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Extraction,Purification and Characterization of Biosurfactant from *Bacillus subtilis*

أستخلاص,تنقية وتوصيف الـ Biosurfactant من بكتريا *Bacillus subtilis*

Ghofran Flaih Abd-Al-Hussan[1],Fatima Abd AL–Hussein Mejbel [1] andHamid Majid Jassim [2]
1-Department of Biology,Faculty of Science,University of Al-Kufa.
2-Department of Biotechnology , Faculty of Biotechnology , University of AL- Nahrain .

Abstract

Bacteria was isolated from soil sample of various automobile work shop of five different areas in Iraq.About 50 isolate suspected to belong to genus *Bacillus*.All these isolates were subjected to microscopic examination and cultural characteristics about 42 isolate belonged to genus *B.subtilis*. Crude biosurfactants produced by the selected isolates were extracted by acid precipitation followed by partially purified with dichloromethane.Biosurfactant has been purified from *B.subtilis* S4 by two purification steps included Ion exchange chromatography using DEAE- Cellulose followed by Gel filtration chromatography using Sepharose-6B.The the characteristics of the purification compouned were studied . Results showed that the molecular weight of biosurfactant produced by *B.subtilis* S4 ranging between (1036-1058 Da) with the HPLC-MS System .

الخلاصة

تم عزل البكتريا من عينات التربه من مختلف محلات تصليح السيارات لخمس مناطق مختلفه من العراق وقد حصل على 50عزلة يتوقع انها عائدة الى جنس *Bacillus* وهذه العزلات تم اخضاعها الى الفحص المجهري واختبارات الصفات الزرعيه وقد حصل على 42 عزله تعود الى *B.subtilis* . تم انتاج Biosurfactant من العزلات المختاره وتم استخلاصه بواسطه الترسيب بالحامض وتبعها تنقيته جزئيا مع مذيب dichloromethane . تم تنقيه Biosurfactant من العزله *B.subtilis* S4 بواسطه طريقتين من التنقيه وهما Ion exchang chromatograghy بأستخدام DEAE-Cellulose وطريقه Gel filtration chromatograghy بأستخدام Sepharose -6B . تم دراسة صفات المركب المنقى ، واظهرت النتائج ان الوزن الجزيئي للـ Biosurfactant المنتج من العزله *B.subtilis* S4 يتراوح بين 1036-1058دالتون بواسطه HPLC-MS System .

1:Introduction

Surfactants are amphipathic molecules that accumulate at interfaces, decrease interfacial tensions, and form aggregate structures such as micelles. Due to these properties, surfactants and emulsifiers are widely used for industrial, agricultural, food, cosmetic and pharmaceutical applications. Surfactants can be derived from both petrochemical feedstock and renewable resources (plants and animal oils, microorganisms). They were originally made from renewable resources like fats and oils, whereas today, the majorities are of petrochemical origin .Some surfactants, known as biosurfactants, are produced by yeasts or bacteria that have some effect on interfaces.They are grouped as glycolipids, lipopeptides, fatty acids,polymeric and particulate compounds.The kinetics of biosurfactant production, its characterization, properties and toxicity were evaluated for the application of this biomolecule in environmental processes (1) .

- **Aim of study**

1. Isolation of *Bacillus* spp.from different localities contaminated with hydrocarbons.
2. Purification and characterization of the produced biosurfactant .

2:Materials

2.1:Soil samples collection

Soil samples were collected from different locations of Najaf , Kufa and Diwaniya governorates .Samples were taken from the oil spilled surface of the automobile workshop , below the soil surface of contaminated soils of machine , hydrocarbons soil beneath cars fuel stations , oil refineries and other contaminated sites from January to March , 2013 (2) .

2.2:Isolation of Bacteria

Isolation of *Bacillus* spp. was performed by adding one g from each soil sample to 9 ml of sterile D.W. ,mixed well and then heated in water bath at 80ºC for 20 min ,with gentle agitation , after cooling 0.1 ml of soil solutions was taken from each sample and spreaded on a nutrient agar plates and then incubated aerobically for 24 hrs. at 30°C. After incubation , colonies appeared with different sizes and shapes were selected for identification (3).

2.3: Identification of bacterial isolates

2.3.1: Morphological and cultural characteristics

The shape, size , color ,edge and appearance of bacterial colonies were studied on nutrient agar plates after 24 hr of incubation (4).

2.4: Biosurfactant production

A portion of 1ml of the inoculum was used to inoculate 100 ml of Jacques medium , in triplicate, and incubated aerobically in shaker incubater at 180 rpm at 30ºC for the periods (24 , 48 and 72 hrs.) .Each culture was then centrifuged at 4 ºC, 10.000 rpm. for 20 mins ,after centrifugation of broth ,cell-free supernatant was taken which contains biosurfactant.

2.5: Estimation dry weight of biosurfactant and Partial purification

Cell-free supernatants of each isolate were treated by acid precipitation adding drops of 6N HCl continuously until to reaching to pH of 2,after this allowing the supernatants for precipitating over night at 4ºC. Precipitation was collected by centrifugation with10.000 rpm at 4ºC for 20min.,then pellets were resuspended by 2 ml of D.W.then adjusted to pH 7 with agitation in order to complete dissolving and finally lyophilized and weighed (5) .

The biosurfactant pellet was resuspended in water and pH was raised to 7 in order to solubilize biosurfactant with agitation to complete dissolving , the water soluble biosurfactant was lyophilized.The dried biosurfactant was resuspended in dichloromethane in a separating funnel and then shaken vigorously,surfactin was recovered in the organic layer at the top,the extraction was performed twice,and the organic layer was pooled and evaporated (5) .

2.6: Methods for detection and purification of biosurfactant

2.6.1: Ion Exchange Chromatography

2.6.1.1: Preparation of ion exchange column (DEAE-Cellulose)

The DEAE-Cellulose was prepared .20 g from ion exchange resin were suspended in one liter D.W. and left in graduated cylinder to stagnate, after that the supernatant was removed.This step was repeated many times, until the supernatant become clear. The ion exchange resin was filtered (without drying the ion exchange resin), then the resin was activated in 250 ml from buffer which contains 0.25M NaoH - 0.25M NaCL for 30 min, the resin was refiltered and washed under vacuum using D.W., then the resin was suspended in 250ml HCL 0.25M,after that, the resin was washed with D.W. under vacuum, then the resin was suspended in potassium phosphate buffer (1mM , pH8) and the ion exchange resin was degassed by using vacuum pump, the resin was packaged gently in glass column , and the equilibration was achieved by the same potassium phosphate buffer.

2.6.1.2:Separation of biosurfactant through ion exchange resin (DEAE -Cellulose)

Ten ml from sample was loaded on ion exchange column prepared, the separated fractions were collected approximately 8ml for each fraction, the wash and the elution was achieved by using potassium phosphate buffer, the flow rate was 5ml / 3min, the protein fractions were measured at wave length 280 nm .

2.6.2: Gel filtration chromatography

Purification procedure was achieved by using chromatographic step to obtain purified biosurfactant, purification procedure was achieved by using chromatographic step to obtain purify protein (6).

2.6.2.1: Preparation of Sepharose-6B Column

Sepharose-6B was prepared according to Pharmacia catalogue as shown below :

1.Gel was washed several times with phosphate buffer then degassed for 10 min and used to filled column . Gel was left to settle down and packed well to give column 2.5×15cm.

2.Column was equilibrated with phosphate buffer over night and flow rate was adjusted to give 5 ml / 3 min and absorption were checked in UV - visible spectrophotometer and read at 280 nm .

2.6.2.2: Loading of sample

Five milliliters of sample were loaded by using pasture pipette. It was eluted from the column by equilibrated phosphate buffer,9ml for each fraction were collected .The absorbance of each fraction was read at 280 nm .The performed peaks were collected separately, concentrated by sucrose.Fractions which showed activity were kept at 4ºC until used .

2.6.3 :Qualitative detection of carbohydrates in biosurfactant

Add 2 ml of purified biosurfactant to 2 drops of α-naphthol solution and pour dropwish 1ml of concentrated H_2SO_4 on the inner wall of test tube , Molisch's reagent giving aviolet or purple colored between the two layers in the precense of carbohydrate (7).

2.6.4 : Determination of lipid content

About 0.5 g of the isolated material was extracted with chloroform: methanol in different proportions (1:1 and 1:2, v/v).The organic extracts were then evaporated under vacuum and the lipid content determined by gravimetric estimation (8).

2.7: Characterization of biosurfactant by UPLC-TOF-MS System

The molecular mass of purity biosurfactant produced by the selected isolate was determined by analysis with Shimadzu (UPLC - TOF -MS system) to purify the compounds by fractionating the samples according to the retention time and high sensitive TOF mass identifying molecular weight of compounds by measuring the time of fly (TOF) for each single atom and suggesting the possibility of compound identification using software data base.The sample injection volume of auto injector was set to 100 µL, the solvent system consisted of water : acetonitrile: methanol (30:35:35 v%) as a mobile phase. The flow rate was 1 ml /min. (9).

3:Results and Discussion

3.1: Isolation of biosurfactant – producing *Bacillus* species

For isolate *Bacillus* producing biosurfactant , samples of heavily oil contaminated soil were collected from automobile work shop, cars fuel stations,oil refineries and surface beneath electrical machine of different area ,from which 50 isolates were isolated suspected to be *Bacillus* The obtained isolates were examined to microscopic examination with gram staining , endospore formation,morphological and cultural characteristics on nutrient agar (3).The result showed about 42 isolates belonged to *B.subtilis* .Occurrence in soil samples were found to be relatively high when

compared to other genus and member of this genus have the ability to produce of biosurfactants which is well documented (10)

3.2:Partial purification of biosurfactant produced by the selected isolates of *B.subtilis*

The eight selected *B. subtilis* isolates were propagated in jacques medium for 72 hrs ,biosurfactant in cell- free supernatant precipitated by acid to produce biosurfactant , the precipitate of each isolate was solubilized in dichloromethane in a separating funnel. Surfactin was recovered in the organic layer at the top .The brown precipitate was lyophilized in lyophilizer and weighed to calculate the yield of biosurfactant (g / l of broth media) in each isolate (figure 1) (11) .

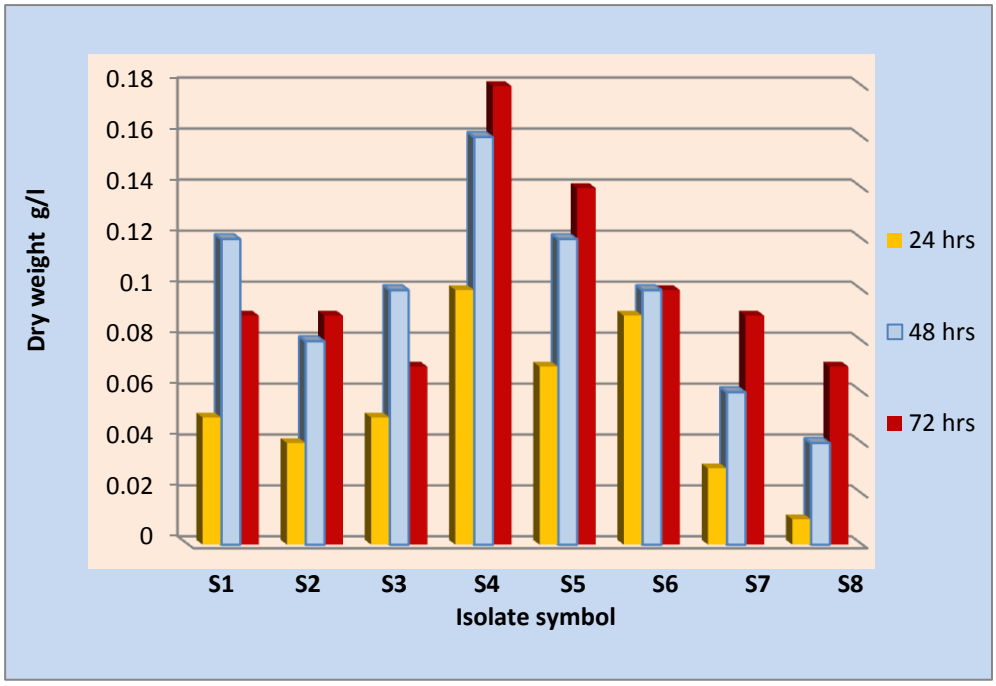

Figure 1: Dry weight of biosurfactant produced by *B. subtilis* isolates propagated in jacques medium for different incubation time .

3.3:Biosurfactant purification

In order to determine the molecular mass of biosurfactant produced by the isolate *B.subtilis* S4 which was chosen depending on the amount of biosurfactant production), relatively pure biosurfactant was conducted followed by determination of molecular weight of protein and analysis by HPLC-MS System .

3.3.1: Ion exchange chromatography

The analysis by DEAE ion exchange chromatography can provide qualitative information about biosurfactants. Many advantages have been found in DEAE-Cellulose chromatography including: high resolution power, easy handling ,high capacity, good separation , and ability of re-activation for using many times besides the simplicity of separation principle which depend on charge differences (12) .Only 25% of the initial activity was recovered by ion exchange chromatography , and the specific activity actually decreased relatively to that in previous purification step. A complete loss of activity was observed when the antibiotic was left in the ion exchange eluent overnight. For this reason the ion exchange protocol was carried out as quickly as possible at 4°C.One peak appeared when reading absorbance at wavelength of 280 nm ,this fraction containing antimicrobial active compounds were collected , concentrated and lyophilized. (13) .

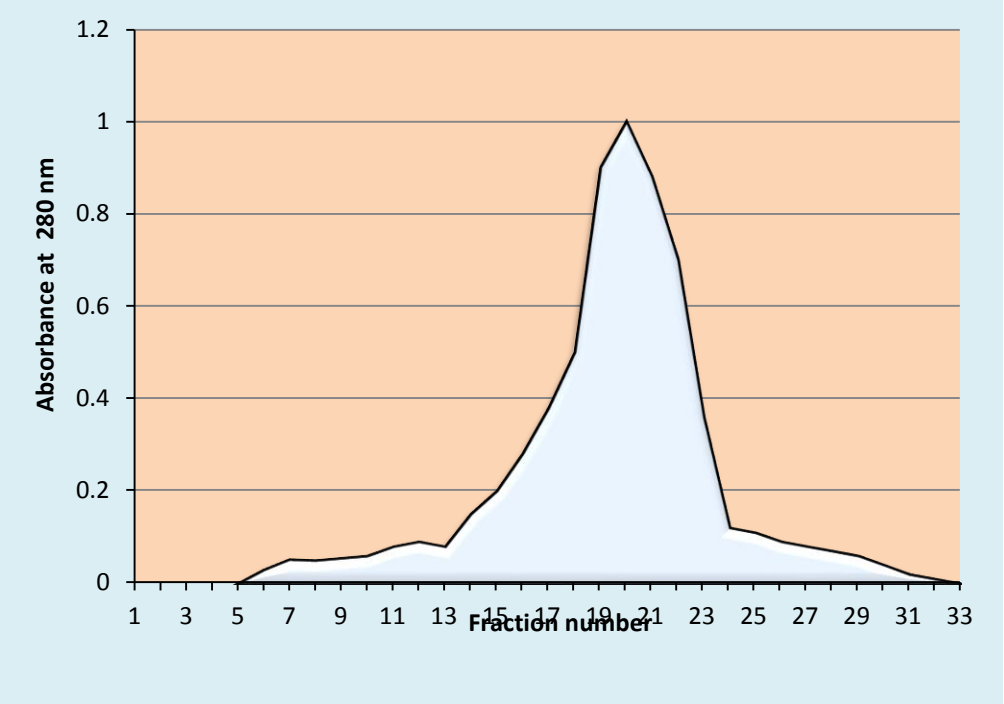

Figure 2 : Ion exchange chromatography of *B.subtilis* S4 by using ion exchange column (2.5 ×10 cm) ,with phosphate buffer , flow rate 100 ml/hr and 8 ml /fraction.

3.3.2: Gel filtration chromatography

The results showed two high activity peaks , the first peak including few column fractions ranging between (5-12) which contain purified biosurfactant that obtained after gel filtration, the second peak , fractions ranging between (19-25) ,all these fractions were collected , pooled and lyophilized for the next experments.

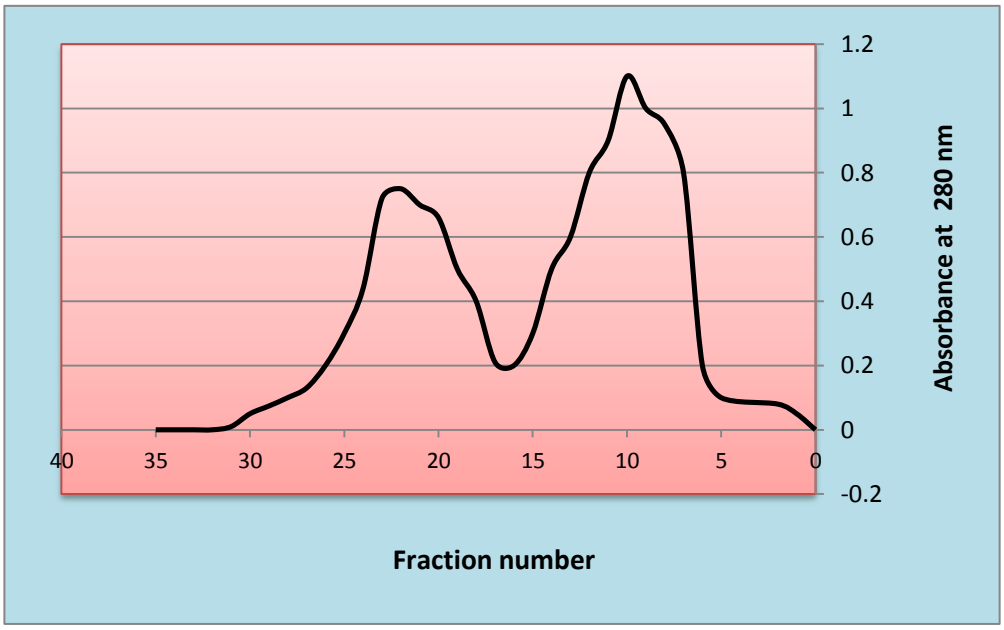

Figure 3: Gel filtration chromatography of *B.subtilis* S4 biosurfactant by using Sepharose-6B column (2.5 ×15 cm) with phosphate buffer , flow rate 100 ml / hr and 9 ml / fraction.

Similar study found that the biosurfactant produced by *B.circulans* and purification by gel filtration chromatography give also two peaks from the fractions (7-12) and (19-22) and the activity was found in these fractions .

3.4 :Preliminary characterization of biosurfactant

3.4.1 :Detection of carbohydrate presence

The surfactants produced can differ widely from one genus to another ,the results showed that the purified lipopeptide do not have any suger moiety as shown molish's .

3.4.2: Determination of lipid content

The crude biosurfactant after extracted with chloroform: methanol solvents it found to have 17 % lipids .

3.5: Detection by HPLC-MASS spectrum

The results of Mass analysis (Figure 4) revealed that a cluster of peaks with mass/charge (m/z) ratios between 1,036 and 1,058, which could be attributed to protonated surfactin isoforms .The peak with a m/*z* ratio 1,049.69 corresponds to the mass of [M+Na]+ ion of surfactin with a fatty acid chain length of 14 carbon atoms (14).

The natural surfactin of *B.subtilis* was mixture of isoforms with slightly different properties as result of substitutions in amino acids and the aliphatic chain (15).This result close to other study showed that the surfactin group of lipopeptides produced by *B.subtilis* K1 within the mass ranging m/*z* between 994 -1,065 (16) .

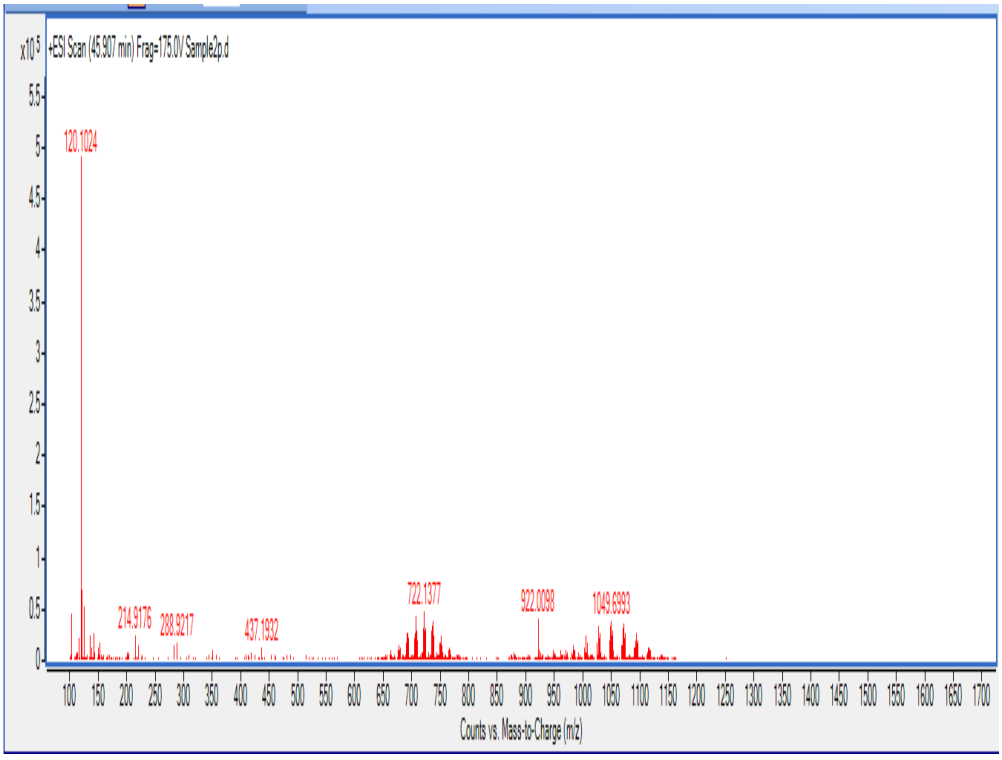

Figure 4: Mass spactrum analysis of purified biosurfactant

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