Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 9795 - 9817
Received 25 April 2021; Accepted 08 May 2021.

Isolation and Purification of Endophytic Actinomycetes Spp from Local Algaein the Central Marshesof Thi-Qar Provence, Southern of Iraq

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ABSTRACT
A total of fifty local green algae samples were collected from three different areas fromcentral marshes of Thi-Qar Provence, southern of Iraq.The current study, which continued to Isolation, purification and characterization endophytic actinomycetes*Streptomycets* sp. from local algaeisolates (chlorophyta), (Chladophora sp. and Ulothrix sp.).The Endophytic Actinomycetes were isolated by starch casein agar(SCA) and Actnomycetes Isolation Agar (AIA) plate method.Various biochemical testes performed for the identification of potent isolates. Identification of the strainswere carried out by a conventional PCR protocol to analyze simultaneously the presence of 16S rRNAgene. Three endohpytic actinomycetes isolates has ben tested for the antimicrobial activities against multidrug resistant pathogens (bacteria and yeasts) by the cross streak technique.The extracellular extract crud of the isolates (S5) MT974060 (Streptomycets) recorded great zone of inhibition than other isolates (17.0, 11.5 and 10.0)mm*against Enterococcus faecalis*, *Bacillus subtilis* and *Klebsiella sp*. respectively. While (14.0, 12.0 and 18.0)mm against *E.colli*,*Proteus sp* and *Pseudomonas sp*. respectively.From the results, it was suggested that use of these isolates to produce new antibiotics against pathogenic bacteria, fungi and other pathogens.
Keywords: Endophytic actinomycetes,extracellular crud , AL Islah marshes, Antibacterial activity,Thi-Qar-Iraq .

INTRODUCTION
Actinomycetes are an ubiquitous group of prokaryotes widely distributed in natural ecosystem and a variety of manmade environments (Ningthoujam,*et al*., 2009). The term actinomycetes was derived from the Greek words “atkis” (a ray) and “mykes” (fungus) (Das et al., 2008).They have a high guanine (G) and cytosine (C) ratio in their DNA (>55mol %) (Goodfellow and Williams, 1983). Marshes environments were recently found to be one of the important sources for the isolation of new endophytic actinomycetes (EA) with potentiality to produce chemically diverse compounds with a wide range of biological activities (Bredholt *et al.*, 2008).Algae are the undisputed primary producers in the aquatic ecosystem and contribute approximately half of the global net primary productivity (Field *et al*., 1998). Several studies show that heterotrophic bacteria play a ubiquitous role in algal growth and survival (Seyedsayamdost *et al.*, 2011; Amin *et al*., 2015).The EA which are associated with plants also play important role in protection of their host from phytopathogenic invasions (Crawford *et al*.,1993).
It has been found also that some endophytic microorganisms can produce valuable pharmaceutical substances of biotechnological interest (Strobel *et al*., 1998).The EA may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicines (Strobel *et al*., 2004). The production of bioactive substances by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to better adapt to plant host and carry out some functions such as protection

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from pathogens, insects, and grazing animals. Endophytes are chemical synthesizer inside plants, in other words, they play a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms (Owen *et al*., 2004).

MATERIALS AND METHODS

Areas of the Study

A study area of this project has covered three different location site of water marshes in Thi-Qar Provence southern of Iraq (Al-Islah , Al-Chibayish and AL-fohood). This area is located at the east of the Nasiriyah city and northwest of Basra province, southern of Iraq (Figure 1) .

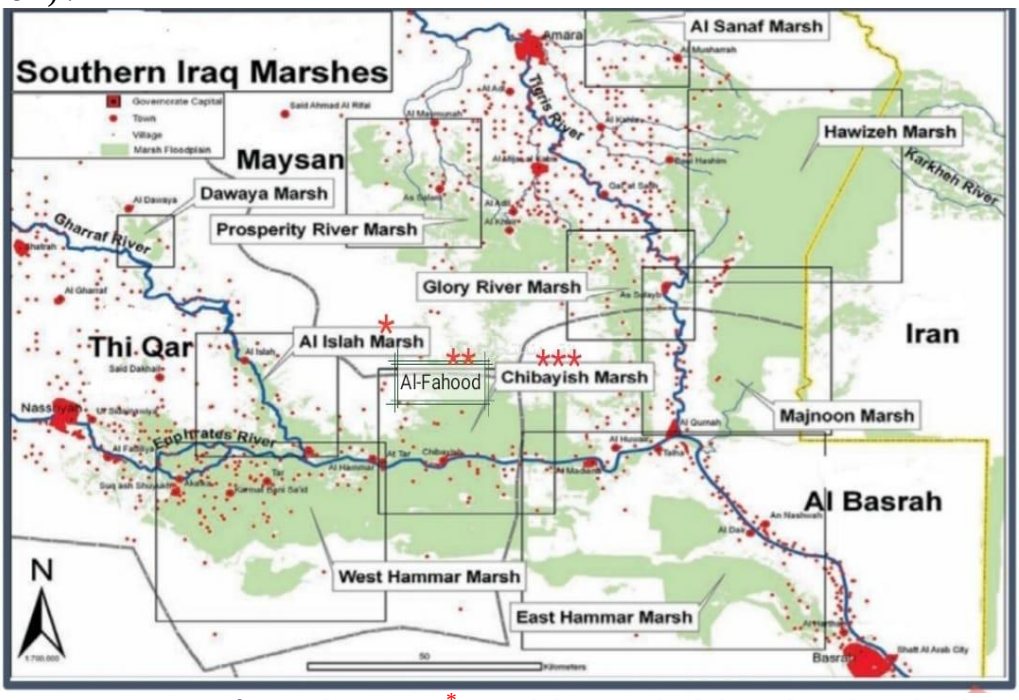

Figure 1: Location of Marshes area (*AL Islah, ***Al Chibayish and**Al Fahood) on the Iraqi map and general subdivision (UNEP 2010).

Collection of samples

Locally Algae samples were collected randomly from water marshes of some areas in Thi-Qar Provence Al Islah, Al Chibayish and Al Fahood (south of Iraq). sealed with sterile plastic cover and kept in ice box, The sample was completely washed with tap water and followed by distilled water for removal of the free floating organisms. Surface sterilization of 70% ethanol was used for removal of epiphytes.Then transferred to microbiology Laboratory in Biology Department/ College of Science in Thi-Qar University.

Isolation of endophytic actinomycetes (EA)

The endophytic actinomycetes was isolated from surface sterilization method was performed by following procedure (Kusari *et al*., 2013;Rajivgandhi *et al*.,2018). The algal samples were thoroughly washed with tap water, and small fragments of leaves, twigs, and buds of approximately 10mm (length) were cut aseptically by using sterile scale pale. Then, the small fragments were surface sterilized by 70% ethanol for 1 min and followed by 1.3M sodium hypochlorite (3-5% available chlorine) for 3min, and 70% ethanol for 30s. Finally, these surface-sterilized tissue pieces were rinsed thoroughly in sterile, double-distilled water for 2min, to remove excess surface sterilants. The excess moisture content was wiped by sterile cotton. The surface-sterilized tissue fragments, thus obtained, were evenly placed (four

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fragments in each plate) in starch casein agar medium (SCA) incorporated with streptomycin (100mg/l) to remove any bacterial growth. All the plates were incubated at 28 °C for 6-7days. To ensure proper surface sterilization and isolation of endophytes, unsterilized tissue fragments (only washed thoroughly in water) were prepared simultaneously, placed in SCA, and incubated under the same conditions in parallel, to isolate the surface-contaminating organisms (differentiated morphologically by both macroscopic and microscopic evaluation) (Rajivgandhi *et al*.,2018). The cultures were monitored daily to observe the growth of endophytic actinomycetes. The endophytic organisms, which grew out from the sample segments over 4-6 weeks were isolated and subcultured onto aactinomycetes isolation agar (AIA) and got into pure culture. To perform proper surface sterilization, surfacesterilized tissue fragments were imprinted simultaneously in SCA and AIA and incubated under the same conditions in parallel (Fouda *et al*., 2015).

Phenotypic characterization

The morphology and biochemical observation of isolated colonies are important for taxonomy of EA Gram staining, biochemical characterization, aerial mass color, melanoid pigments, spore chain morphology and some minerals such as carbon, nitrogen sources (Jagan mohan *et al*., 2013). were performed to determine the taxonomy of actinomycetes.

Biochemical characterization and Gram stain of (EA)

The potent EA were characterized by morphological methods consist of macroscopic and microscopic methods,Gram's staining: Crystal violet, gram's iodine, 95% ethyl alcohol and safranin were used in Gram staining. (Shrivastava *et al*.,2015). The mycelium structure, color and arrangement of conidiophores and arthrospore on the mycelium were observed through the oil immersion (100X). The observed structure was compared with Berge's manual of determinative Bacteriology and the organism was identified. Various biochemical tests performed for the identification of potent isolates are as follows: Starch hydrolysis, fermentation of citrate, nitrate reduction and IMVI C tests.

Molecular identification

Thirteen selected isolates were identified using PCR amplification of 16S RNA gene. The genomic DNA used for the PCR was prepared from the single colonies grown in the Actinomycetes isolation broth (AIB) or 7 days. Molecular identification of the potent isolates has been performed by extracting the genomic DNA using a standard bead beating method (Stephen *et al*., 1996). After analyzing qualitatively and quantitatively via a spectrophotometer, the extracted DNA has been separated via running an electrophoresis on a 0.8% (w/v) agarose gel. The 16S RNA gene was then PCR amplified using universal bacterial 16S RNA the gene fragment was amplified using the following primers, 27F (5'AGAGTTTGATCCTGGCTCAG-3')and (5'- GGTTACCTTGTTACGACTT- 3') (Mohseni *et al*., 2018) .

The thermo cycler was programmed to denature the molecule at 95 °C for the first 5 min followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C, and 2 min at 72 °C with a final extension step for 5 min at 72 °C. The PCR products were purified using a GeneJet PCR purification kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. Purified products were sequenced by G-spin DNA extraction kit (intron biotechnology/Korea).

Primary screening of antimicrobial activity of actinomycetes isolates

The isolated actinomycetes species were subjected to primaryscreening by cross streak method (Oskay,2009).Endophytic actinomycees isolation agar(AIA) plates were prepared, inoculated with the EA isolatesby a single streak in the center and incubated at 28 °C for 10–14 days(Kumar, 2014). Subsequently, the plates were inoculated with the human pathogensby a single streak at 90° angles and the plates were incubatedovernight at 37 °C for bacteria. Remarkably, Staphylococcus aureus,Bacillus circulans, Bacillus subtilis,

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Pseudomonas aeruginosa, and Escherichiacoli were used as test microorganisms(Kumar *et al*., 2014; (Salehghamari *et al.*, 2015). Further,the subculturedtest organisms were streaked perpendicular to the actinomycetesisolates. Afterwards, the plates were incubated for 24 h at 30 °C forbacteria. Eventually, the zone of inhibition was measured and recordedafter incubation.

Extraction of crude extracts

Based on the primary screening, the most potent isolates were selected and grown in actinomycetes isolation broth (AIB) as a production medium for the extraction of crude compounds. Besides, the active strains were inoculated individually in AIB and incubated for 5–7 days in a shaker incubator at 28 °C(Ahmed, 2007).

Subsequently, the broth with EA was centrifuged for 20 min at 10,000 rpm and the supernatant collected was mixed with ethyl acetate. Afterwards, ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and incubated in shaker for 1hr for complete extraction (Ahsan *et al*., 2017). After incubation, the bioactive compounds were separated from the aqueous phase. Eventually, the extracted crude compounds were dried to get dry powder by using heating mantle at 40 °C.

Secondary screening

Three active isolates (S5) MT974160, (S6) MT974161 and (S7) MT974162were inoculated on fermentation broth medium according to after incubation for 10-15 days at 28°C, the cultures were filtered by Waksman No.1 filter and the antimicrobial agent extracted using organic solvent Ethyl acetate (V:V), then tested for its inhibitory activity by agar well diffusion method (Ahmed, 2007).

Solvent Extraction of Extracellular Antimicrobial Metabolites

The supernatant obtained from fermented broth, after filtering it by Whatman No.1 filter paper, was used to extract antimicrobial metabolites by using different solvents methanol, ethyl acetate, chloroform. In order to achieve the maximum ability of solvents to dissolve the antimicrobial metabolites and determine their antimicrobial activities by agar well diffusion protocol. 1ml of extracellular crude extract (supernatant) is to be added after filtering through Whatman No. 1 into a test tube and mixed with 1ml of the above mentioned solvents gently shacked in a shaking plate for 60 minutes, and then spun at 10000 rpm for 10 minutes. After centrifugation, the mixture was separated into two phases: the upper phase containing the dissolved antimicrobial metabolite was collected by micropipette from test tubes into the sterilized petridish and the plates were kept in hot air oven at 45± 2°C. After the solvents completely vaporized the remaining residues was resuspended in 500 ul of the sterilized distal water. This mixture was treated as a solvent extracellular extract for detecting antimicrobial activity, by agar well diffusion procedure mentioned previously against the reference strains, (Hemashenpagam, 2011).

Intracellular Extraction of Antimicrobial Metabolites

After centrifugation separated the extracellular crude extracts, intracellular extracts were obtained. A bacterial cells pellet with its intracellular antimicrobial metabolites was found in the tube's residual. These components were used to determine the intracellular antimicrobial activity by agar well diffusion as follows: the pelleted cells were re-suspended in the test tube containing lysis bufer 1ml TE buffer "Tris 200ml and 50ml EDTA, 60 ul of 10% SDS and 6 ul of proteinase K, with a gently shaking, the mixture were incubated at 37°C for 60 minutes. That caused bacterial cell walls disruption as well as intracellular metabolites liberation. Six hundred µl of the intracellular crude metabolites was taken and mixed with 600 µl of methanol. The mixture was gently mixed and left for 60 minutes.

Then the tubes were spun at 1000 rpm for 10 minutes at room temperature. The mixture was separated into two phases, the upper phase methanolic phase containing dissolved metabolites, was collected and transferred to the sterilized petridish, then kept in a hot air

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oven 45°C for 24 hrs to dry the dissolved intracellular crude extract. Finally, the dried I ntracellular crude extracts were dissolved in double volume of sterilized distal water 1200ul. Agar well diffusion procedures as mentioned previously were used to determine the antimicrobial activity against tested microbial pathogens, (Khan and Patel, 2011).

Test organisms

The multidrug resistant strains (MDR) bacterial pathogens were obtained from different hospitals in Al-Nasiriyah city for used in this study, *Enterococcus faecalis*, *Bacillus sp*, *Klebsiella sp* and *S. aureus* and that is *Escherichia coli*, *Proteus sp*, *Citrobacter sp*, *Pseudomonas sp* and *Candida albicans* as model of yeast . The collected uropathogens were maintained in glycerol stock and stored at(-20°C) (Rajivgandhi *et al*., 2016). The Marshes EA strains were identified and isolated from local algae samples then, screened for their antimicrobial activities (Gurung *et al*., 2009) .

RESULTS

Isolation and identification of marshes endophytic actinomycetes (EA)

Interestingly, a total of fifty young healthy green algae samples were collected from three different areas of Thi-Qar marshes province , this samples distributed as follows: 19(38%) samples collected from Al-Islah marshes, 16(32%) from Al-Fahood marshes and 15(30%) from Al-Chibayish marsh the data presented in (Figures 2 andTable: 1).

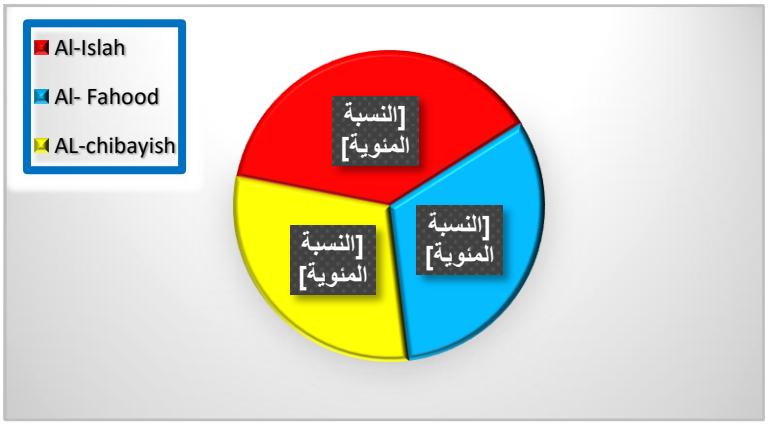

Figure 2: Percentages of samples distributed depending on their locations .

algae samples cultured on a special medium (Chu 10 Medium and BG-11 Medium) then subculture on Starch Casein Agar (SCA) and Actinomycetes Isolation Agar (AIA) medium for Marshes Endophytic Actinomycetes (EA) cultivation. Invalidation, no microbial colonies were observed in the Starch casein agar (SCA), actinomycetes isolation agar (AIA), M-protein and Nutrient agar (N.A) medium plates and the result noticed that the sterilization was good. After validation, 13 locally algae samples (26%) were suspected EA growth pure, fine, ribbons like powdery white color colonies, the distribution of these isolates illustrated in (Table 1).

Table (1): Distribution of EA samples according to their location.

The area	No. of isolates	(%)	P. value
Al-Aslah	**4**	**30.7 %**	**0.926**
AL-Fahood	**5**	**38.4 %**	

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AL-Chibayish	**4**	**30.7 %**	
Total	**13**	**100 %**	
χ^2 = 0.154 df=2			

These active strains were chosen for further study for the production of bioactive compounds as in (Figure 3).

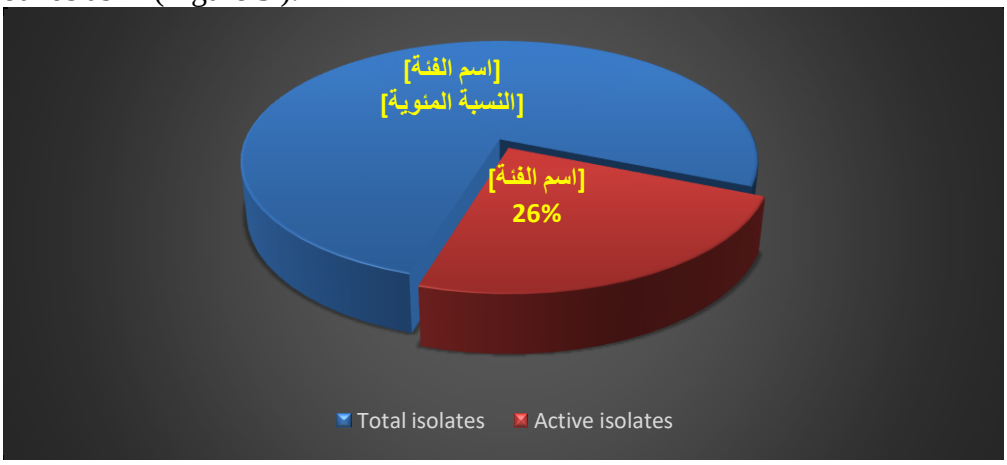

Figure 3: percentage of active marshes Endophytic Actinomycetes isolates

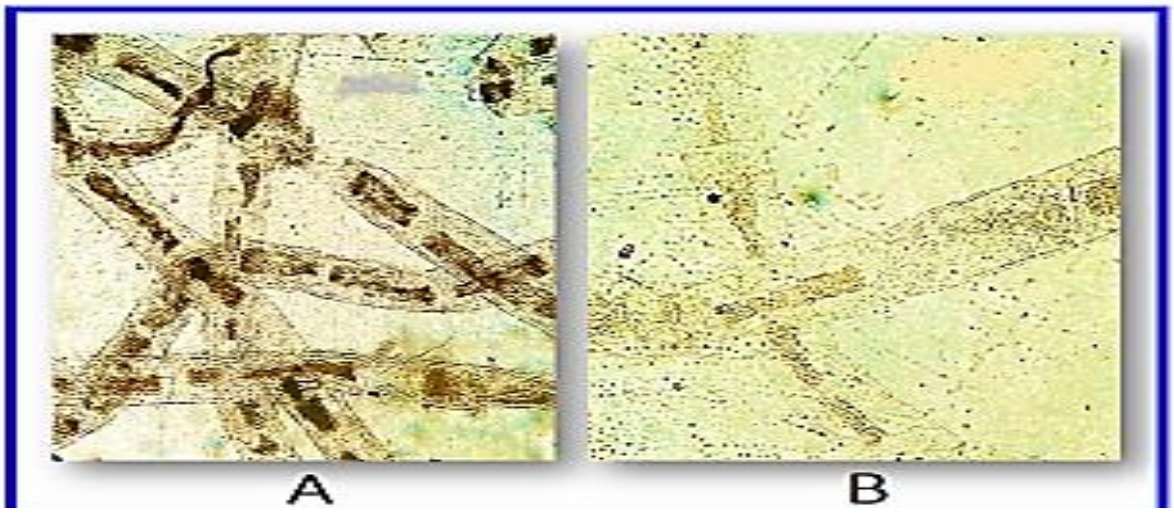

Figure 4: Collection of local brown Algae from the marshes water . (1) *Chladophora* sp. under the microscope (40X)

(2) *Ulothrix*sp under the microscope (40X)

Isolation ofendophytic actinomycetes (EA)

The curent study showed that ther are four more active isolatse of EA bacteria (S5) MT974160, (S6) MT974161, (S7) MT974162 and (S8) MT974163 , should be emphasized that all the strains were pure, fine powdery ribbons white or yellow color colonies with white or yellow Arial mycelium. All the isolates were identification on the bases of microscopy culture media, biochemical examinations (Figure 5 and Table 2).

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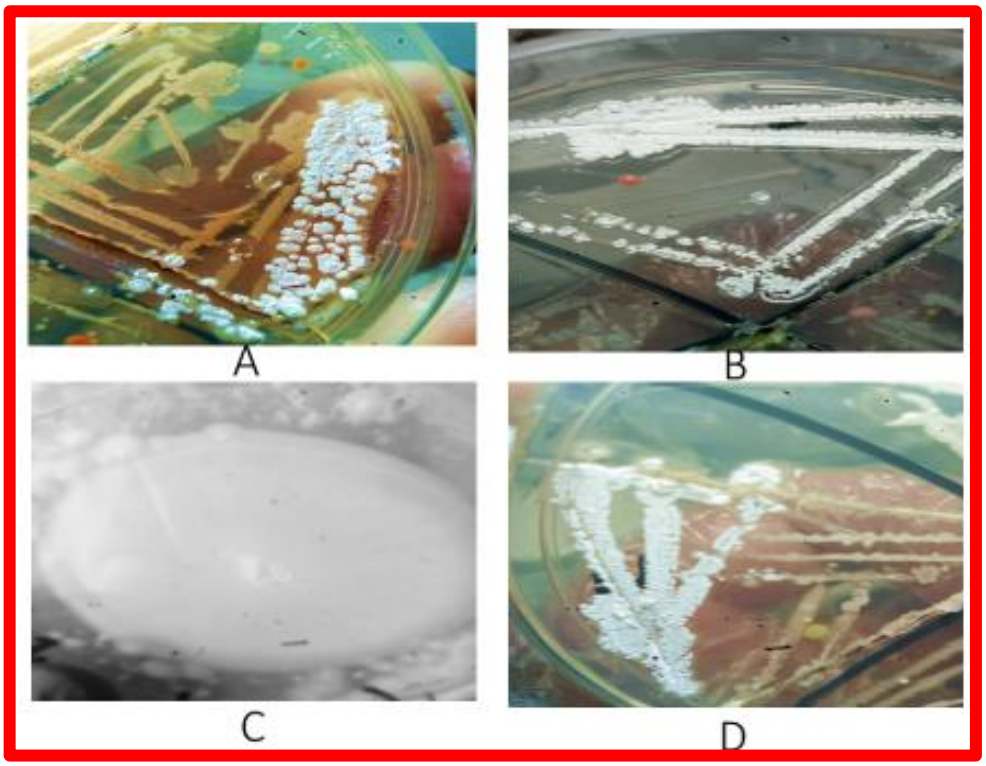

Figure 5: isolation and identification of Marshes Endophytic actinomycetes Streptomycets sp. on different media plates (A: Actinomycetes Isolation Agar (AIA), (B:M-protein agar, (C: Starch casein agar (SCA), (D: Nutrient agar (NA).

Table (2): Morphological and physiological characteristics of EA*Streptomycets* sp. isolates on different media.

Media	**(S5) MT974160**		**(S6) MT974161**		**(S7) MT974162**		**(S8) MT974163**	
	Aerial mycelium	Substrate mycelium	Aerial mycelium	Substrate mycelium	Aerial mycelium	Substrate mycelium	Aerial mycelium	Substrate mycelium
Starch casein agar	White	White	White	White	White	White	White	White
Actinomycete isolation agar	White	White	White	Yellow	White	Yellow	White	Yellow
Nutrient agar	White	Yellow	White	Yellow	White	Yellow	White	White
M-protein agar	White	White	White	White	White	White	White	White

Microscopic and biochemical studies

The biochemical tests are used in the current study included IMViC test, Catalase , Oxidase, H_2S production test, Ureas and fermentation of citrate for the identification of the potent isolates, but it was unable to identify the EA up to species level. This isolates were Gram positive filaments and break into coccid which examined by cover slip method and gram stain procedure, It performing the Gram staining test, it showed purple color indicating the positive result. The data summarized in (Table 3) included morphological and biochemical examinations of four active isolates as well as showed the Indol test was

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positive for (S6) MT974161 and (S7) MT974162, while it was negative for (S5) MT974160 and (S8) MT974162. The oxidase test was positive for all isolates nevertheless , the catalase test, Ureas test and H_2S test were negative for all isolates. Methyl Red test was negative for (S5) MT974160, (S6) MT974161 and (S8) MT974163 However, (S7) MT974162 was positive. Voges-proskauer test has been negative for all isolates except (S1) MT974160. Citrate test all isolates can be utilize citrate except (S8) MT974163. Urease test used to check the ability of bacteria to produce an exoenzyme, called urease, all four active isolates were urease negative.

Table (3): Biochemical tests for EA sp. isolates.

Test	(S5) MT974160	(S6) MT974161	(S7) MT974162	(S8) MT974163
Gram stain	+	+	+	+
Oxidas	+	+	+	+
Catalase	—	—	—	—
H_2S production	K/K No gas	K/K No gas	K/K No gas	K/K No gas
Indole	—	+	+	—
Methyl red	—	—	+	—
Voges - proskauer	+	—	—	—
Citrate	+	+	+	—
Urea	—	—	—	—

'+' indicates positive result of the test. '--' indicates the negative result of the test S= sample .

Molecular identification

Identification Endophytic Actinomycetes *Streptomycets* sp. by using 16S rRNA gene. One and a half µl of genomic DNA was used for each PCR reaction. A conventional PCR protocol was used to analyze simultaneously the presence of 16S rRNA gene. The presence of the 16S rRNA gene was identified by 1250 bp, as shown in (Figure 6). The 16S rRNA sequence (1250 bp) of the strains has got the confirmed the identification of the strain at species level.

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Figure 6: Agarose gel electrophoresis for *16S rRNA* gene (1250bp). Bands were fractionated by electrophoresis on a 2% agarose gel (2 h., 5V/cm, 1X TBE) and visualized under U.V. light after staining with red stain. Lane: 1 (M: 100bp ladder).

Primary screening for antagonistic activity

A total of thirteen locally isolated EA that obtained from fifty algae sources were tested for the antimicrobial activities against some of multidrug resistant pathogens (bacteria and yeasts) by the cross streak technique *Enterococcus faecalis*, *Bacillus sp*, S. aureus *Escherichia coli*, *Proteus sp*, *Pseudomonas sp*, *Klebsiella sp*. and *Candida albicans* as model of yeast as showed in (Table 4).

Primary screening showed that 8 strains were able to produce antibiotics, Out of those, 3 strains (S5) MT974160, (S6) MT974161 and (S7) MT974162 had great potential for antibiotic production with large zone of inhibition the activity of each were done against each microbial pathogen Interesting, (S5) MT974160 exhibit excellent inhibitory activity against most the tested pathogenic bacteria than other strains. The data presented in (Figure 7) were applied to conclude the antimicrobial activity.

The data in (Table 5) summarized the details of primary screening results of the locally isolated EA activities against the tested microbial pathogens, which were selected to complete the secondary screening program . However, (S5) MT974160 exhibit excellent inhibitory activity against most the tested pathogenic bacteria than other strains . To achieve this potential, all isolates were streaked as a straight line on actinomyceteisolation agar (AIA) media separately, and incubated at 28 °Cfor 7 days. After completing their growth, different strains of microbial pathogens were streaked at right angle, without touching each other, in addition to being incubated at 37 °C for 24 hours.

Table (4): Thirteen EAproduce antibacterial agents which are active against some pathogenic bacteria by cross-streaking method.

Isolates	**Gram positive bacteria (mm)**			**Gram negative bacteria (mm)**				
	Entero. faecalis	***Bacill. subtilis***	***S.aureus***	***E. coli***	***Proteus***	***Pseudo-monas***	***Klebsiella***	***C.albecans***
(S1) MT974156	**+ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**-ve**	**-ve**
(S2) MT974157	**+ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**
(S3) MT974158	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**	**-ve**	**-ve**
(S4) MT974159	**-ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**
(S5) MT974160	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
(S6) MT974161	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**

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(S7) MT974162	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**+ve**	**+ve**	**-ve**
(S8) MT974163	**+ve**	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**-ve**	**-ve**
(S9) MT974164	**-ve**	**-ve**	**-ve**	**+ve**	**-ve**	**+ve**	**+ve**	**-ve**
(S10) MT974165	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**
(S11) MT974166	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**-ve**
(S12) MT974167	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**	**+ve**	**+ve**	**-ve**
(S13) MT974168	**+ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**	**-ve**

Table (5):

No. Sample	Strain	Antagonistic activity
5	MT974160	excellent activity
6	MT974161	Good activity
7	MT97162	moderate activity

Identification of marshes endophytic actinomycetes (EA).

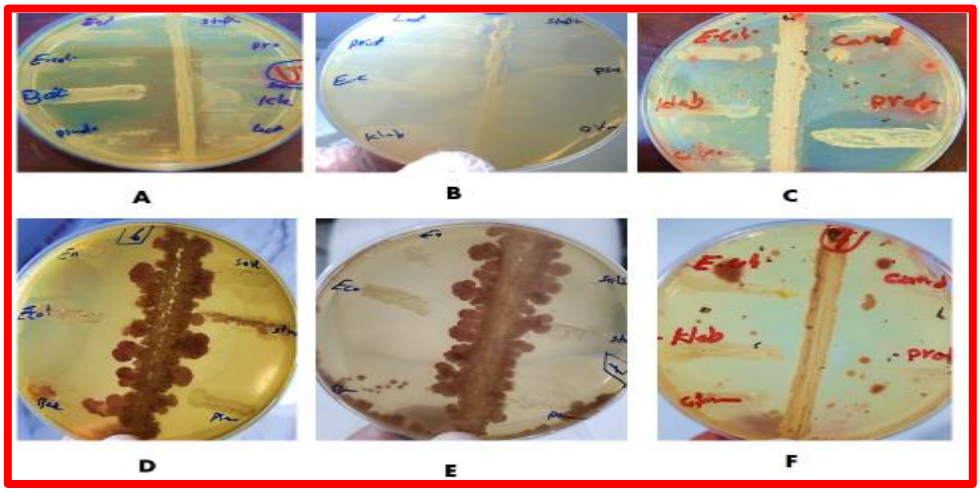

Figure 7: Streak-plating technique to screen the antibacterial activity of isolated Streptomyces, plate A, B for isolate(S5) MT974160 a highly active strain , Plate C, D for isolate (S6) MT974161 a moderately active strain, while E, F for isolate (S7) MT974162 a low active strain the test organism *Enterococcus. faecalis*(1), *Bacillus subtilis* (2), *staphylococcus auraus* (3), *E.coli* (4), *Proteus* (5), *Pseudomonas* (6),*Klebsiella sp*. (7), *candida albecans*(8), *Salmonella typhi***i.** (9), *citrobacter sp***.**

Secondary Screening Methods

Locally isolated EA successfully prevented or inhibited the growth of tested human microbial pathogens *Enterococcus faecalis*, *Bacillus subtilis*, *Staphylococcus aureus* and *Klebsiella sp*. *E. coli*, *proteus sp.*, *pseudomonas aeruginosa* and *candida albicans* which collected from patients in the hospitals of AL-Nasiriyah city by secondary screening programs (S5) MT974160, (S6) MT974161 and (S7) MT97162 as presented in (Table 5). They were selected to perform the secondary screening programs in order to evaluate several parameters best media composition for obtaining maximum antimicrobial compounds, best organic solvents for extracting secondary metabolites , extraction of intra

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and extracellular antimicrobial compounds, comparison between synthetic antibiotic discs with extracellular crude extract. pressively, 3 EA strains showed strong antimicrobial activity as confirmed by primary screening. Furthermore , the isolated strains of EA were tested for the antimicrobial activity against various bacterial human pathogens which showed various range of activities.

The isolate (S5) MT974160 showed excellent activity of inhibition zone against human pathogenic bacteria, Gram positive and Gram negative but there was no activity against candida albecans, the results recorded that zone of inhibition was (17.0 , 11.5 and 10.0)mm *Enterococcus faecalis*, *Bacillus subtilis* and *Klebsiella sp.* respectively . While against Gram-ve bacteria, was (14.0, 12.0 and 18.0)mm *E.colli*,*Proteus sp* and *Pseudomonas sp.* respectively, at the same time did not exhibit any activity against G+Ve bacteria, *Staphylococcus aureus*,as showed in (Table 6) and (Figure 8). followed by the isolate (S6) MT974161 that showed moderate activity of inhibition zone against human pathogenic bacteria and yeas hence, showed mean zone of inhibition (15.0, 0 and 10.0) mm against G+ve bacteria, *Enterococcus faecalis*, *Bacillus subtilis* and *Klebsiella sp*. respectively . while, when it tested against G-ve bacteria showed mean zone of inhibition(15.0, 7.0, 11.0) mm against *E. colli*,*Proteus sp.* and *Pseudomonas sp*. respectively, while there was no inhibition zone against *candida sp*. (Table 6).

Table (6): Antimicrobial activities of locally isolated EA (extracellular crude alone), fermented on AIB.

0= no inhibition zone

Isolates	Gram positive bacteria hibition zone (mm)				Gram negative bacteria inhibition zone (mm)			Yeast zone(mm)
	En.fae.	*B.sub.*	*St.aur*	*Kleb.*	*E.coli*	*Prote.*	*Pseud*	*Candida*
(S5)MT974160	17.0	11.0	0	10.0	14.0	12.0	18.0	0
MT974161(S6)	15.0	0	0	0	15.0	10.0	11.0	0
(S5) MT974162	0	0	0	0	12.0	8.0	0	0

Table (7): Antimicrobial activities of locally isolated E A (intracellularcrude alone), fermented on AIB .

Isolates	Gram positive bacteria inhibition zone (mm)				Gram negative bacteria inhibition zone (mm)			Yeast inhibition zone (mm)
	En.fae.	*B.sub*	*St.aur*	*Kleb.*	*E.coli*	*Prote.*	*Pseud.*	*Candida*
(S5) MT974160	19.0	13.0	0	0	16.0	11.0	19.0	0
(S6) MT974161	14.0	8.0	0	0	13.0	0	13.0	0
(S7) MT974162	0	0	0	0	14.0	10.0	0	0

0= no inhibition zone

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The data in (Table 7) summarize the antimicrobial activitiesof the intracellular extract against reference G+*ve* human pathogenic bacteria *Enterococcus faecalis, Bacillus subtilis, Staphylococcus aureus* and *Klebsiella sp.* andG-*ve bacteria Escherichia coli, protus sp. and pseudomonas sp.* and *Candida albicans* as yeast model.

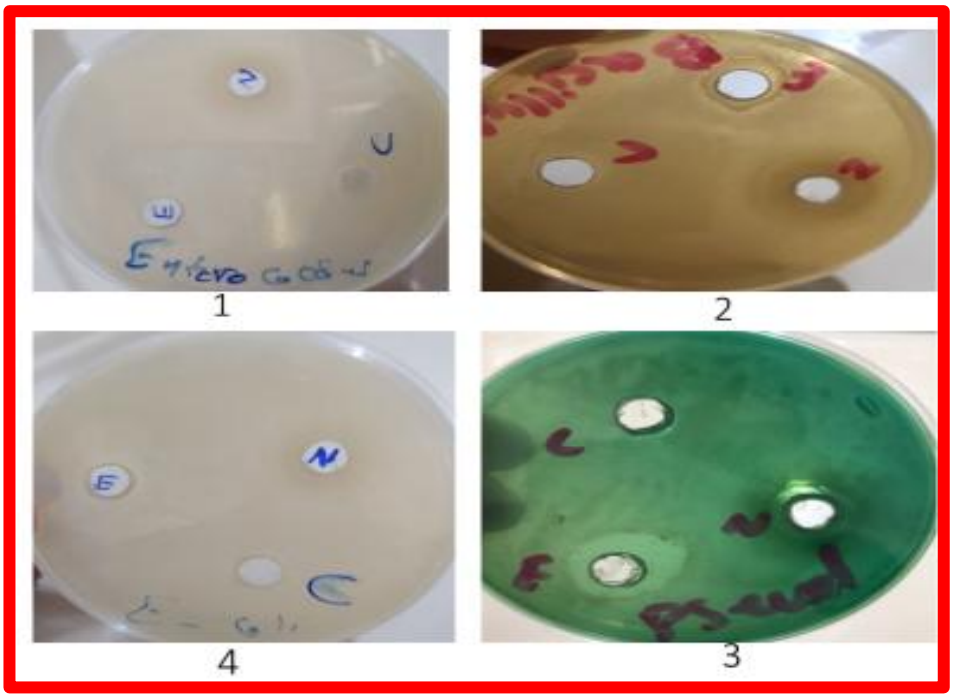

Figure 8: Secondary screening program for isolated actinomycetes against human pathogens, extracellular crude extract (E), Intracellular crud extract (N) activities against and (C) negative controls.
(1) Enterococufaecalais (2) *Bacillus subtilis*
(3) *Escherichia coli* (4) *Pseudomonas sp*.

DISCUSSION

Isolation and identification of Marhses Endophytic Actinomycetes (EA).

The current study showed that out of thirteen isolates, only four showed excellent growth on a Starch casein agar (SCA) Interestingly, this isolates were isolated based on the Gram staining and colony morphology. All the isolates were Gram +ve with different morphological structures, this in agreement with that described by Saadoun *et al.* (2015). Ramazani *et al.* (2013) described EA colonies being slow growing, glabrous or chalky, aerobic, piled, as well as with the different color of aerial and substrate mycelium. This study are in accordance with the earliest finding of Passari *et al*. (2016) reported that the hypochlorite was the better choice for surface sterilization to algae, plant derived E A. The study was in agreement with the finding of Portillo *et al.* (2009) concerning the isolation process that each plate was often contained one or few colony types ranging from two to four colonies, and from similar habitats the EA diversity exhibited few different colony types. A similar report of branched, filamentous and microspore chain colonies with smooth spore surface morphology has been reported by Ullah,*et al*. (2012). The aerial mass of the strains were white color powdery growth in almost, and few strains were showed whitest grey color, these results were correlated with their finding (Sathiyaseelan and Stella, 2011, Ravikumar *et al*. 2011).

Actinomycetes isolation

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four isolates (S5) MT974060, (S6) MT974061, (S7) MT974062, and (S8) MT974063 were indicated as (EA)and confirmed, this agrees with result of (Hayakawa and Nonomura 1987) which mention that a number of selective media were developed for isolation of actinomycetes, SCA and AIA were selected, because in this medium the development of bacterial and fungal colony was very much suppressed, allowing only the actinomycetes to grow. Rashad *et al*. (2015) reported that EA bacteria can be isolated from algae that spread in the fresh water or the ocean because 70% of the earth surface are made of marine environment and the ocean has an unexploited source for many potential drugs and secondary metabolites .Rajivgandhi *et al*. (2018) mentioned that the EA can be used in the medical field and treat some diseases, because, the algae derived marine E.A has better inhibitory activity against MDRS pathogens.

Microscopic and biochemical studies

The morphological method consists of macroscopic and microscopic characterization. Microscopic characteristics using light microscopy and Gram stain properties were also performed and observed that the spore chains under light microscope, showed various spore characters like straight or flexuous chains, extended, long and open coils according to the method described previously Kumar *et al*. (2011). Gram staining was used to study EA cell morphology and spore chain morphology was examined with a light microscope (Nolan *et al*., 1988).Most the EA contain colonies of whites gray or black powdery surface whereas bacterial colonies are of different colors shiny surface and gummy appearance. Generally bacteria have colonies can be observed without microscope and have a distinct hollow with a darker interior while bacteria do not. Actinomycetes colonies are firmer than bacteria when picked with a sharp needle (Mohamed *et al*., 2014). The potent EA isolates selected from primary screening were characterized by morphological, biochemical and physiological methods. The observed morphology of the isolates was compared with the Actinobacteria morphology provided in Bergey's Manual for the presumptive identification of the isolates. Different biochemical tests like Citrate utilization test, Indole test, methyl red test, Vogus Proskauer test, catalase test were performed to characterize actinomycetes till genus level (Pallavi and Padma Singh, 2019).

Many biochemical tests were performed, to identify but it was unable to identify the *Streptomycets* up to species level due to the lack of other tests. Apart from proper identification of genera and species of *Streptomycets* besides morphological and physiological properties (Kuster, 1972). This study, used various biochemical tests such as IMViC test, Catalase , Oxidase, H_2S production test, Ureas and fermentation of citrate were performed for the identification of the potent isolates (Abbas, 2006).The indol test were positive for (S6) MT974161 and (S7) MT974162 , because, the bacteria can cleaving indole from tryptophan.There are many researchers found the same result such as (Kowsalya *et al*., 2017; Tsujibo, 2003), While in (S5) MT9760 and (S8) MT974163 was negative this find agree with result of (Vyawahare *et al*., 2013).

oxidase test was positive for all isolates because this bacteria use oxygen as a terminal electron acceptor in respiration and produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain, this contrast to (Vyawahare *et al*., 2013). However, catalase test was negative for all isolates this agree with finding of (Kowsalya *et al*., 2017). Some studies mention that the catalase test must be positive for EA bacteria (Vyawahare *et al*., 2013), most pathogenic bacteria are catalase-positive such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Campylobacter jejuni*, that make catalase to deactivate the peroxide radicals, thus allowing them to survive unharmed within the host(Srinivasa. *et al*., 2003). H_2S test was negative for all isolates, many previous studies reported same this finding (Williams, 1989 and Mincer *et al*., 2002). Methyl Red test: was negative for (S5) MT974160, (S6) MT974161, (S8) MT974163, except (S7)

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Received 25 April 2021; Accepted 08 May 2021.

MT974162 it was positive, this finding agreement with (Al-Saadi, and Noora, 2013) While some studies showed the opposite (Vyawahare *et al*., 2013). Voges-Proskauer test: was negative for all isolates except (S5) MT974160 this finding agree with (Vyawahare *et al*., 2013).

Citrate test all isolates can be utilize citrate except (S8) MT974163. this meaning, the bacteria metabolized citrate and produced an acid end product, While in (S8) MT974163 negative because, the bacteria cannot use citrate as sole source of carbon and energy, many previous study agree with this result (Kowsalya *et al*., 2017). Urease test check the ability of bacteria to produce an exoenzyme, called urease, all four active isolates were urease negative that meaning it cannot urease production, this finding agree with (Fatim**a***et al.*, 2017).

Molecular identification

many previous studies use molecular identification method for characterization and identification of EA bacteria that including Polymerase chain reaction technique (PCR) , the 16S rRNA sequence (1250 bp) of the strains has got the confirmed the identification of the strain at species level. previous studies confirmed that characterization of the molecular level was established by 16S rRNA sequencing using sequence results (Farris and Oslon, 2007).

Primary screening for antagonistic activity

All the bioassays for antibacterial and antifungal activity were carried out in actinomycet isolation agar (AIA) plates, because this media very specific for the isolation of EA, as only organisms (actinomycetes) those are capable of degrading the polymers in the media are able to grow.Previous studies mentioned that if the microbial pathogens were susceptible to the antimicrobial compound produced by the isolated actinomycetes; it would not allow their growth near the actinomycetes Rana and Salam (2014). The process was done for all 13 locally isolated EA, instead, most of the isolates had a power against the tested pathogens, but our designed protocol concentrated on choosing the most active and broad spectrum activity. Only 3 strains (S5) MT974160, (S6) MT974161 and (S7) MT974162 has been showed potential for antibiotic production with large zone of inhibition the activity of each were done against each microbial pathogen Interesting, the strain (S5) MT974160 showed excellent inhibitory activity against most the tested pathogenic bacteria than other strains.

For this, the results by positive and negative result (+ve and -ve) indicated capability or incapability to prevent the growth of the tested microbial pathogens respectively, so that most of the isolates were ignored because of their incapability to prevents the growth of microbial pathogens. Finally, the isolates achieved a high activity to produce a large amount of antimicrobial compounds as secondary metabolites which prevent the growth of tested microbial pathogens. The study of Ravi *et al.* (2015) investigated that screening for microorganisms belonging to the EA family, revealed from the soil sources, can serve as an important source for obtaining novel antimicrobial compounds and for finding new chemicals used for achieving new therapeutic agents. Many studies conducted for produce antibiotic from aquatic sources, a study by Rajan and Kannabiran, (2014) mentioned the EA was frequently identified from various marine sources and it produced new classes of antibiotics against various clinical disorders this, encouraged us to use local algae from water marshes as sources to produce the antibiotic from the Streptomyces bacteria. The differences in results between primary and secondary screening among suspected EA colonies may be due to appearances of EA as fragmenting mycelia and filamentous mycelia when growing in liquid media and solid media respectively, in addition to showing the difference in the morphology of EA as described by Bushell (1993).

Moreover, Reza Dehnad *et al.* (2010) and Ramazani *et al.* (2013) also investigated that some EA species were producing antimicrobial metabolites on agar media and primary screening but the field in liquid media and secondary screening meant that flask shaking

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 9795 - 9817
Received 25 April 2021; Accepted 08 May 2021.

methods obtained the fewest amount of antimicrobial because of the significantly breakage of the hyphal component of some isolated EA occurring in case of liquid culture rather than on agar media in plates. Metabolites bearing antibiotic activity can be defined as low molecular weight organic natural substances made by microorganisms that are active at low concentrations against other microorganisms (Guo *et al*., 2008).

Marshes Endophytic Actinomycets were believed to carry out a resistance mechanism to overcome pathogenic invasion by producing secondary metabolites (Tan and Zou,2001). So far, studies reported a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids (Yu *et al*., 2010). The discovery of novel antimicrobial metabolites from EA is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens, the insufficient number of effective antibiotics against diverse bacterial species, and few new antimicrobial agents in development, probably due to relatively unfavorable returns on investment (Song, 2008). The antimicrobial compounds can be used not only as drugs by humankind but also as food preservatives in the control of food spoilage and food-borne diseases, a serious concern in the world food chain (Liu, 2008). Many bioactive compounds, including antifungal agents, have been isolated from the genus *Xylaria* residing in different plant hosts, such as "sordaricin" with antifungal activity against *Candida albicans;* "mellisol" and "1,8 dihydroxynaphthol 1-O-a-glucopyranoside" with activity against herpes simplex virus-type 1 "multiplolides A and B" with activity against *Candida albicans* (Pittayakhajonwut *et al*., 2005). The compound have wide-spectrum inhibitory activity against several microorganisms including *S. aureus, E. coli, S. typhia, S. typhimurium, S. enteritidis, A. hydrophila, Yersinia* sp, *V. anguillarum, Shigella* sp,*V. parahaemolyticus, C. albicans, P. expansum,* and *A. niger,* especially to*A. hydrophila*, andwas suggested to be used as natural preservative in food (Liu *et al*.,2008). Endophytic actinomycets bacteria are able to produce necessary enzymes for the colonization of plant tissues, and to use, at least *in vitro*, most plant nutrients and components (Firakova *et al*., 2007). Therefore, more recently, EA have received attention as biocatalysts in the chemical transformation of natural products and drugs, due to their ability to modify chemical structures with a high degree of stereo specificity and to produce known or novel enzymes that facilitates the production of compounds of interest.

Secondary Screening Methods

The current study exhibit that the strains (S5) MT974160, (S6) MT974161,(S7) MT974162 has been wide spectrum antibacterial activity against G+ve human pathogenic bacteria, thane G–ve, Bode *et al.* (2002) explained that the antimicrobial compounds secreted to the culture medium (fermented broth) can be easily extracted when using one of the most suitable organic solvents. The results of the optimal nutritional media showed that antibiotic production was higher in the medium containing glucose. The importance of glucose in the nutritional medium for the synthesis of a wide range of antibiotics by different EA *Streptomyces* species has been reported by many investigators (Singh *et al*., 2009). The current study about effectiveness of EA, particularly *Streptomyces* strains and their ability to production of antibiotic consisted with previous study of Thumar *et al*. (2010) which mention that during the course of screening of bioactive compounds for the isolation of new antibiotics each year, thousands of EA, particularly *Streptomyces* strains, are screened by pharmaceutical research laboratories as sources for novel antimicrobial compounds . All so agreement with study of Mariana *et al*., (2010) explained that thediscovery of novel antimicrobial metabolites from EA is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens .

Yu *et al*. (2010) reported that a large number of antimicrobial compounds isolated from EA, belonging to several structural classes like alkaloids, peptides, steroids, terpenoids,

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 9795 - 9817
Received 25 April 2021; Accepted 08 May 2021.

phenols, quinines, and flavonoids. secondary screening results clearly showed that (S5) MT974160 possessed high antimicrobial activity against most the tested human pathogens. Oskay *et al*, (2009) screened 50 actinomycetes isolated from soil against several human pathogens, It was found that 34% of strains produced antibiotics. Arasu *et al*, (2009) isolated a strain of *Streptomyces sp*. from soil sample and reported that it was very effective against *S. aureus, S. epidermidis, Xanthomonas sp.* and *C. albicans* at 0.25 mg/ml concentration. This study recorded that the isolate (S7) MT974162 did not exhibit any activity against G +ve pathogenic bacteria, while have strong activity against Gram -ve bacteria tested, the zone of inhibition 12.0 mm and 8.0 mm respectively, *E.colli, Proteus sp.* while there was no activity against *Pseudomonas sp*. This results may be attributed to this isolate of *Streptomyces* was capable of inhibiting the growth of Gram negative bacteria rather than Gram positive bacteria, even though it is not clear if one or more antimicrobial compounds are responsible for this activity. Results of this study are not consistent with finding of Singh*et al*, (2016) which mention that EA strains usually exhibit stronger antimicrobial activity against Gram-positive rather than Gram-negative bacteria. The results showed that the strains(S5) MT974160,(S6) (S6) MT974161, (S7) MT974162 have good antibacterial activity against candida albecans . The antagonistic potential of Actinomycetes in fungal test involved the production of extracellular hydrolytic enzymes and moreover Secondary antifungal compounds (Prapagdee *et al*., 2008).Antifungal compound has classified based on their mechanisms of action, including: covering inhibitors of the synthesis of cell wall components (glucan, chitin, and monoproteins), sphingolipid synthesis (serine palmitoyl transferase, ceramide synthase, inositol phosphoceramide synthase and fatty acid elongation) and protein synthesis (sordarins) (Vicente *et al*., 2002).

This finding agreement with study of Hozzein *et al.* (2011) which obtained result similar to our results from the isolated strain D332 which recorded 30mm zone of inhibition against *Candida albicans* .While contrast with result of Attimarad et *al.* (2012) which reported that the actinomycetes do not show any activity against *Candida albicans*. No zone of inhibition was observed in the control well. The result of this study was in agreement with finding of Abdul Wahab *et al.*(2015) who obtained 15mm zone of inhibition , and than the isolate strain of actinomycetes by Attimarad et *al.* (2012) which do not show any activity against *Candida albicans* , while Hozzein *et al.* (2011) obtained better results than our results from the isolated strain D 332 which recorded 30 mm zone of inhibition against *Candida albicans.*

Earlier several studies reported that most of the antimicrobial secondary metabolites were from extracellular actinomycetes (Berdy,2005). microorganisms by the actinomycetes species was highly specific, so this was selective, and not only depending on the strain and species of the isolated actinomycetes, but also the medium composition was used for growing it and the condition of the fermentation remained the powerful point for production of inhibitory substances. The isolates (S5)MT974160 and (S6) MT974161 showed the highest antimicrobial activities against *Enterococcus Facials and Bacillus subtilis*. all so showed excellent activity against *Escherichia coli, protus sp. and pseudomonas sp.* while the isolate (S7) MT974162 didn't showed any activity against G+ve bacteria while showed moderate activity against G-ve bacteria and *Candida albicans*. In general, the extracellular crude extracts exhibited comparatively better activity than intracellular crude extracts at the basis of their register positive cases against tested microbial pathogens, in which recording 12 and 11 cases for extracellular cellular extract alone and intracellular extract alone activities, respectively. Recently, Rajivgandhi *et al.*(2018) reported that algae derived marine EA DMS3 has better inhibitory activity against MDRs pathogen. The EA was frequently identified from various marine sources and it produced new classes of antibiotics against various clinical disorders(Gogineni *et al*., 2018). The supportive evidence ofZaitlin *et*

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 9795 - 9817
Received 25 April 2021; Accepted 08 May 2021.

al.2006 also evidenced, marine derived actinomycetes compound has highest inhibition against Gram negative bacteria and 23, 25, 19 and 24 mm zone of inhibition against *S. aureus*, *E. coli*, *P. aeruginosa* and *B. subtilis* were observed.

Best Organic Solvents for Extracting Extracellular Antimicrobial Metabolites

Extraction of bioactive compounds by ethyl acetate seems good enough because of observing a proper growth inhibition of indicators when disc diffusion method has been applied. Ethyl acetate can dissolve biomolecules with moderate polarity which can be used as drug in human body, all so, this solvent is not miscible with water, which results in easy separation of organic from water phase (Mohseni *et al*., 2013 ; Gebreyohannes *et al*., 2013). In several reports, ethyl acetate was mostly used as excellent an extraction solvent to isolate the crude extracts from EA isolates (Kavitha *et al*., 2010). Extracellular extract crud of the MT974160 isolate with ethyl acetate showed that highest zone of inhibition against *Enterococcus Faecalis* 20.0mm. All so highest of zone inhibition against *E.coli* was 18.0 mm . There are many results that support our results of which finding obtained by Abdul Wahab *et al.* (2015), who documented that ethyl acetate provided the best solvent for the extraction of antimicrobial metabolites followed by chloroform, while methanol solvents were unable to extract the antimicrobial metabolites.

Study conducted by Vijakumar *et al*. (2010)reported that the extraction with ethyl acetate gave the maximum antimicrobial activity against the tested microorganisms, while extracting with other solvents showed a moderate activity or no activity as antimicrobial compounds. Selvameenal *et al.* (2009) stated that the extracellular crude extract from fermented cultures was prepared for secondary screening protocol and when extracting their bioactive antimicrobial metabolites, different organic solvents were used on the basis of their differentiation in their polarity to organic solvents against antimicrobial metabolites presented in EA. Extracellular antimicrobial metabolites with ethyl acetate of the isolate (S6) MT974161 showed highest zone of inhibition against *Enterococcus Faecalis* 21.0 mm. All so the highest zone of inhibition against *E .coli* was 18.0 mm. this results indicated that extracellular extract of the isolate (S6) MT974161 had higher powerful activities against *Entero. faecalis* and Escherichia coli. than other pathogenic bacteria similar results were earlier obtained by Charoensopharat *et al*. (2008); Kekuda *et al*. (2012) and Lertcanawanichakul *et al*. (2015).This was in disagreement with finding of Khan and Patel (2011) who recorded that chloroform was the best solvent for extracting the extracellular metabolites than other organic solvents . Results of this study were most accordance with earliest study of Gorajana *et al*. (2007) reported that ethyl acetate was better solvent for production of active metabolites from actinomycetes strains. Ethyl acetate extract of rsk5 was dried to get yellow crude extract which was further assayed against S. aureus to confirm the heat stability of metabolites (Kumar *et al*., 2012).Extracellular antimicrobial metabolites with ethyl acetate of the isolate (S7) MT974162 showed highest zone of inhibition against *Enterococcus Faecalis* 17.0 mm. All so the highest zone of inhibition against *E.coli* was 16.0mm while showed .

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Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 9795 - 9817
Received 25 April 2021; Accepted 08 May 2021.

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