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MOLECULAR IDENTIFICATION OF DETERGENTS DEGRADATION FUNGI IN WASTE WATER

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ABSTRACT

The present study was conducted to test the ability of some fungal species which isolated from waste water of the Khosr River in Mosul city to remove the high concentration of the active substance detergent (linear alkyl-benzene sulfonate, LAS) from the wastewater. The amplification of ITS gene was used to diagnose the fungi showed the ability to breakdown the active substance in wastewater from the Al-Khosr river. The selected fungi were cultivated in Potato Sucrose Broth (P.S.B)medium supplemented with Surfactants (LAS). The results showed changes of physicochemical and biochemical parameters such as pH and dry weight of biomass, five species of fungi which included: *A. niger*, *F. solani*, *T. asperellum*, *P. chrysogenum* and *M. verrucosa* were identified and the highest rate of degradation of the Surfactants (LSA)was 33% showed by the fungus *A. niger*.

Keywords **:** Molecular identification, detergents, fungi, waste water

Introduction

The detergents that we use in our daily washing are identified as one of the most important pollutants responsible for water quality changing and pollution (Arotupin *et al*., 2018). Detergents are chemical compounds that contain constituents, which are surface-active substances and formed compounds such as phosphates and small proportions of deficient substances, optical brighteners and enzymes, but the surfactants substances mainly responsible for cleaning operations. The types of active surface used as a major ingredient in commercial cleaners are linear alkyl-benzene sulfonate, (LAS) (Abu Lebda, 2016). Surfactants have harmful effects on the aquatic environment, including the eutrophication and toxic effect on living organisms, as well as the influence of ventilation processes in the wastewater treatment station and other damages (Rebello *et al*., 2014). The need to develop safe, effective and economical methods for degradation and removing pollution with detergents and reducing their harmful effects leading to use some bioremediation and degradation of detergents by a mixture of chemical and physical methods; Such as removal by activated carbon and various other methods. The use of biodegradation to remove contaminants is a typical, less expensive method than physical and chemical methods (Jakovljević and Vrvić, 2017). The importance of fungi in biological treatment, due to its high enzymatic ability to analyze pollutants and their massive biomass, these specifications made it more favorably than bacteria. Biological treatment is the less harmful way to the aquatic environment (Singh *et al*., 2020). Many method and studies were used to identify the most important fungi to degradation the detergent, using the molecular method which depends on DNA sequences is more accurate than using methods which depending on morphological and classic methods. Fungi were used in bioremediation as they have many enzymes systems and active metabolism made them a very useful tools for pollution control and treatments (Altaee, 2018).

Materials and Methods

The fungi used in this work were isolated from wastewater, which contain different detergents. The wastewater samples were collected from Al-Khosr river on the left side of the city of Mosul, 1 mlof water samples were added and placed in a sterile Petri dish in 3 replicates for each sample which contained Potato Dextrose Agar (PDA) amended with 250 mg/Lof chloramphenicol to prevent bacterial growth. The plates were incubated at 2+52ºC for 7-21 days. After the end of incubation period, the colonies we retransferred separately to PDA plates for purification. After checking the purity of the fungal colonies, they were again subcultured to PDA plates. (Khimmy, 2007).

Experimental procedure and culture conditions

The fungi were grown in 250 mL Erlenmeyer flasks with liquid nutrient medium Potato Sucrose Broth (P.S.B) (control). The pH value of this liquid media was about 4.80 (adjusted with 0.1 M HCl). The medium with the addition 16 mg\l of Surfactants (LAS), the pH values of these media were measured and reached to 9. All flasks were sterilized at 121°C in an autoclave for 15 minutes. After cooling the liquid medium at room temperature, 10 plugs of each fungi \200 mL of liquid medium were inoculated. Inoculated flasks were incubated in a shaking incubator (Labnet) at 150 rpm at 30 C temperature for 10,15 days respectively, After the end of incubation period, the samples measurements were made, (Jakovljević and Vrvić, 2018).

Calculation of biodegradationrate of surfactants (linear alkyl benzene sulfonate)

The fungal surfactants (linear alkyl-benzene sulfonate) degrader included; *A. niger*, *F. solani*, *T. asperellum*, *P. chrysogenum* and *M. verrucosa.* The primary biodegradability of synthetic detergent was confirmed by the Methylene Blue–Active Substance (MBAS) method, (Ojo and Oso, 2009). Percentage of surfactants degradation (LAS) was calculated by the following formula (Chaturvediand Tiwari, 2013):

$$\%\ \text{detergent degradation} = \frac{\text{Optical density of unknown after inoculation}}{\text{Optical density of sample (LAS)}} \times 100$$

DNA Isolation: Ready kit were used "Genomic DNA mini Kit of fungi" for DNA extraction by following the instructions of the company protocol.

Determination of molecular sizes DNA: The molecular sizes of the DNA were estimated by running the electrophoresis for the samples on the agarose gel using the Molecular Ladder in size 100 bp, DNA was mixed with loading dye and running in 2% of agarose gel.

Amplification of the high conserved ITS region in fungi using PCR : The PCR reaction was performed using universal primers which amplify ITS regions in genomic DNA, Table (1).

Table 1 : Universal

Primer	Sequence
Forward (ITS1)	TGAATCATCGACTCTTTGAACGC
Revers (ITS4)	TTTCTTTTCCTCCGCTTATTGATAT

Then the reaction tubes were inserted into the Thermocycler using the program, as shown in the following table (2)

Table 2: PCR program

No.	Stage	Temperature	Time	Cycle number
	Initial denaturation	95	5 min.	1
	denaturation	95	45 sec.	35
	Annealing	55	1 min.	
	Extension	72	1 min.	
	Final extension	72	7 min.	1

DNA extraction from agarose gel

The ITS bands amplified for each isolate were extracted from the gel and sent to gene company for sequencing by using Gene analyzer.

Results and Discussion

Isolation of fungi from wastewater:

Five fungal genus where isolated from wastewater and identified by using PCR to amplify the ITS region, figure (1).

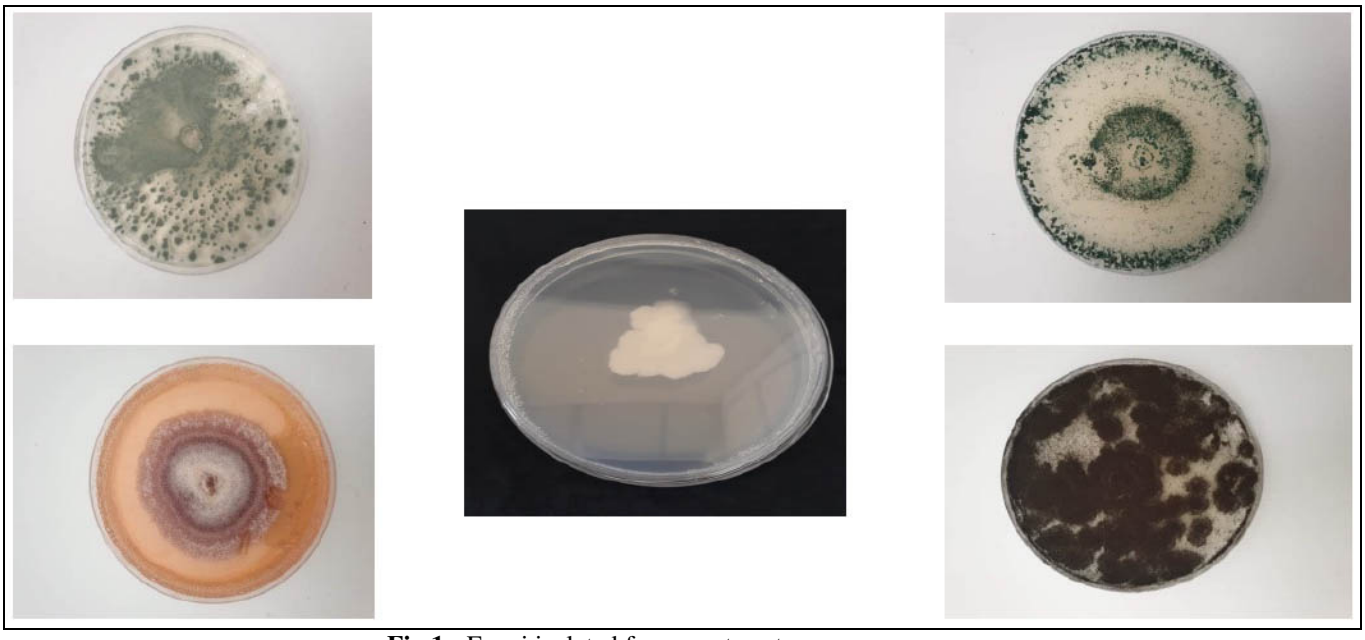

Fig 1 : Fungi isolated from wastewater

The five fungal were *Aspergillus niger*, *Fusarium solani, Tricoderma asperellum*, *Penicillium chrysogenum* and *Mycelliophthora verucosa*

The ability of fungi to degrade the detergent linear alkyl-benzene was studied by comparing the fungi activity between the control treatment and different active substance (LAS) during of the two period incubation 10 and 15 days, as shown in Table (3). After 15 days of incubation, the fungi were able to degrade the detergent at highest level by strains; *A. niger, F. solani, T.asperellum, P.chrysogenum and M. verrucosa* (33%, 16%, 11.7%, 10%, 5.11%) respectively. The difference in the percentage of degradation depends on the physiological and genetic characteristics of the different fungi (Jakovljević *et al*., 2015). *A. niger*able to degrade the highest rate of detergent among the tested fungi due to the many enzymes that it produces outside the cell (Mitidieri *et al*., 2006). biodegradationrateis increasing when there a abundance of nutrients which offer energy and sources of

growth such as sulfates and carbonates, the fungi obligate to degrade the linear alkyl benzyl sulfonate by converts it to carboxyl acids leading to opens the aromatic ring and removes sulfur and converts it to CO_2, H_2O and inorganic salts (Ojoand Oso 2009), Table (3) shows a decrease in biomass (dry weight) compared to the control- Linear alkyl benzyl sulfonate can cause an inhibitory effect on the enzymes secretion involved in the metabolism pathways of fungi according to the type of fungi tested (Garon *et al*., 2002). Also, the pH values decreased from the value 9 after the incubation period 10 and 15 days. The reason may be due to their impact on the absorption of ions and cations from the medium by the fungal cells and the impact rate according to the type of fungi and the excretion of organic acids in the medium (Vieira and Volesky 2000).

Table 3 : Bioremediation of LAS

Fungi	pH (control)	pH (LAS)	Biomass dry weight (control)	Biomass dry weight (LAS)	Biodegradatio LAS %	Day
A. niger	5.11	5.2	0.86	0.53	20	10
F. solani	5	6.5	2.03	1.02	7.6	
T. asperellum	5.25	5.9	2.11	0.75	6.5	
P. chrysogenum	5.15	5.8	2.99	0.82	3.5	
M. verrucosa	5.24	5	1.77	0.79	6.5	
A. niger	5.01	5	1.55	0.95	33	15
F. solani	5.41	6.2	3.55	1.44	16	
T. asperellum	5.18	6.25	2.68	1.53	11.7	
P. chrysogenum	7.47	6	4.13	1.46	5.11	
M. verrucosa	5.28	6.23	3.86	1.38	10	

Identification of fungi isolated from wastewater: After obtaining the fungal isolates, a molecular diagnosis was made to identify the species of fungi.

DNA extraction: The use of DNA extraction kits has many advantages, including the fast of extraction and obtaining high-purity DNA, after the DNA isolation and extraction process. Electrophoresis was carried out with using 2% of agarose, figure(2).

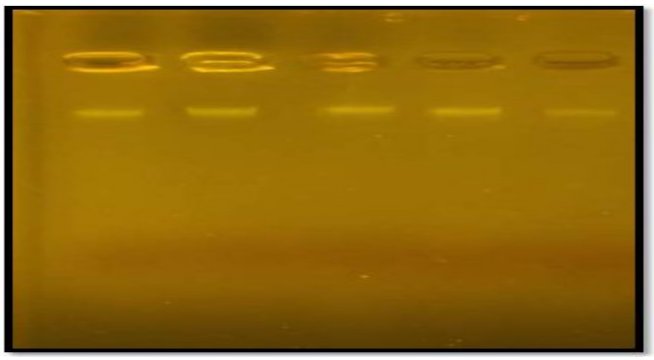

Fig. 2 : Extracting the Genomic DNA with high purity and concentration.

The results of the amplification of genomic DNA by using universal primers amplify the ITS region, the amplified band were in sizes 300 bp, Figure (3).

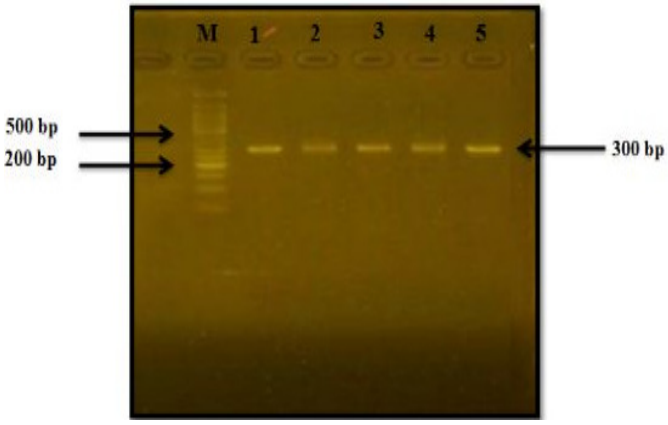

Fig. 3 : PCR reaction result for fungi samples at ITS4 site with a reaction result of 300 bp since M is the DNA ladder and samples (1,2,3,4,5) are related to fungi

After extracting the amplified bands from the agarose gel by using the gel extraction kit and sending it to the Macrogen biotechnology company for sequencing , the nucleotide bases of the first fungi isolate as shown in figure (4).

ARRSCCCRGGGAWTCGGGGGGGMAWGCCWGTCCGAGCGTCATTGCTGCCCTCAA
GCCCGGCTTGWGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGGACGGGCCCGAAAG
GCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTG
TAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGARRAAAAGAAA

Fig. 4 : Sequence of nucleotides for the first isolation

The amplified nucleotides sequences were identified submitting and blast in the National Center of Biotechnology Information (NCBI) website, the strain was *Aspergillus niger* which showed 96.34% of identical, as shown in Figure (5).

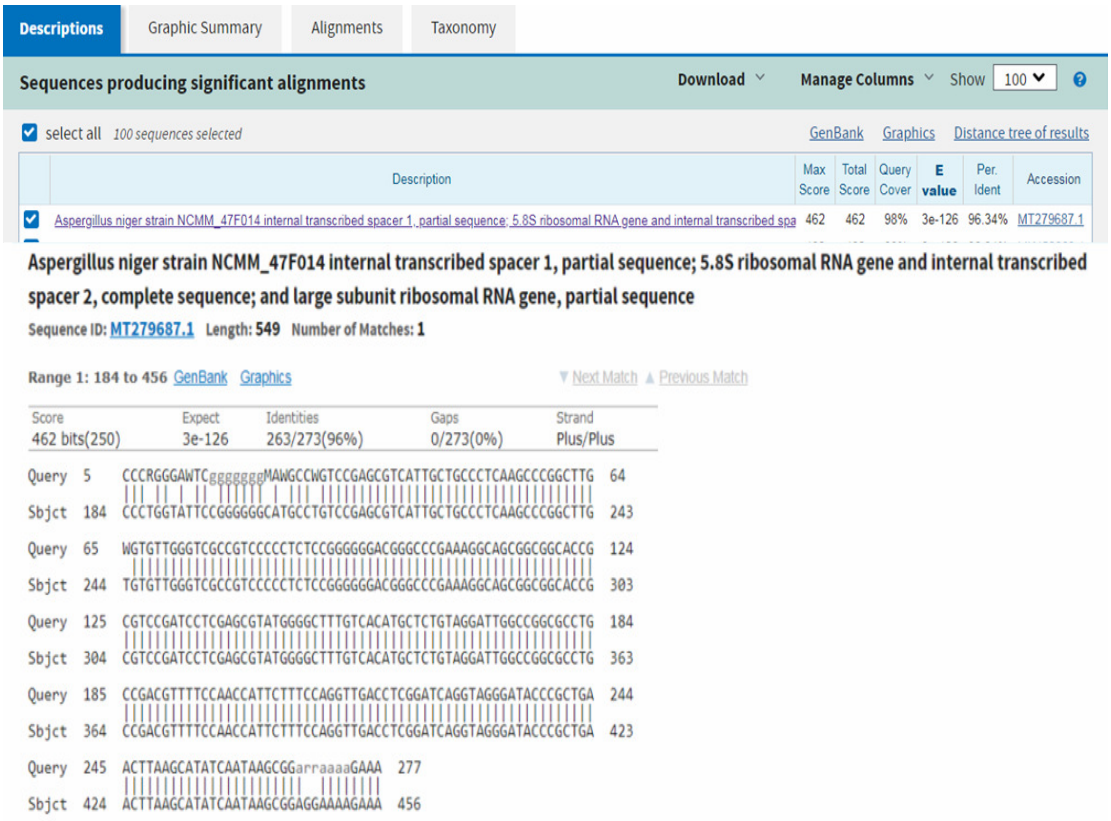

Fig. 5 : sequence of *Aspergillus niger*

The second strain sequence of nucleotides sequences, figure (6):

AWCGWCAGTATCTGGYGGGCWGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGG
GCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTCCCCCAAATACA
GTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAGAGCG
GCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAAT
ACCCGCTGAACTTAAGCATATCAATAAGCGGRGGGAAAAGAAA

Fig. 6 : Sequence of nitrogenous bases for the second isolation

The amplified nucleotides sequences for the second strain was identified by submitting and blast in the National Center of Biotechnology Information (NCBI) website, the genus was *Fusarium solani*, which showed a match rate of isolation of 97.48%, as shown in Figure (7).

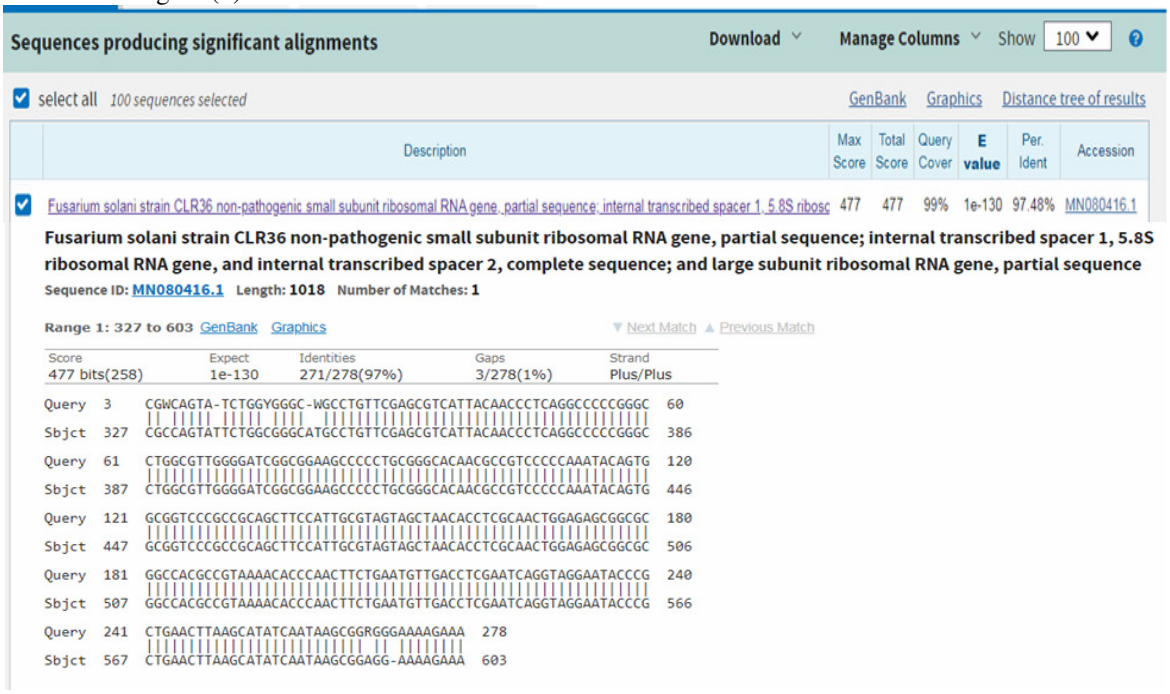

Fig. 7 : Sequence of *Fusarium solani*

The results of the third isolation sequence of nucleotides as figure (8):

GSSYSYCSGTSWTKCAGGCGCRTATCTGGCGGGCAGCCTGTCCGAGCGTCATTTCAACCCTC
GAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCTAA
ATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGA
GCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAATAAGCGARGGAAAAGAAA

Fig. 8 : Sequence of nitrogenous bases for the third isolation

The amplified nucleotides sequences for the third strain was identified by submitting and blast in the National Center of Biotechnology Information (NCBI) website, the genus was *Trichoderma asperellum*, which showed a match rate of isolation of 98.89%, as shown in Fig. (9)

Fig. 9 : Sequence of nitrogenous bases of *Trichoderma asperellum*

The fourth isolation sequence of nucleotides as figure(10)

ACCCAGMGCAWACGGTRGGCATGCCTGTCCGAGCGTSATTGCTGCCCTCAAGCACGGCTTGT
GTGTTGGGCCCCGYCMTCCRATCCCGGRGGACGGGCCCRAAAGGCARCGRCGGCACCGCGTC
CGGTCCTCGASYKTATGGGGCTTTGTCACCCGCTCTGTARGCCCGGCCGRCGCTTGCCGATCA
ACCCAAATTTTTATCCAGGTTGACCTCGGATCRGGYASSGAWACMCGCTGAACTTAAGCATA
TCAATAARSRGAGGAAAAGAAA

Fig. 10: Sequence of neucleotides of the fourth isolate

The amplified nucleotides sequences for the fourth strain was identified by submitting and blast in the National Center of Biotechnology Information (NCBI) website, the genus was *Penicillium chrysogenum*, which showed a match rate of isolation of 91.43% as shown in Figure (11)

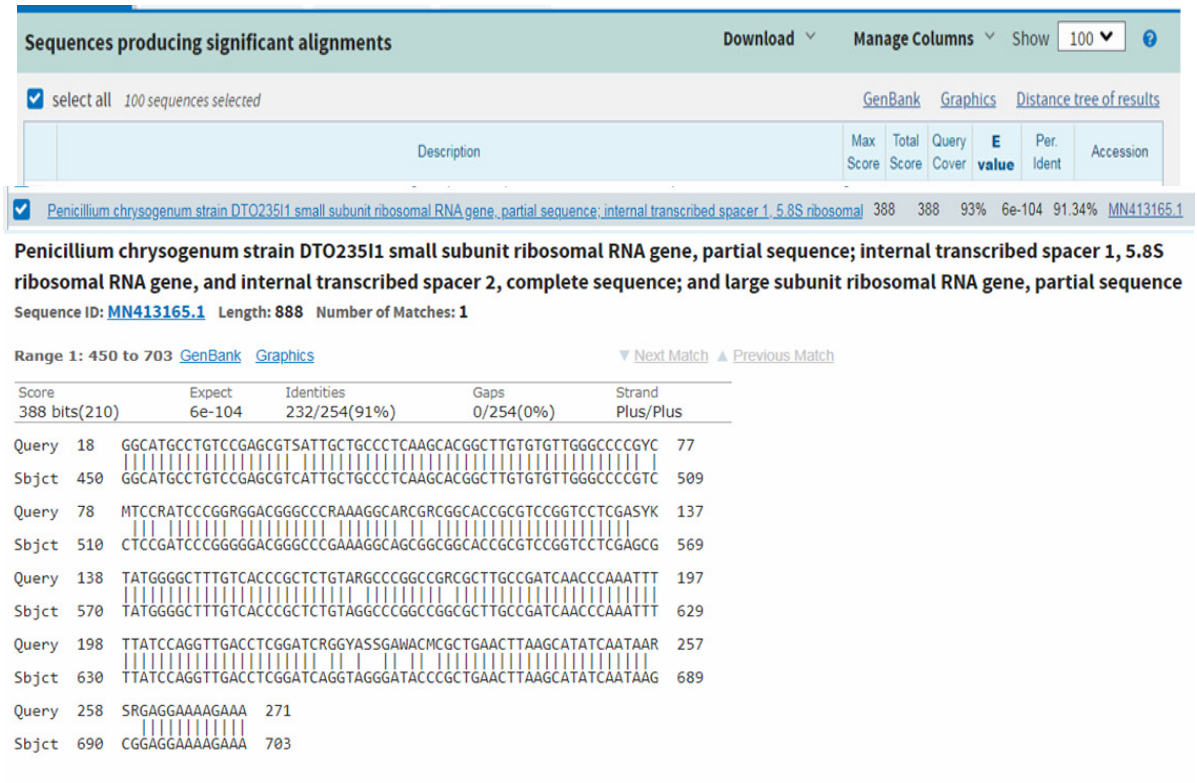

Fig. 11 : Sequence of neucleotides of *Penicillium chrysogenum*

The fifth isolation sequences of nucleotides as shown in figure (12):

ACCGCAGATCTGGCGGGCWGCCTGTTCGAGCGTCATTTCAACCATCAAGCCCCCGGCTTG
TGTTGGGGACCTGCGGYTGTCCGCAGGCCCTGAAAACCAGTGGCGGGCTCGCTAGTCACA
CCGAGCGTAGTAGCATACATCTCGCTCAGGGCGTGCTGCGGGTTCCGGCCGTTAAACGAC
CTTCATAACCCAAGGTTGACCTCGGATCAGGTAGGAAGACCCGCTGAACTTAAGCATATC
AATAAGCGGAGGAAAAGAAA

Fig. 12 : sequence of neucleotides for the fifth isolation

The amplified nucleotides sequences for the fifth strain was identified by submitting and blast in the National Center of Biotechnology Information (NCBI) website, the genus *Myceliopthora verrucosa*, which showed a match rate of isolation of 97.67% as shown in Figure (13).

Fig. 13: Sequence of nucleotides *Myceliopthora verrucosa*

Conclusions

Using the fungi in bioremediation offer and effective tools to remove the pollutants from different environments, wastewater contain a very difficult compound which effects of different marine life, using fungi which have a high enzymatic system can degrade these compounds and decrease their complexity. Techniques based on DNA amplification can reveal fungal DNA unique sequences as they are rapid and sensitive to screening, and thus can overcome the limitations of conventional diagnoses that are slow or insufficiently sensitive (Czurda *et al*., 2016) Where the diagnosis of fungi may be mistaken by conventional methods, molecular diagnostic methods compare the sequence of nucleotides bases of the unknown species of fungi with known genera within a database based on the amplification of the ITS region within the DNA Because of the accuracy of this method, it can diagnose different types of fungi within genus as well as strains within the type that are difficult to distinguish depending on themorphological of the fungus (Altaee 2013).

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