Biodegradation of diesel oil wastes for production of biosurfactants by two Actinomycetes strains

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Abstract

Biotechnological studies on production of bio-surfactants by actinomycetes strains that were isolated from Egyptian soil were carried out using some diesel oil waste samples. Two actinomycetes strains were found to give bio-surfactant production up to 40 - 45% when tested with emulsification test. Effect of carbon and nitrogen sources on bio-surfactant production were tested using urea, ammonium nitrate, ammonium chloride and potassium nitrate. Best results for bio-surfactant production were observed with urea and ammonium nitrate as the main nitrogen source in the biological medium. Best results for bio-surfactant production were observed when using Bushnell Hass medium supplement with 1.5% hydrocarbon waste. Ammonium sulfate precipitated samples were further applied on Sephadex LH-20 column to obtain purified products. Results obtained from isolated strains show optimistic results for a successful bioremediation of diesel oil wastes for production of commercial active bio-surfactants that could be used in both industrial and pharmaceutical fields.

Keywords: Bio-surfactants, Biodegradation, Actinomycetes, LH-20 column

Introduction

Bio-Surfactants are surface active agents produced by certain species of micro organisms upon growing on sugars, oils, alkanes and wastes (**Thampayak, I., *et al*., 2008**). These molecules are amphiphilic agents which reduce surface and interfacial tension by accumulating at interface between immiscible phases. Reduction of interfacial tension caused by the bio-surfactant increases the solubility and emulsification of the immiscible phases and bioavailability of the insoluble substrate for microorganism (**Khopade, A., *et al*., 2012**). Bio-surfactants were tested for enhanced oil recovery since these molecules are able to form micro emulsions where hydrocarbons can be solublized in water. Bio-surfactants are complex molecules with wide variety of chemical structures,

such as glycolipids, lipopeptides, fatty acids, polysaccharides-protein complexes, peptides, phospholipids and neutral lipids (**Sahoo, S,. *et al*., 2011**). In general, surfactants produced by microbes are classified into two main groups, low molecular weight (bio-surfactants) such as lipopeptide and glycolipides, and high molecular weight (bio-emulsifiers). Bio-surfactants have many properties such as lowering surface and interfacial tension, wetting and penetration, spreading , hydrophylicity and hydrophobicity action, microbial growth enhancement, metal sequestering, antimicrobial activities, antitumor and anti adhesive activities (**Kosaric, N., 2001**), (**Khopade, A., *et al*., 2012**). Bio-surfactants are mainly consisted of lipopeptides. For example, surfactin produced by *Bacillus subtilis* is the first and the most well-known member of this category. Other antimicrobial lipopeptides include fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis*. Lichenysin and pumilacidin produced by *B. licheniformis* and *B. pumilus*. Production of these antimicrobial compounds by Bacillus probiotics is one of major mechanisms by which they inhibit the growth of pathogenic microbes in the gastrointestinal tract (**Das, P., *et al*., 2007**). Bio-Surfactants have many advantages, they are non toxic, biodegradable and highly specific agents. They are also effective in sever conditions as high PH, temperature and salinity (**Kokare, CR., *et al*., 2007**). Bio-surfactants are useful in bioremediation of sites contaminated with heavy metal by-products of industries such as tanneries, leather industries, sugar mills, fertilizer industries and textiles (**Deepika, L., and Kannabiran, K., 2010**). Biosurfactants are also used commercially in food, microbiological, pharmaceutical agricultural and cosmetic industries (**Salihu, A., *et al*., 2009**). Bio-surfactants are also used for degradation of pesticides in soil and water environment (**Jennings, E.M., and Tanner, R.S., 2000**). Bio-surfactants producing Actinomycetes has been reported in few cases, Emulsan is an extracellular polymeric bio-emulsifier produced by *Acinetobacter calcoaceticus* also glycolipids from *Nocardia erythropolis*, *Rhodococcus erythropolis* producing glycolipids (**El-sersy, N.A, Abou-Elela, G.M, 2006)**, (**Kokare, CR., *et al*., 2007**). Scientific research is now increasing towards studying chemical and physical properties as well as production of bio-surfactants from microorganisms. This kind of research may offer the industry a cheap, safe and very useful group of compounds that have multiple applications in many fields.

Materials and Methods

Growth media

Growth medium used for isolation and purification of Actinomycetes was starch nitrate medium with the following composition (g/1): Starch 20; KNO_3 1; K_2HPO_4 0.5; $MgS0_47H_20$ 0.5; NaCl 0.5; $FeSO_4$ 0.01; agar 15 in distilled water. (**Küster, E., and Williams, S.T., 1964**). The same medium was supplemented

with diesel oil to be used for Screening for hydrocarbons biodegrading strains. Tributyrene agar medium was used to screen for lipase production. The medium composition (g/l) was: peptone 0.5 ; yeast extract 0.3 ; tributyrene 1; Calcium carbonate 1 and agar agar 20 in distilled water. pH was adjusted to 7.4. Blood agar medium was used to test for hemolytic activity. The medium was prepared using 5% human blood and blood agar base. Bushnell Hass medium was used for bio-surfactants production. The medium composition (g/l) was: magnesium sulphate 0.2g; calcium chloride 0.02g; monopotassium phosphate 1.0 g; dipotassium phosphate 1.0 g; ammonium nitrate 1.0 g; ferric chloride 0.05 g and Agar 15.0 g. pH was adjusted to 7.0 ± 0.2.

Isolation of Actinomycetes strains

Samples were collected from different Egyptian soil areas, 1 gm of each sample was mixed with 9 ml sterile saline solution, serial dilutions were made for these samples. From each dilution 0.1 ml was taken and spread evenly with sterile L-shaped glass rod over the surface of Starch Nitrate Medium (SNM) then incubated at 30°C for 7 days, Streak plate method was used for purification of Actinomycetes **(Williams, S.T., and Cross, T., 1971)**. The developed colonies can be individually purified on Starch Nitrate Medium (SNM) then subculture to insure their purification. pure cultures were preserved on starch nitrate medium slants at 4°C for further analysis (**Ellaiah, P., and Reddy, A.P.C., 1987**) .

Screening for Bio-Surfactants Producing Actinomycetes

Screening for hydrocarbons biodegradation

Isolated strains were tested for biodegradation of diesel oil by growing them on starch nitrate medium supplemented with diesel oil. After incubation for 7 days at 30°C the hyper bio-surfactant producing strains were selected.

Screening for Lipase production

Isolated actinomycetes strains were tested for lipase production by growing them on Tributyrene agar medium. Cultures were incubated for 7 days at 30°C. After incubation period clear zone of hydrolysis around the colonies were observed, positive strains were screened for emulsification activities (**Karthik, L., *et al*., 2010**).

Hemolytic Activity

Hemolytic activity were tested using blood agar medium. Media were supplemented with 5% human blood. Actinomycetes isolates were streaked on

blood agar plates then incubated for 7 days at 30°C. Clear zone around the colonies showed the hemolytic activity of different strains. Positive strains were screened for emulsification activity (**Maneerat, S., and Phetrong, K., 2007**)

Emulsification index *(E_{24})*

Emulsification index was estimated by adding 2 ml of cell free supernatant centrifuged at 10,000 rpm for 20 min at 30°C with 2 ml kerosene then vortex at high speed for 2 min, after 24 hrs emulsification index was calculated by measuring (a) emulsion height divided by (b) total height multiply by 100 where EI= (A/B)*100 this essay performed in the same size glass tube (**Saravanan, V., and Vijayakumar, S., 2012**)

$$\text{Emulsification index (E24)} = \frac{\text{Height of emulsified layer}}{\text{Total height of the liquid column}} \times 100.$$

According to screening results, hyperproducing strains were grown on Bushnell Hass medium for bio-surfactant production.

Identification of Bio-Surfactants producing strains
Morphological and Physiological characterization
The micro morphology of isolated Bio-surfactants producing Actinomycetes strains gram staining, shape and size were done under light microscope (**Vimal, V., *et al*., 2009**). Culture characterization such as colony morphology like elevation, surface, density, aerial and substrate mycelium and pigment production was carried out (**Kenneth, L.K., 1958**). Physiological characterization of isolated Actinomycetes strains were carried out by performing organisms growth at different temperature range 10°C - 60°C, PH range from 4 to 10, and the growth under aerobic conditions (**Reddy, N.G., *et al*., 2011**).

Biochemical Characterization
Biochemical tests were carried out for identifying the isolated strains these tests including Indole test, Nitrate reduction, Methyl red ,Voges-Proskaur, citrate utilization and other enzymatic activities such as Starch hydrolysis, casine hydrolysis, gelatin hydrolysis, catalase production and urease production (**Reddy, N.G., *et al*., 2011**).

Genotypic characterization of Isolated Actinomycetes strains

Bacterial isolates were submitted to genotypic characterization through 16S rRNA gene technique. The 16S rRNA gene from the strain was amplified using universal primers (27F; 5-AGA GTT TGA TCC TGG CTC AG-3 and 1492R; 5-GGT TAC CTT GTT ACG ACT T-3). The PCR products were purified and sequenced by the GATC-Biotech. Company (Germany). The sequences were compared with known sequences in the Gene Bank nucleotide database and identified as the nearest phylogenetic neighbor with the highest similarity percent (**Hentschel *et al.*, 2001**).

Bio-surfactants Production

One hundred ml of Bushnell Hass medium were inoculated with isolated strains. The culture was incubated for 7 days at 30°C with continuous shaking at 150 rpm.

Purification of Bio-surfactants.

Gel filtration of bio-surfactants

Liquid cultures were centrifuged at 10,000 rpm to remove cells content. Extracellular bio-surfactant sample was then precipitated by 65% ammonium sulfate. The floating materials resulting from the treatment of ammonium sulfate were collected by centrifugation, and then dissolved in a small fraction of water. Chilled acetone was added to the solution to remove the protein and acetone-insoluble materials. 3 ml of sample was applied to Sephadex LH-20 column with a diameter of 1.5 cm and a length of 40 cm equilibrated with 90% methanol. The column was eluted with the same solvent at a flow rate of 2 ml/min. After elution, collected fractions were tested for their extracellular bio-surfactants activity by the oil spreading technique carried out by (**Youssef, N.H., *et al*. 2004**).

Oil spreading technique

Distilled water was added to the Petri dish (90 mm × 15 mm) followed by addition of olive crude oil to the surface of water. Then, 10 µL samples for each fraction were dropped onto the crude oil surface. The diameter of the clear zone on the oil surface was measured and compared with those on the negative and positive controls. A calibration curve was made with different concentrations of surfactin ranging from 50 to 250 mg/l.

Results

Twenty five Actinomycetes strains were isolated from different Egyptian environments. All isolates were identified with morphology and light microscopic examination. All strains were found to be positive for Gram staining. Biochemical tests showed also that all isolates were positive to both methyl red and catalase tests. Isolates showed positive results when tested for their ability to hydrolyze both starch and casein.

Screening for Bio-Surfactants producing strains.

Hydrocarbon Biodegradation.

The previous 25 Actinomycetes strains were screened for their ability to biodegrade hydrocarbons using a medium containing diesel oil as the only carbon source. Sixteen isolates were able to grow on the diesel oil medium.

Lipase Production.

In a second screening step, the 16 strains were tested for growth on the selective Tributyrene agar medium. Isolates were again selected for their lipase production after measuring clear zone around each single colony. Six isolates (AA04, AA05, AA06, AA07, AA08 and AA15) were found to give a clear zone diameter of more than 2.7 cm (Table 1). These hyper lipase producers were selected for the rest of study.

Table (1): Lipase production test for sixteen Actinomycetes isolates from different Egyptian soil areas. All isolates were grown on Tributyrene agar medium for 7 days at 37^{o}C. Clear zone around each colony was recorded in cm.

Isolate	Clear zone diameter (cm)
AA01	2.1
AA02	2
AA03	1.8
AA04	3.2
AA05	3.5
AA06	3.1
AA07	2.8
AA08	2.9
AA09	2.6
AA10	2.5
AA11	1.8
AA12	2
AA13	1.6
AA14	2.3
AA15	2.7

Hemolytic Activities.

Carrillo et al. recommended blood agar lysis test as a method for bio-surfactant activity screening. So, previously selected strains were tested for their hemolytic activity by growing them on blood agar medium. All isolates showed a hemolytic activity (Table 2 and Figure 1). The highest two isolates in their production were selected for further studies on bio-surfactant production.

Table (2): Hemolytic activity of selected isolates. Strains were grown on blood agar medium for 7 days at 37°C. Clear zone around each colony was recorded in cm.

Isolate	Clear zone diameter (cm)
AA04	5.2
AA05	5.6
AA06	4.6
AA07	4.9
AA08	4.8
AA15	5

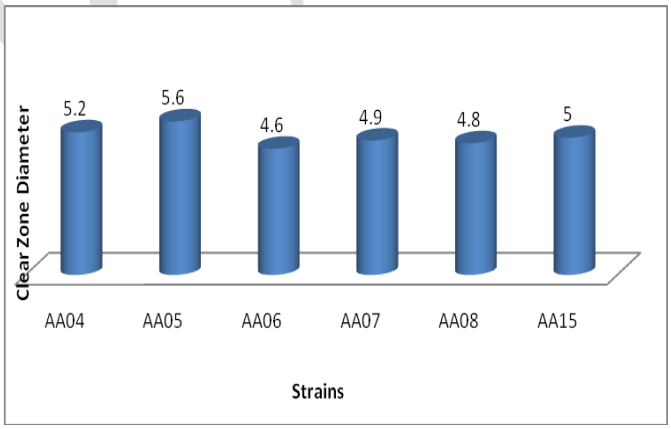

Figure (1): Hemolytic activity of isolated Actinomycetes strains represented in clear zone diameter (cm) around each colony upon growing them on blood agar medium for 7 days at 37°C.

Emulsification Index.

The previously selected 6 strains were tested for their emulsification activity by growing them on Bushnell Hass Broth medium that contained 1gm diesel oil. After incubation at 30°C for 7 days at 150 RPM, the emulsification index for each isolate was measured according to the method mentioned by Saravanan, V., and Vijayakumar, S., 2012. Results of table (3) and figure (2) showed that the previously selected AA04 and AA05 isolates gave the highest emulsification index of all, making them the best candidates for bio-surfactants production among all the previously tested isolates.

Table (3): Emulsification index of Actinomycetes isolates. The index was estimated by adding 2 ml of cell free supernatant centrifuged at 10,000 rpm for 20 min at 30°C with 2 ml kerosene then vortex at high speed for 2 min, after 24 hrs emulsification index was calculated by measuring (a) emulsion height divided by (b) total height multiply by 100 where EI= (A/B)*100 this essay performed in the same size glass tube.

Emulsification Index	
Strain	E 24
AA04	15%
AA05	17%
AA06	10%
AA07	12%
AA08	12%
AA15	14%

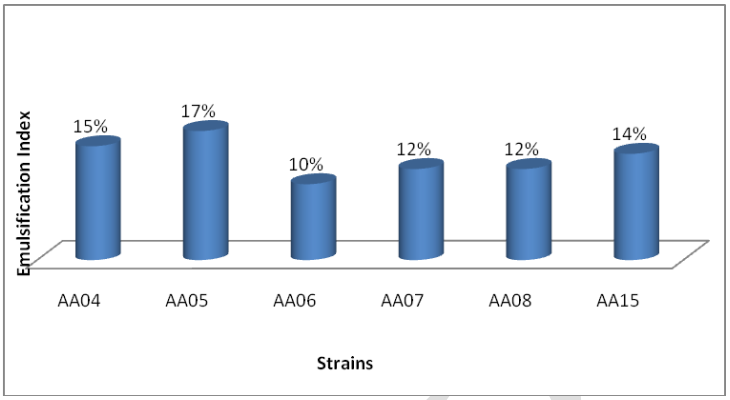

Figure (2): Emulsification Index of different Actinomycetes isolates. Highest results were recorded by strains AA04 and AA05.

Bio-surfactant producing strains

For complete identification, the two selected lipase-hyperproducing Actinomycetes isolates (AA04 and AA05) were further tested physiologically. The two strains grown on incubation temperature that was ranged from 20^{o}C to 50^{o}C with temperature optima of 30^{o}C and 35^{o}C respectively (Table 4). Optimum pH for growth was pH 7 but both isolates showed a positive growth over a pH range from pH 4 to pH 9 (Table 4) which gives them a very useful character especially when these microbes are used industrially where the pH is often unstable. Other morphological and physiological characters of both isolates are listed in table (4).

The two isolates were both negative to indole test and citrate test, but positive to nitrate reduction and Voges-Proskaur tests. Biochemical and enzymatic tests for both isolates are shown in table (5). Genotypic characterization of the isolated Actinomycetes strains was carried out through 16S rRNA gene technique. The 16S rRNA gene from both strains was amplified using universal primers (27F; 5-AGA GTT TGA TCC TGG CTC AG-3 and 1492R; 5-GGT TAC CTT GTT ACG ACT T-3). The PCR products were purified and sequenced. Figure (3) and figure (4) show the phylogenic tree for strains AA04 and AA05 respectively after the PCR products were compared with known sequences in the Gene Bank nucleotide database and identified as the nearest phylogenetic neighbor with the highest similarity percent. According to Bergey's manual of Determinative Bacteriology, Ninth edition by Holt (2000) and 16s RNA gene technique the isolated strains were identified as *Streptomyces xanthophaeus* and *Streptomyces viridochromogenes.*

Table (4):Morphological and physiological characters for isolated Actinomycetes strains.

Morphological and Physiological Characters		
Test	**AA04**	**AA05**
Colony		
Gram Stain	Positive	Positive
Shape	Rods	Rods
Size	Long Rods	Very Long Rods
Color	Ash Gray	Gray with greenish tinge
Elevation	Raised	Raised
Surface	Wrinkly	Wrinkly
Density	Opaque	Opaque
Mycelium	Aerial	Aerial
Pigment	Yellow to Brown	Brown
Growth at different temperature range		
at 10°C	Negative	Negative
at 20°C	Positive	Positive
at 25°C	Positive	Positive
at 30°C	optimum	Positive
at 35°C	Positive	Optimum
at 40°C	Positive	Positive
at 45°C	Positive	Positive
at 50°C	Positive	Positive
at 60°C	Negative	Negative
Growth at different PH range		
PH 4	Positive	Positive
PH 5	Positive	Positive
PH 6	Positive	Positive
PH 7	Optimum	Optimum
PH 8	Positive	Positive
PH 9	Positive	Positive
PH 10	Negative	Negative

Table (5): Biochemical tests for Actinomycetes isolates.

Biochemical tests		
1- Indole Test	Negative	Negative
2- Nitrate Reduction	Positive	Positive
3-Methyl Red and Voges-Proskaur Test	Positive	Positive
4- Citrate Utilization	Negative	Negative
Enzymatic Activities		
1- Starch Hydrolysis	Positive (Rapidly)	Positive
2- Casein Hydrolysis	Negative	Negative
3- Gelatin Hydrolysis	Negative	Positive
4- Catalase Production	Positive	Positive
5- Urease Production	Positive	Positive
6- Cellulase Production	Negative	Negative

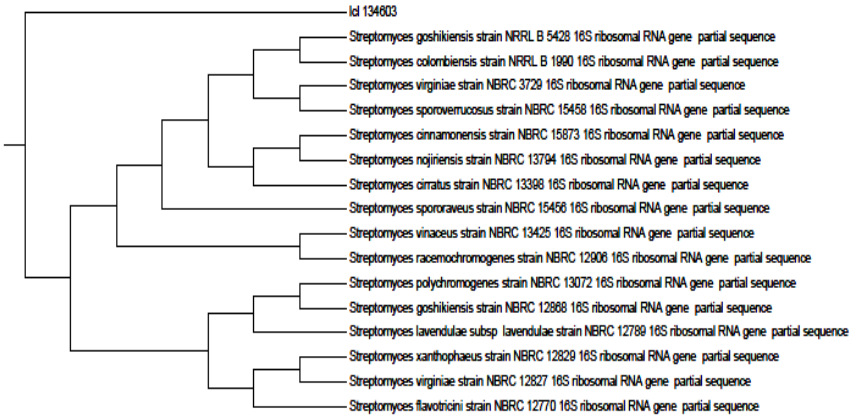

Figure (3): phylogenic tree for strain AA04. According to Bergey's manual of Determinative Bacteriology, Ninth edition by Holt (2000) and 16s RNA gene technique the isolated strains were identified as *Streptomyces xanthophaeus*

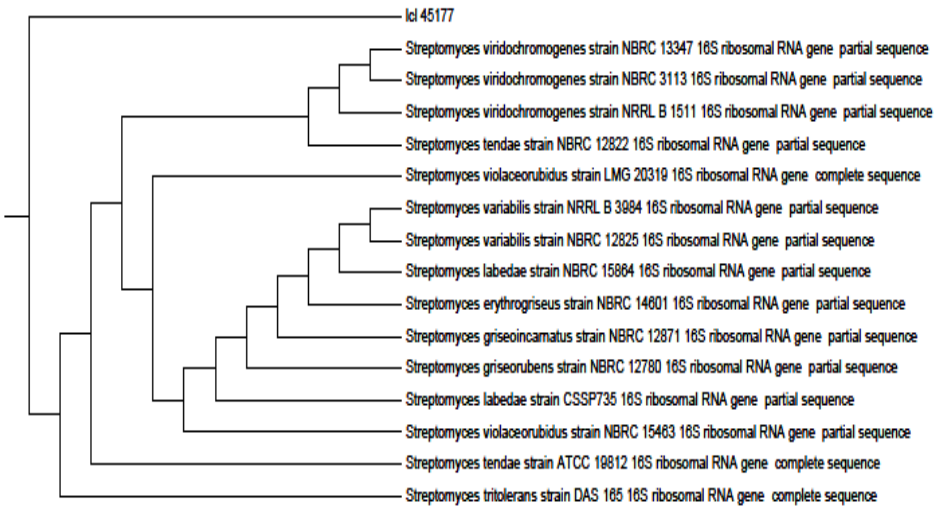

Figure (4): phylogenic tree for strain AA05. According to Bergey's manual of Determinative Bacteriology, Ninth edition by Holt (2000) and 16s RNA gene technique the isolated strains were identified as *Streptomyces viridochromogenes*.

Purification of extracellular bio-surfactants.

The two identified Streptomyces strains were tested for their ability to produce bio-surfactants upon growing them on Bushnell Hass medium supplemented with 1% petroleum oil. After liquid culture was incubated for 7 days at 30°C with continuous shaking at 150 rpm, cell-free supernatant was salted out to be further purified using gel filtration. Figure (5) shows the fractionation pattern of *Streptomyces xanthophaeus* extracellular bio-surfactant after sample application on Sephadex LH-20 column. Purified sample gave a biosurfactant activity of more than 400 mg per ml when tested with the oil spreading technique discussed by Youssef, N.H., *et al*. 2004. In a similar way, Figure (6) shows the fractionation pattern of *Streptomyces viridochromogenes* extracellular bio-surfactant after sample application on Sephadex LH-20 column. Purified sample gave a biosurfactant activity of more than 600 mg per ml when tested with the oil spreading technique which makes it a hyper producer strain when even compared with the previous *Streptomyces xanthophaeus* production.

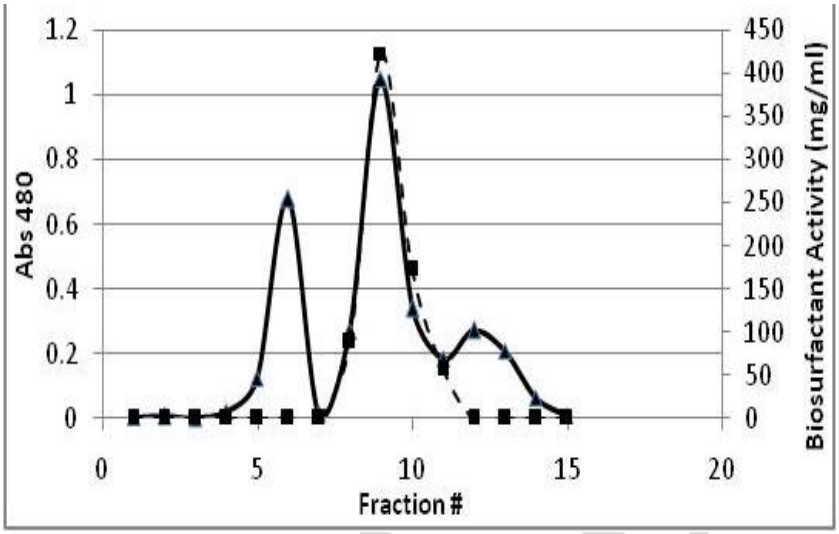

Figure (5): Fractionation of ammonium sulfate partially purified sample produced by *Streptomyces xanthophaeus* after applying it on Sephadex LH-20 column. ▲ represents spectrophotometric measurement of fractions, while ■ represents extracellular bio-surfactant activity in mg/ml.

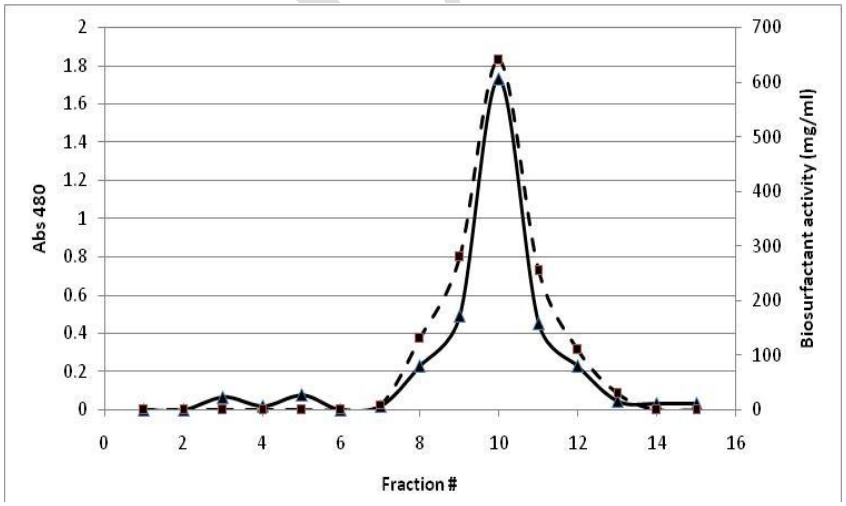

Figure (6): Fractionation of ammonium sulfate partially purified sample produced *Streptomyces viridochromogenes* after applying it on Sephadex LH-20 column. ▲ represents spectrophotometric measurement of fractions, while ■ represents extracellular bio-surfactant activity in mg/ml.

Discussion

To search for biosurfactant producing micro-organisms, a screening test was made to isolate biosurfactant producing microbes from the Egyptian soil. Study concentrated on areas that were highly contaminated with hydrocarbons, especially from petroleum oil source. Two Actinomycetes isolates were selected according to their lipase production and hemolytic activies. The two strains were genetically identified as *Streptomyces xanthophaeus* and *Streptomyces viridochromogenes.* Physiological and biochemical tests for both strains showed a high range of stability for both temperature and pH variations which makes them a good choice for bio-surfactant production especially in uncontrolled pH liquid culture tanks. Results of biodegradation of petroleum oil waste samples that were added to the culturing media for both *Streptomyces xanthophaeus* and *Streptomyces viridochromogenes* gives us a very optimistic point of research especially in a developing country like Egypt where environmental oil wastes as well as wastes that are originated from petroleum industries represent a huge environmental problem. From a bioremediation point of view, these kind of strains are not only good bioremediators, but also are producers of a high commercially important group of compounds. Purification results by Sephadex LH-20 column for partially purified extracellular bio-surfactants produced by *Streptomyces xanthophaeus* showed a 29 fold increase in activity, while 41 fold increase in activity was recorded for partially purified extracellular bio-surfactants produced by *Streptomyces viridochromogenes* after application on the same column. Salting out and gel filtration results offer a method to get the produced bio-surfactants in a purified form that is ready to be used in further study and application steps.

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