Cross Current International Journal of Medical and Biosciences
Abbreviated Key Title: Cross Current Int J Med Biosci
ISSN: 2663-2446 (Print) & Open Access
DOI: 10.36344/ccijmb.2024.v06i03.005

Volume-6 | Issue-3 | May-Jun, 2024 |

Original Research Article

Evaluation of the Effectiveness of *Streptomyces* sp. Against Biofilm-Forming Bacteria and Starch-Protein degradation

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| **Received:** 12.05.2024 | **Accepted:** 26.06.2024 | **Published:** 29.06.2024 |

Abstract: *Streptomyces* sp. play important role in controlling pathogenic bacteria by using different mechanisms so this study aims to diagnose the *Streptomyces* sp. and select strains having the ability to inhibit biofilm-forming bacteria and degradation proteins and starch. The capacity of bacteria to produce biofilms was examined by staining with 1% crystal violet. The ability to inhibit biofilm-forming bacteria, proteolytic and amylolytic, were determined by bacterial drop and well diffusion methods. The bacteria capable of creating biofilms were isolated from urinary tract infection and identified by biochemical reactions and sequencing the 16S rRNA gene. The Selected 6 Streptomyces strains which were isolated from soil are capable of inhibiting the growth of *Escherichia coli* and capable of degrading protein and starch. The strain STV1 was selected for 16S rRNA gene sequencing with standard isolates. Isolation of Escherichia coli capable of forming biofilm from urinary tract infection. Selection of *Streptomyces* sp strains capable of inhibiting biofilm-forming bacteria. The research is the first step to studying the application of *Streptomyces* sp in inhibiting biofilm-forming bacteria and in treating organic matter.

Keywords: Oral candidiasis, candida albicans, removable dental prosthesis, prosthetic biofilm.

INTRODUCTION

Biofilm-forming bacteria are resistant to antibiotics at concentrations thousands of times higher than free-living bacteria [8, 14, 17], and biofilms are also a hiding place for many pathogens [21]. Therefore, creates a community of pathogenic bacteria that is difficult to destroy, and this is the cause of prolonged epidemics [19]. Some bacterial strains capable of forming biofilms were isolated such as, *Vibrio cholerae*, *S. aureus*, *S. epidermidis* [7, 16]. Besides Vibrio spp. and Staphylococcus spp. which can cause disease and create biofilms, *Escherichia coli* is also being evaluated as a new pathogen in aquaculture. *Escherichia coli* are Gram-negative rods and some biofilm-forming strains are known to cause disease in humans, but recent studies have found that they also cause disease in aquatic animals. Escherichia coli, identified as a pathogen in some cases by Yang *et al.,* [22] is capable of causing hemorrhage in the gonads, bladders, intestines, and enlarged spleens.

Streptomyces sp. has long been studied and applied for biological control of bacterial and fungal pathogens. They can control pathogenic bacteria by different mechanisms, such as competing for accommodation and adhering to intestinal mucosal epithelium to prevent the adhesion of pathogenic bacteria. Competing for nutrients, *Streptomyces* sp. decomposes and uses organic matter in the environment thereby controlling free-living bacteria. *Streptomyces* sp. also secretes antimicrobial substances, Tran *et al.,* [18] mentioned that there are 47 compounds produced by *Streptomyces sp* that have antimicrobial activity with identified mechanisms. These compounds act on cell walls, plasma membranes, intracellular processes, and other metabolites. Besides, *Streptomyces sp* also can decompose protein, starch, and other organic substances. This study aimed to isolate biofilm-forming bacteria from urinary tract infection and isolate *Streptomyces* sp capable of inhibiting them and evaluated the proteolytic and starch-degrading ability of *Streptomyces* sp. isolates.

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Citation: Ahmed Majeed Abdzaid, Mohammed Mudhafar Alkhuzaie, Abbas Mayar Hezam (2024). Evaluation of the Effectiveness of *Streptomyces* sp. Against Biofilm-Forming Bacteria and Starch-Protein degradation. *Cross Current Int J Med Biosci*, *6*(3), 77-83.

MATERIALS AND METHODS

Samples collection

Soil samples was collected at 3 locations (beginning, middle, and end), 100 gm each. Soil samples at different locations were homogenized then moved to the Microbiology laboratory for the purpose of isolating Streptomyces bacteria. Pathogenic bacterial samples were isolated from urinary tract infection patients in Diwaniyah city, Iraq.

Isolation of biofilm-forming bacteria

Bacterial isolation: After the samples were transferred to the addiction laboratory, the bacteria were isolated on a tryptone soya agar (TSA) medium. 10 mL of sample added to 90 mL of tryptone soya broth (TSB) medium and incubated at 120 rounds per minute (rpm) at room temperature (27℃±3) for 24 h. to increase the number of bacteria present in the sample. After incubation, aspirate (suck) 100 µL of sample solution, spread on the TSA medium, and incubate at room temperature. After 24 h. select individual colonies with different characteristics on the TSA medium.

The ability to form biofilms: Bacterial suspension (20 µL) and 180 µL TSB medium were poured in the wells (microplate) and kept at room temperature under static conditions. After 48 h, remove the culture from the wells and stain with 200 µL of 1% crystal violet solution for 15 min. Then, remove the dye solution and wash it with distilled water (until the water is translucent and has no color of the dye) and allow it to dry naturally. Then, added 200 µL of 95% ethanol into the wells to solubilize the biofilm and measure the OD at 600 nm. The biofilm-forming capacity is directly proportional to the measured OD value (the larger the value, the higher the amount of biofilm produced). Select bacterial strains with high biofilm-forming ability. The strains that were determined to have the ability to form biofilms were stored with glycerol at -20℃ (12).

Isolation of *Streptomyces* spp.

The isolation method was achieved as done by Han *et al.*, [11] with some modifications. Samples were heated for 20 min at 60℃ in a water bath, 100 µL of supernatant, spread on TSA medium, then kept at room temperature for duration of 24 h. After that, select individual colonies with different characteristics and then puree (spread) on TSA medium. Cultured strains were stored with glycerol at -20℃.

Screening for the ability to inhibit biofilm-forming bacteria

Bacterial droplet method: the method was achieved according to the description of Qian *et al.* (15). The biofilm-forming bacteria were cultured overnight in TSB medium, then spread 100 µL of bacterial suspension on TSA medium and allowed to stand for 15 min. *Streptomyces* strains were inoculated into plates with a volume of 2 µL, then incubated for 48 hours at room temperature. The inhibitory capacity was determined by the inhibition halo diameter in mm.

Cell-free supernatant (CFS) method: the method described by Çadirci and Çitak, (6) with some modifications. *Streptomyces* was cultured overnight in TSB medium, the culture then centrifuged for 10 min (4℃) at 12000 rpm, removing the cells. Aspirate 10 µL of the supernatant onto an agar well on TSA medium coated with biofilm-forming bacteria and kept for 48 hours at room temperature. The inhibitory capacity was determined by halo diameter in mm.

Evaluation of the ability to degrade organic compounds

Test for proteolysis and starch degradation by bacterial droplet method: *Streptomyces* strains were inoculated with a volume of 3 µL on plates of Skim milk agar (SMA) and Starch agar (SA) and incubated for 24 hours at room temperature. Determination of bacterial strain resolution according to the diameter of the halo ring. The proteolytic power is determined by zones of clearing diameter (mm), the larger the diameter, the stronger the bacteria.

Test for proteolysis and starch by CFS: *Streptomyces* was cultured overnight in TSB medium, then the bacterial culture was centrifuged at 12000 rpm using cold centrifuge (4℃) for 10 min, removing the cells. Aspirate 10 µL of the CFS onto agar well on Skim milk agar (SMA) and Starch agar (SA).

Identification of bacteria isolates

The bacterial isolates with superior organic matter degradability and antibacterial properties were selected for 16S rRNA sequencing. PCR analysis was conducted by Abbasiliasi *et al.*, [1] and Al-Amran *et al.*, [3] with some modifications (Table 1 and Table 2). The amplification process was conducted using a C1000 thermocycler manufactured by Bio-rad in the United States. The PCR results underwent separation by 2% agarose electrophoresis for a duration of 45 minutes at a voltage of 50V. The resulting bands were then made visible using the Run-Safe dye, manufactured by Cleaver Scientific in the United Kingdom. The amplicons that exhibited distinct bands and lacked any non-specific products were sent to GenLab for sequencing analysis. The raw sequence was analyzed using Bioedit software version 7.2.3.0. The quality value is utilized to validate the precision of the sequencing process, whereas the chromatogram is employed to verify the presence of unidentified nucleotides. The National Centre for Biotechnology Information (NCBI)'s BLAST program was utilized to conduct similarity searches on sequencing data.

Table 1: Primers' oligonucleotide sequence

Primers	Sequence	bp
27F	5'-AGAGTTTGATCCTGGCTC-3'	18
1492R	5'-TACGGTTACCTTGTTACGACT-3'	21

Table 2: PCR cycles' conditions

	Steps	Temperature	Time	Cycles number
Step1	Initial denaturation	95 °C	5min	1
Step2	Denaturation Annealing Extension	95 °C 55 °C 72 °C	45sec 80sec 80sec	35
Step3	Final extension	72 °C	5min	1

Statistical analysis

Each value is presented as the means ± SD of three replications. Minitab 16.2.3.0 software was used to statistically process the results using the ANOVA approach. To determine whether there was a significant difference among results at the significance level of $P<0.05$, Tukey's test was used.

RESULT AND DISCUSSION

Isolated and identification biofilm-forming bacteria

E. coli was found capable of forming biofilms (Figure1). After observing colony morphology, cell morphology, biochemical characteristics (Table 3), and the results of molecular biology determination, the *E. coli* was identified. *E. coli* is Gram-negative rod, pink colonies, lactose ferment. The sequence of the 16S rRNA gene region (Figure 2) of *E. coli* is 99.77% similar to Standard isolates of *Escherichia coli* when compared with sequences on the NCBI database.

Our findings corroborated those of [2, 4], who identified E. coli using the 16S rRNA gene. Our results, however, were not the same as those of [10]. There are several variables to consider, such as the sample size and the number of hospitals included in the research, the techniques used to isolate the bacteria, and the timing and manner of sample collection, might be blamed for the increased prevalence of E. coli in urinary tract infections.

Table 3: *E. coli* identification Results by biochemical tests

Test	Results
Citrate utilization	-
Triple sugar iron	+
Hemolysin test	+
Voges-proskauer	-
Methyl red	+
Oxidase	-
Catalase	+
Urease	-
Indole production	+
lactose ferment	+

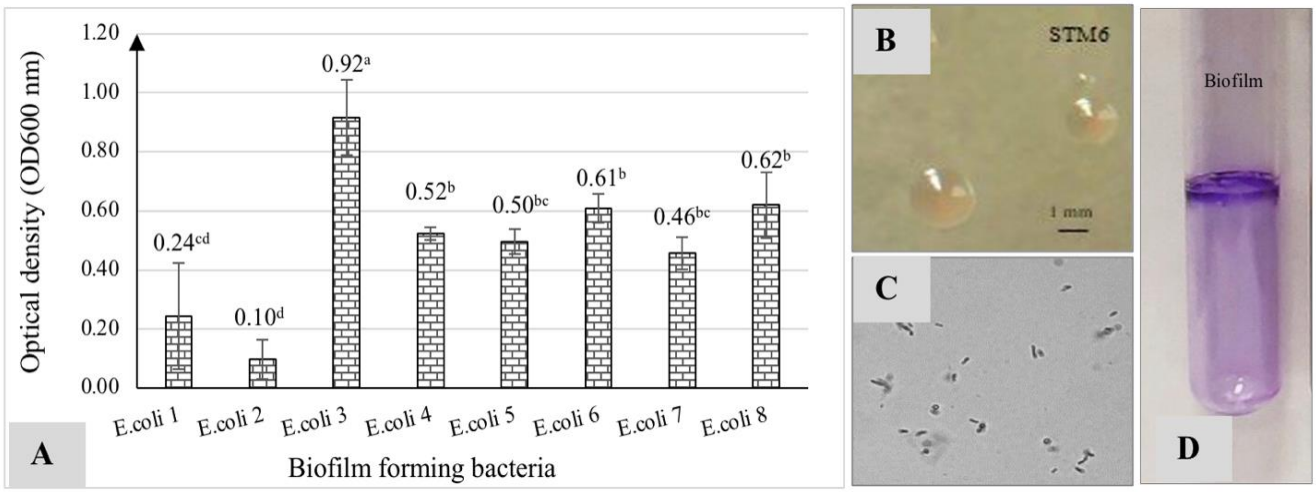

Figure 1: Biofilm forming ability and morphology of *E. coli.* Each treatment was replicated three times. Means followed by the same letter is not significantly different at the 5% level in the same map using Tukey's test. (A) The biofilm-forming ability of bacteria is expressed as an OD600 nm value when staining the biofilm with crystal violet. *E. coli* has a strong biofilm-forming ability (B) Colonies of *E. coli* on TSA medium observed after 24 h of culture (C) Bacteria have rod cells when examined under an optical microscope at 1000X magnification and Gram-negative. (D) Biofilm testing

Score	Expect	Identities	Gaps	Strand
824 bits (446)	0.0	454/455(99%)	0/455(0%)	Plus/Plus

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Query  929   CATTAAAAGGTAAGCGTCCATTATGGATTGGCACATCATGCTCTTTGTTGCATAGTCCAA  988
Sbjct  1     ............................................................  60

Query  989   TTGATTTAAGTGCGGAGACTAAGCTTGATGATGAAGTGAAAAGCTGGTTTGCCTTTGCGG  1048
Sbjct  61    ............................................................  120

Query  1049  TGCAAAAATGTGCAGAAGTGGCGTTACTCGCAAAAGCATTAAATGCACCAGAAGGTGAAT  1108
Sbjct  121   ............................................................  180

Query  1109  ATGACGAACAACTTGCCCAATACAGTGCCCCCATTCGTCAACGTGAACACTCTAGTCGTG  1168
Sbjct  181   ............................................................  240

Query  1169  TACATAATGCAAAAGTGGCCGCACGCTTGCAGGCGATTAATGCACAAGACGGTGAAAGAA  1228
Sbjct  241   ............................................................  300

Query  1229  CATCGCCTTATCAACAACGAGCTAAAGTGCAACGCGCTCGGTTTAATTTCCCTCTGTGGC  1288
Sbjct  301   ............................................................  360

Query  1289  CAACGACAACCATAGGTTCATTCCCACAAACTACCGAGATCCGCACAGTACGTTTAGACT  1348
Sbjct  361   ............................................................  420

Query  1349  TTAAAAAAGGGCGTATTGATACCACTGCTTATCGC  1383
Sbjct  421   ...................................  455
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Figure2: The NCBI (BLAST) analysis of genetic variation analysis for 16S rRNA gene in *E. coli* compared standard isolate in the NCBI-Genbank

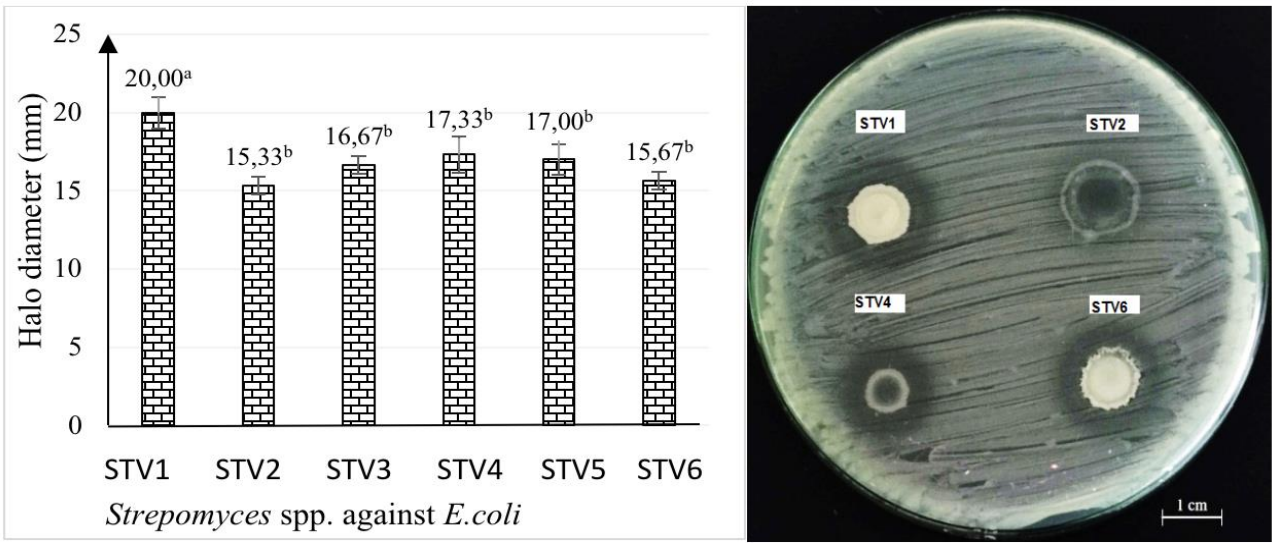

Figure 3: Ability to control biofilm-forming bacteria. Using Tukey's test, the means in the same map that are sequenced by the same letter didn't differ substantially at the 5% level

During the growth of *Streptomyces*, they can change the pH but cannot decrease it to the extent that inhibits the growth of other bacteria such as *Lactobacillus*. The main antimicrobial mechanism studied by *Streptomyces* is the secretion of antimicrobial metabolites. In this study, when examining bacterial cultures after 48 h, no inhibitory activity was observed, although these *Streptomyces* strains were identified as having the ability to inhibit bacteria.

Ability to degrade organic compounds

Proteolytic activity of *Streptomyces* spp.

In general, all 6 *Streptomyces* strains are capable of proteolytic activity and different *Streptomyces* strains can degrade differently (Figure 4A and Figure

6A). The bacterial STV1 strain showed strong proteolytic power but there was no difference compared with STV4 and STV6 strains with resolution diameter of 18 mm. The STV2 strain exhibits the lowest resolving (protein degrading) power compared to the other 5 strains. The cell-free culture of *Streptomyces* is also capable of proteolytic activity (Figure 4B and Figure 6B). The resolution (proteolysis) of the culture broth in the bacterial strains was the same from 14 mm, but the STV2 culture still showed the lowest proteolytic ability. After 48 h of culture, these strains were able to secrete extracellular protease enzymes to degrade proteins.

The operator in *E. coli* consists of a group of genes, which is responsible for encoding the enzymes involved in protein degradation; a mutation in this gene inhibits proteolytic activity [20].

Amylolytic activity of *Streptomyces* spp.

The starch-degrading activities of *Streptomyces* strains were lower than their proteolytic ability. The lowest starch resolution (ability) was 10,667 mm in the STV2 strain (Figure 5A and Figure 6C) and all of these strains were able to produce extracellular amylase (Figure 5B and Figure 6D).

Amylases are classified into two categories: Exo-amylases specifically act on the non-reducing end of starch, while endo-amylases catalyze the hydrolysis of glycosidic bonds within starch molecules as stated by Al-Amran *et al.*, [3]. Amylase, a crucial enzyme in biotechnology, is predominantly sourced from microorganisms and possesses various industrial applications, as highlighted by [9].

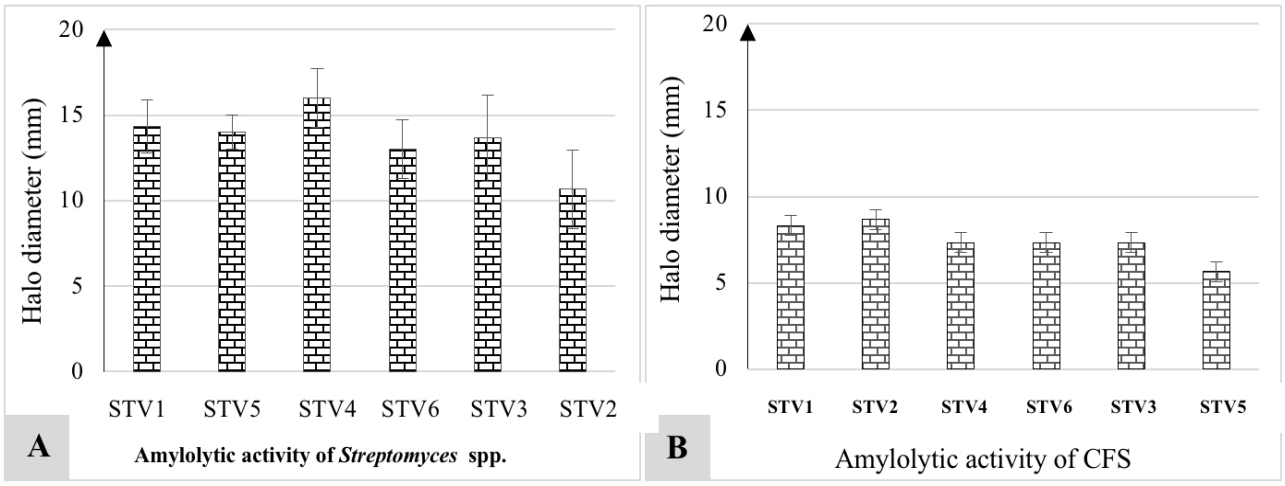

Figure 4: Proteolytic activity: Each treatment was replicated three times. There is no statistically significant difference at the 5% level between means that are followed by the same letter in the same map using Tukey's test. "A" proteolytic ability of *Streptomyces*, "B" proteolytic ability of CFS

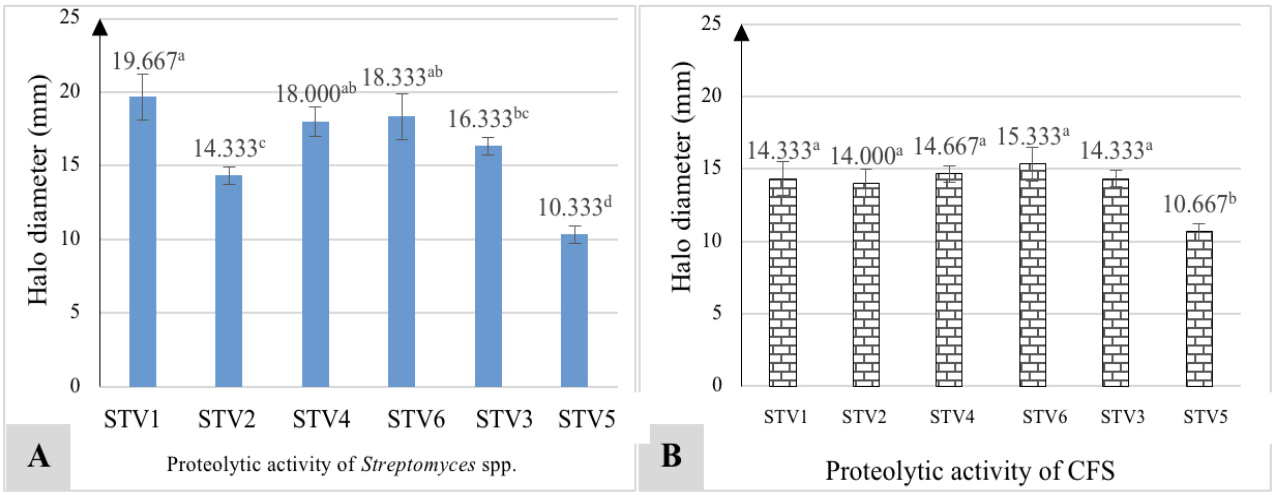

Figure 5. Amylolytic activity: A. Amylolytic ability of Streptomyces. B. Amylolytic ability of CFS

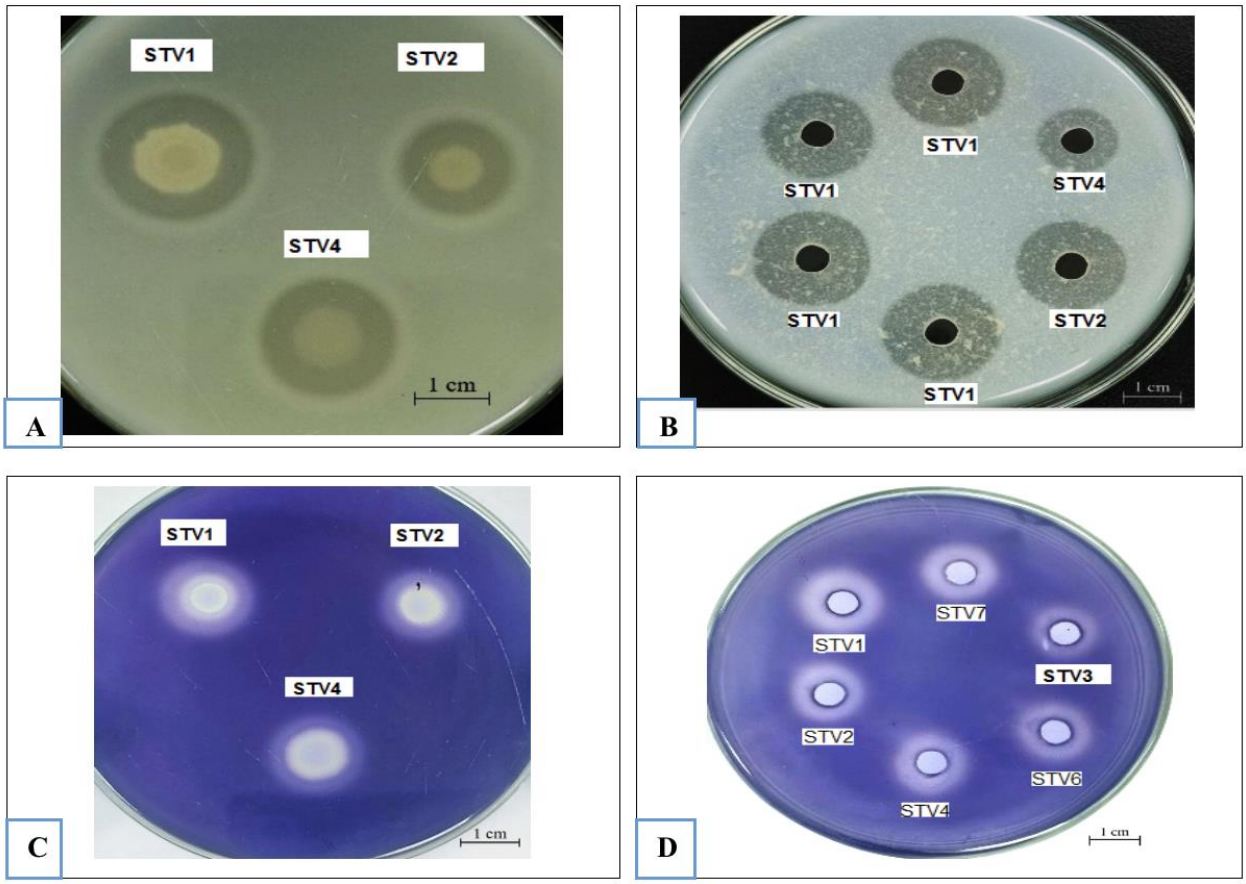

Figure 6. The ability to degrade organic compounds of *Streptomyces* spp. and CFS: A. proteolytic ability of *Streptomyces* B. proteolytic ability of CFS C. amylolytic ability of *Streptomyces* D. amyololytic ability of CFS

Tablet 4: The characteristics of the *Streptomyces* spp

Test	Results
Bacterial morphology	Rod shaped
Gram	+
Catalase	+
Urease	+
H2S	-
IMVC	-
Fermentation of Glucose and Maltose	+
Fermentation of Sucrose	+/-
Fermentation of Lactose	+
Hydrolysis Starch	+

(+) positive (-) negative

CONCLUSION

The study isolated bacteria capable of forming biofilms and identified as *E.coli*. In addition, the study also selected 6 Streptomyces isolates of bacteria capable of inhibiting the growth of *E.coli* and capable of degrading proteins and starches. The STV1 strain has the strongest ability to inhibit *E. coli* and has the ability to degrade protein and starch.

ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation to the staff members of the microbiology lab. at the University of Al-Qadisiyah, College of Science, for their invaluable help throughout the time the duration of this research endeavor.

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