial isolates with the highest enzymatic activity is shown in Table (4).

Table (4) Antimicrobial activity of *Streptomyces* S13 isolate against pathogenic microorganism.

Microorganisms	Inhibition zone(mm)
Escherichia coli	18
Staphylococcus aureus	15

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Bacillus subtilis	20
Aspergillus niger	30
Candida albicans	25

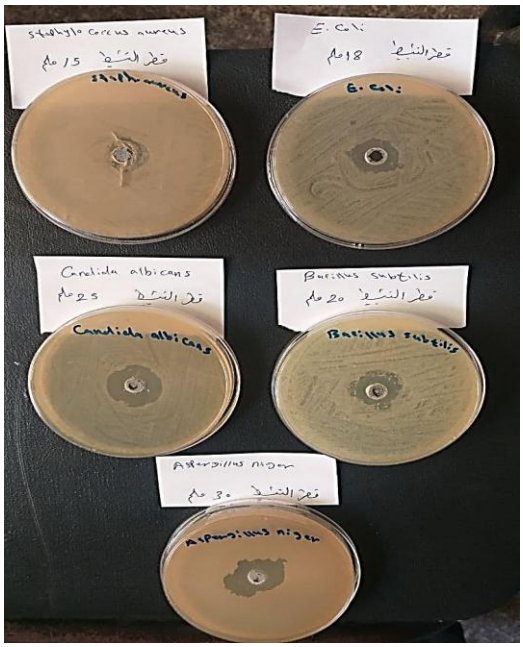

Figure (4) Microorganism-inhibitory behavior of a Streptomyces S13 strain isolated locally.

Molecular Identification of Isolated Streptomycetes

Genomic DNA extraction:

The genomic DNA of the local isolate was extracted using three replicates S13, S13, S13. The results of the agarose gel electrophoresis, which clearly showed three distinct DNA bands of the bacterial isolate in figure 5, that confirmed the purity of the extraction.

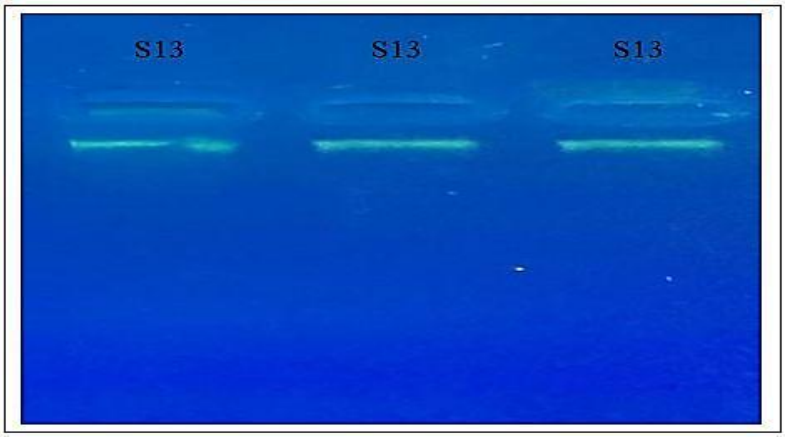

Figure (5) 1: Genomic DNA bands of local isolate on 1% agarose gel

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Amplification of DNA by Polymerase Chain Reaction (PCR):

The polymerase chain reaction (PCR) technique was used for the 16S rRNA test .The primers for the genes were utilized for amplification. Figure (6) shows the result of amplified product of 16S rRNA gene on 1.5% agarose gel electrophoresis with three replicates (S13, S13, S13) as green bands and the size of 16S rRNA region was 1250 a base pair. The primer bound to the target gene 16S rRNA without the other parts of the DNA extracted from the isolated bacteria, according to these findings. The study also agrees with the most of other studies that have confirmed the use of the 16S rRNA test for the diagnosis of *Streptomyces* sp for local isolate in a study that follows the nitrogenous bases of DNA. (Tatar et al., 2014; Lamilla et al., 2018; Ramirez-Rodriguez et al., 2018; Kusuma et al., 2020).

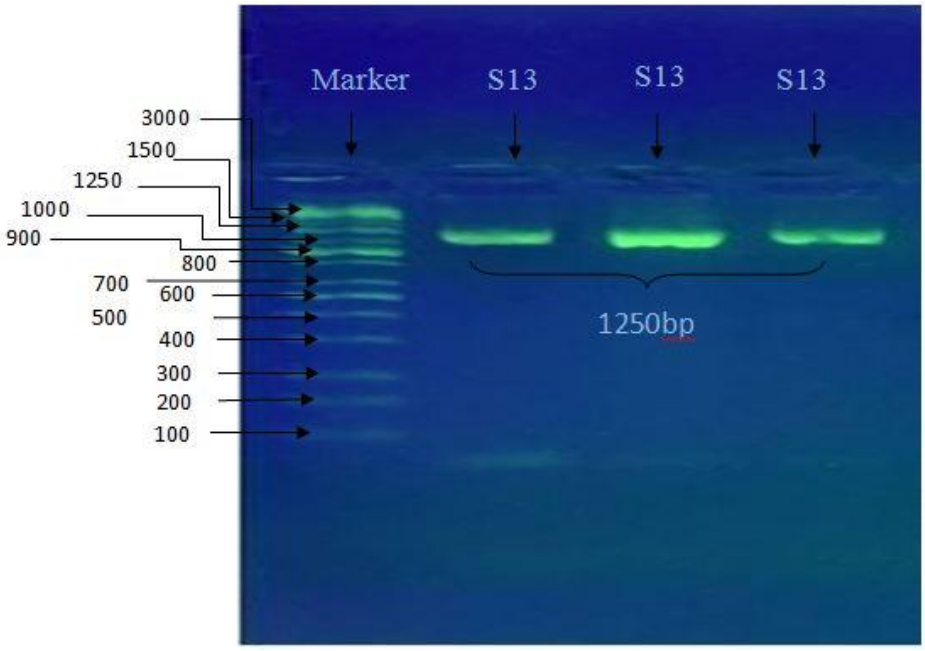

Figure (6) PCR reaction amplified product of 16S rRNA gene on 1.5% agarose gel electrophoresis.(left side is marker 3000 bp DNA ladder)

Sequence Analysis of Amplified DNA products

Most studies have difficulty diagnosing the species of *Streptomyces* . therefore, The conventional taxonomy methods are not satisfactory for the analysis of species of *Streptomyces*, for this, the 16S rRNA gene sequencing tests are one of the optimum methods for the identification of *Streptomyces* species. The results were then compared with other sequences deposited using the BLAST software to analyze the data. Depend on 16S rRNA gene sequence, *Streptomyces* S13 was related closely to *Streptomyces smyrnaeus* SM3501 NR_ 134201.1(99.51%).and Genbank accession numbers are given (Accession No LC495904). The phylogenetic tree has been shown in Figure (7).

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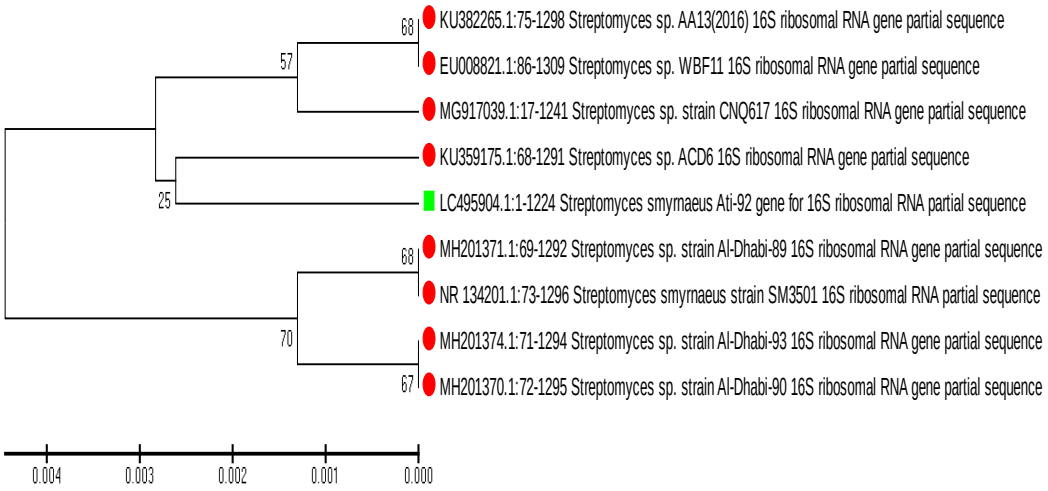

Figure (7)Phylogenetic tree show position of the local *Streptomyces* S13 among the strains of its closest related species in international databases

Conclusion

Depend on the results were obtained in the current study, the conclusion was that the Basrah soil isolates *Streptomyces* spp. have potential great potential for further investigation on transglutaminase production. Among the 31 *Streptomyces* isolates selected based on their ability to produce enzyme quantitatively(data not show), the isolate S13 isolated from the soil of Basrah city, Iraq, exhibited the highest MTGase activity. This isolate S13 was identified at morphological, microscopic, biochemical characteristics, and antimicrobial activity, the isolate S13 was found to be a member of the genus *Streptomyces*. Moreover, The analysis of 16S rRNA gene sequence similarity and the phylogenetic tree showed that *Streptomyces* S13 isolate was closely related to *Streptomyces smyrnaeus* (99.51%).

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