JOINT INSTITUTE FOR NUCLEAR RESEARCH
Department of Raman Spectroscopy, Frank Laboratory of Neutron Physics

FINAL REPORT ON THE SUMMER STUDENT PROGRAM

Raman and CARS microspectroscopy of biofilms.

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Tables of contents

Table of Figures

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Shimaa Husien

Abstract

Biofilm is a phenomena that most of organisms use to live under stress conditions where micro-organisms grow on a surface and covered themselves with Extracellular polymeric substance (EPS). Since, micro-organisms is everywhere around us, therefore, understanding of biofilm composition and structure is an essential matter to protect the world from their harmful effects or in some other cases to benefit from this phenomena. Raman spectroscopy is a type of characterization is a non-destructive technique for identification of biofilm composition and structure. In this study Raman microscopy was used to analyze the Sulphate Reducing bacteria (SRB) biofilm and *Anabaena* sp. algal strain. At first biofilm formation conditions were prepared and leaved nearly three weeks for both of them. Raman spectra of SRB biofilm was detected at the 3^{rd}, 7^{th}, 8^{th}, 10^{th}, 12^{th}, 15^{th}, 22^{nd}, and 25^{th} days periodically, whereas algal biofilm was analyzed after three weeks. It was recorded a formation of lipids, proteins, nucleic acids, and polysaccharides in the SRB biofilm. Algal biofilm showed weaker spectra because it needs more time and special conditions to grow. Furthermore, CARS imaging was recorded, IR, and atomic force microscope in order to further characterization of biofilms.

1. Introduction

Raman spectroscopy is a non-destructive technique that provides fingerprint information about an analyte of interest. Raman technique depends on the inelastic scattering of monochromatic light such as laser source. Inelastic scattering means that the photons frequency in monochromatic light changes through interaction with a sample where photons of the laser light were absorbed by the sample then re-emitted. After that the frequency of the re-emitted photons were shifted up and down in contrast to original monochromatic light frequency, that known as Raman Effect. Raman spectroscopy can be used for studying liquid, solid and gaseous samples. Raman spectroscopy has been used by biologists and chemists to identify chemical compounds, their functional group and conformation determination of complex biomolecules, such as DNA and protein. Furthermore, it can be rapidly determine the structure and the chemical composition of the sample in all states solid, liquid, gas or gel [1, 2].

1.1. Principle of Raman spectroscopy

Raman spectroscopy principle is based on inelastic light scattering of molecules that known as Raman Effect, was defined previously. In the Raman scattering process, photons of light interact with a molecule and then scattered in the surroundings in all directions. After that photons loses or gain energy which is then detected and analyzed.

Raman shift is the change in the frequency between the emitted and incident light and the change magnitude was determined by the various vibrational modes of the molecules in the sample. The Raman shift is a wavelength shift so that it was measured by cm^{-1}. Raman shifts were determined according to the incident and scattered light as follow:

most of incident light were scattered elastically with no change in frequency and this scattered known as (Rayleigh scattered). Whereas, a small portion of light (nearly 10^{-8} of the incident

beam) were scattered in-elastically and this type was identified as Raman scattering. On the other hand when the scattered light had high energy than the incident light where (□□*f* is positive), difference in the energy destruct the photon and the vibrational state of the molecule have been changed and the anti-stock lines were detected. The anti-stoke lines have high energy than more than the incident light that makes it have shorter or more blue wavelength. Whilst, stoke lines occur when the scattered photons has less energy than the incident photons in the state of negative (□□*f*) and the difference in energy known as a phonon. This stoke lines contain more red wavelength than the incident light. Generally, the intensity of the stoke lines is more than anti-stoke at standard temperature, therefore, the lowest vibrational states have more occupation probability. As a result of the previous fact, the Raman spectra that appeared in the dissertation will contain the stoke lines only, see Fig (1).

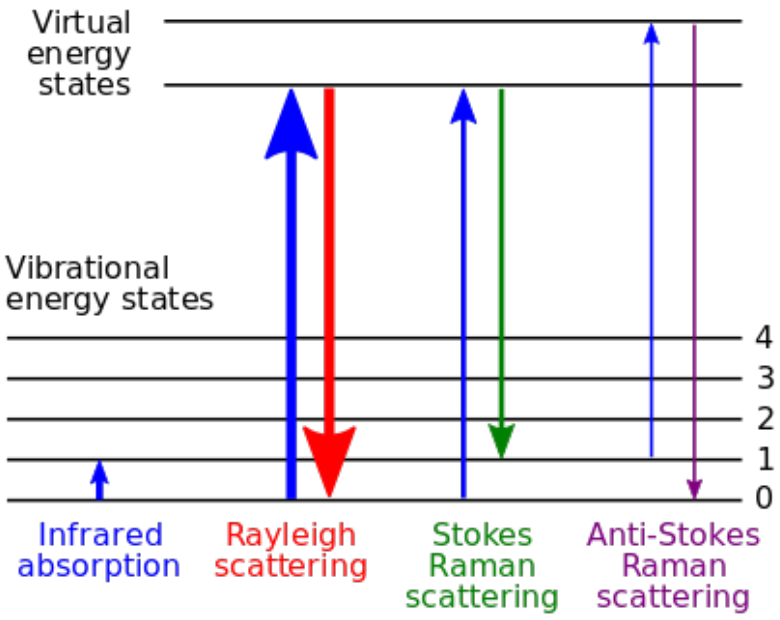


Fig (1): Energy-level diagram.

1.2. Types of Raman spectroscopy

There are a number of advanced types of Raman spectroscopy that will be discussed as follow:

1.2.1. Resonance Raman spectroscopy

Resonance Raman spectroscopy in which the excitation wavelength is matched to an electronic transition of molecules or crystal, so that enhancement for excited electronic state with the vibrational modes were noticed. Resonance Raman spectroscopy is useful for studying large molecules such as polypeptides and association of normal modes with their observed frequency shifts [3, 4].

1.2.2. Tip Enhanced Raman "TERS"

TERS is based on using metallic usually silver/gold-coated AFM and STM tip to enhance the intensity of the Raman signals of molecules [5].

1.2.3. Stimulated Raman spectroscopy

Stimulated Raman Scattering (SRS) provides amplification of the week spontaneous Raman signals by > 10,000x enabling high-speed label-free chemical imaging. SRS has many advantages such as no dyes or labels needed, high-speed (up to 30 frames/s), chemically specific, deep tissue imaging with near-IR lasers, three-dimensional resolution without physical sectioning, reduced auto-fluorescence background, See Fig (2b).

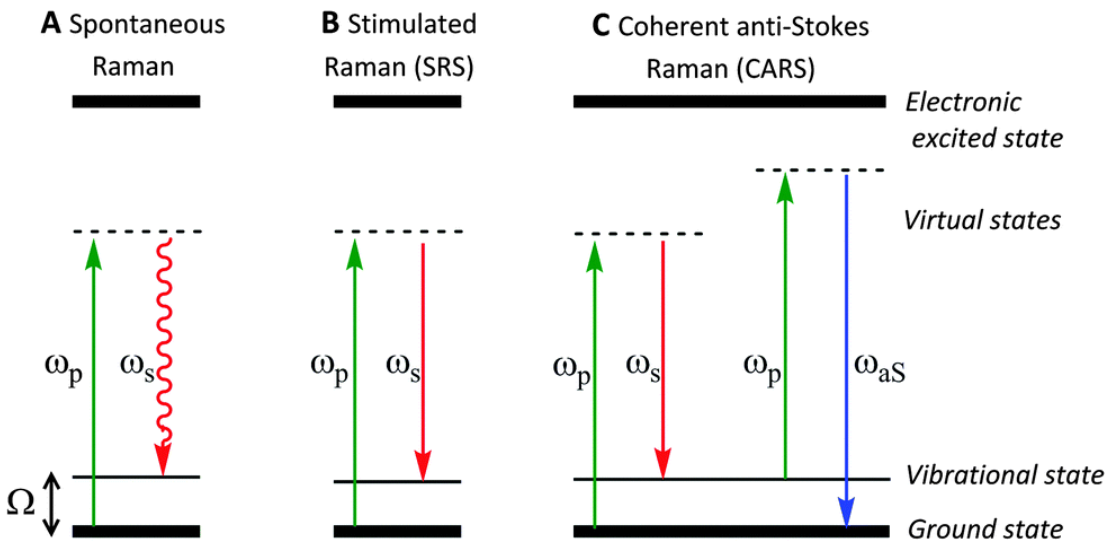


Fig (2) Energy diagram of (A) spontaneous raman, (B) SRS, and (C) CARS.

1.2.4. Transition Raman

Transition Raman permits probing of a significant bulk of turbid material such as capsules, powders, living tissue. It can be used in medical diagnostics applications.

1.2.5. Coherent Anti-stoke Raman (CARS)

CARS is a non-linear four wave mixing process that is used to improve the weak spontaneous Raman signals. In CARS technique, both of pump laser beams and stokes laser beam interact together and produce the anti-stoke at frequency $\omega_{CARS} = 2\omega_{pump} - \omega_{Stokes}$, See Fig (2C) [6].

1.2.6. Surface Enhanced Raman Spectroscopy (SERS)

SERS type of spectroscopy is normally performed on silver or gold colloid or a substrate containing silver or gold NPs. The substrate was excited by a laser resulting in an increase in the local electric fields surrounding the metals. In this state Raman intensity are proportional to the electric field and the measured signals increase highly up to 10^{11} times and even more [7, 8].

1.2.7. Shell-isolated NP Raman

Shell-isolated NP Raman spectroscopy in which the tip is replaced by a film of gold- core silica-shell nanoparticles ($Au@SiO_2$). Since each core of gold acts as a tip, the equivalent of hundreds or even thousands of tips are excited in the laser spot at the same time. The chemically inert di-electric shell prevents the interaction between the gold core and the system under study. This interaction prevents adsorbing of some molecules on the signal amplifier that can't be adsorbed on the silica. Furthermore, it prevents contamination of gold core with the system under study. Therefore, this new technique was called "shell-isolated nanoparticle-enhanced Raman spectroscopy" or "SHINERS". It had many applications such as it has been used to probe structure and processes on materials that has different composition and morphology from metal single-crystals to semiconductors and from food samples to living cells [9].

1.3. Raman spectroscopy application

Raman spectroscopy has many applications such as:

- In chemistry where the vibrational information is related to the chemical bonds and asymmetry of molecules. Therefore, it provides a spectral fingerprint by which the molecule can be identified.
- Another field of Raman is the Raman gas analyzer which may be used in medicine for real-time monitoring of anesthetic and respiratory gas mixtures during surgery.
- In solid state chemistry and bio-pharmaceutical industry, Raman spectroscopy can be used not only to identify (ID) active pharmaceutical ingredients (APIs), but also to identify multiple polymorphic forms and it can also be used to identify the polymorphic form of the API.
- In physics, Raman is used to measure temperature, characterize materials, and find the crystallographic orientation of the samples.
- Raman scattering by an anisotropic crystal gives information on the crystal orientation.
- Lately, Raman is used for characterization of live and biological cells.

1.4. Raman spectroscopy of biofilm

Raman scattering is typically a non-destructive technique, that employ low energy laser irradiation, and there is lower interference from water in aqueous samples. Therefore, Raman is very suitable for imaging of live cells. Therefore, in this study Raman spectroscopy was used for SRB and algal biofilm characterization as living cells in order to characterize the matrix of the biofilm and other composition.

1.4.1. Biofilm definition

Biofilm is a phenomena that micro-organisms form it under stress in order to protect themselves from hazardous effects. It is an aggregation of microbial cells that covered themselves with

extracellular polymeric substances (EPS) that are formed from proteins, lipids, polysaccharides, genetic materials and humic-like substances. The role of EPS is providing the micro-organisms irreversible attachment to the surface and protecting them from environmental stress conditions. Fig (4) shows stages of biofilm formation.

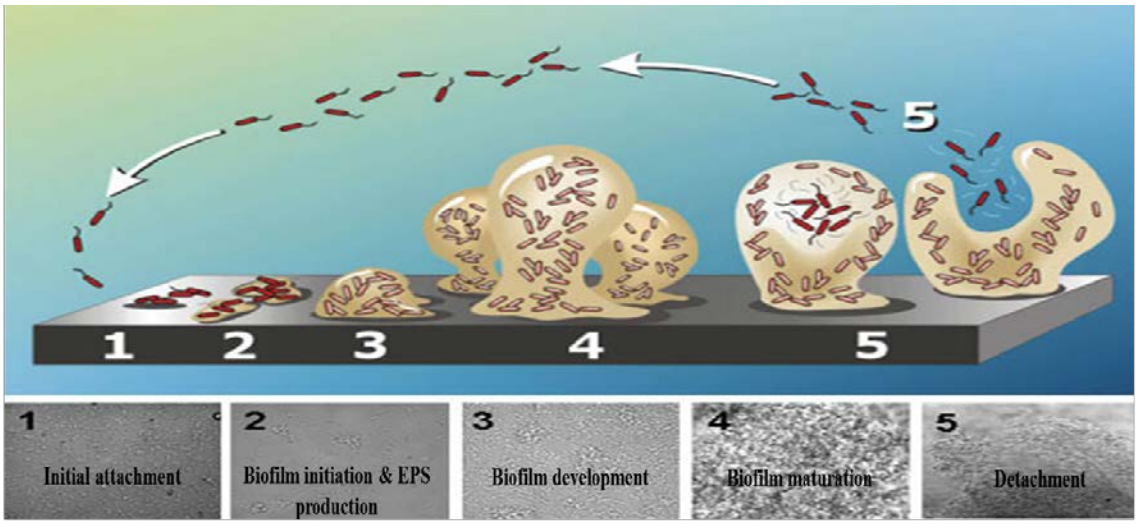


Fig (4): Biofilm formation stages.

1.4.2. SRB Biofilm (Sulphate Reducing Bacteria)

Sulphate reducing bacteria is a type of bacteria that is considered the main cause of corrosion and it grows under an aerobic conditions. Fig (5) illustrates the process of corrosion formation by Sulphate reducing bacteria that form biofilm on the surface of the steal. The reaction occur through different oxidation state as follow:

$4Fe \rightarrow 4Fe^{2+} + 8e^{-}$ (Anodic reaction)

$4Fe + SO_4^{2-} + 4H_2O \rightarrow 3Fe(OH)_2 + FeS + 2OH^{-}$ (Overall reaction)

$SO_4^{2-} + 8H\,(ads) \rightarrow S^{2-} + 4H_2O$ (Cathodic depolarization)

$8H^{+} + 8e^{-} \rightarrow 8H\,(ads)$ (Cathodic reaction)

$8H_2O \rightarrow 8OH^{-} + 8H^{+}$ (Water dissociation)

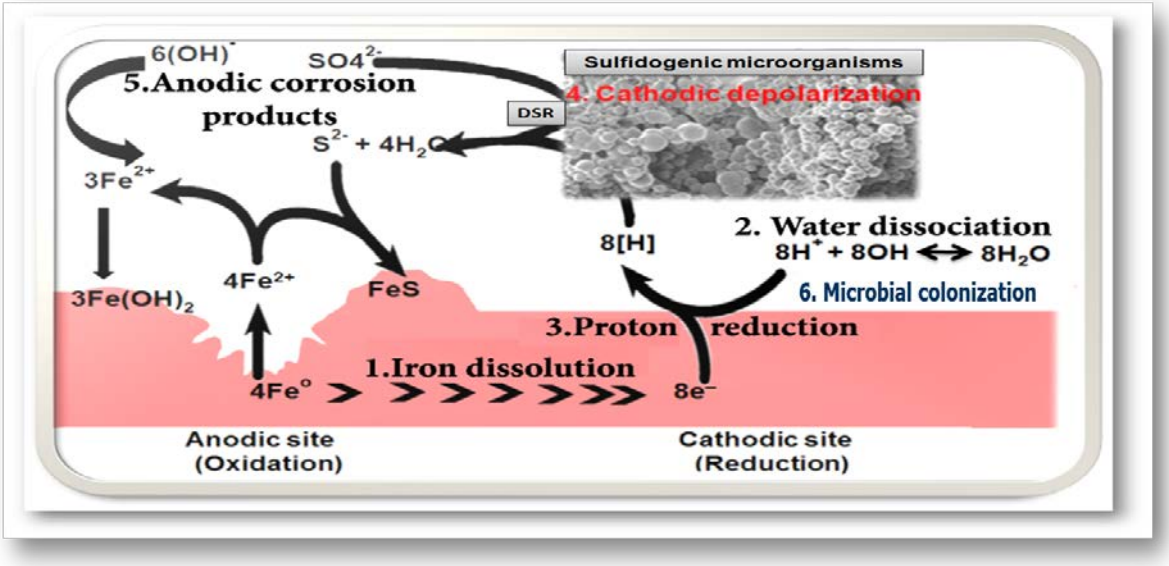


Fig (5): Corrosion formation.

1.4.3. Raman spectra of biofilm

The initial reports that performed Raman spectra of biofilm focused on the investigations of diffusion and distribution processes of certain molecules in the biofilm structure. Lately, in the last two decades, Raman microscope were used to analyze microbial colonies structure and the extracellular polymeric substances compositions of the biofilm [10-12]. Raman microscopy imaging provides information about the spatial distribution of different molecular species within heterogeneous biofilm samples and this enables it to produce the chemical images. Therefore, it has been used for analyzing the spatial distribution and quantification of the microbial composition of the biofilm biomasses [11]. Furthermore, it was used for studying the chemical heterogeneities of mono-species biofilms and further for characterization of multispecies biofilm matrix including microbial constituents and EPS [13]. Additionally, the companion between CLSM and RM can provide us with deeper insights into the composition and the structure of the biofilm matrix, in our state, we did CARS imaging[14].

1.4.4. Importance of Raman spectra of biofilm

Identification the colonies structure and EPS composition of biofilm has many advantages where it helps clarifying the interaction between the environment and the microbial growth.

Furthermore, it helps understanding the molecular dynamics of the biofilms own chaotic system. Moreover, Raman spectra were used in bacterial cells identification and identifying the attachment behavior of bacteria on different surfaces and substrates [15]. Additionally, identification of biofilm composition has many application in industry and medicine.

1.5. Aim of the work

Raman, IR and Atomic Force Microscope (AFM) characterization of biofilms (SRB and algal biofilm).

2. Material and methods

Two types of biofilm were investigated through the study, bacterial and algal biofilm. At first bacterial biofilm were prepared according to the following steps.

1. (Postgate) medium were prepared.

2. By using Elisa plate, media was poured in the plate covered with a layer of vacuum pump oil to prevent air due to SRB bacteria is anaerobic bacteria and steal was put in the media.

3. Strain of sulphur reducing bacteria was inoculated in the plate and leave in incubator at 36 ^{o}C.

4. Different Raman, IR, atomic force microscope were measured at different times starting from the third day. Furthermore, some CARS imaging were recorded and the layout of CARS microscope was shown in Fig (2).

Algal biofilm was prepared by preparing Z-media and fixation of polystyrene at the bottom of the flask followed by inoculation of *Anabaena sp*. After that, the medium was left at room temperature for three weeks and Raman spectra was detected.

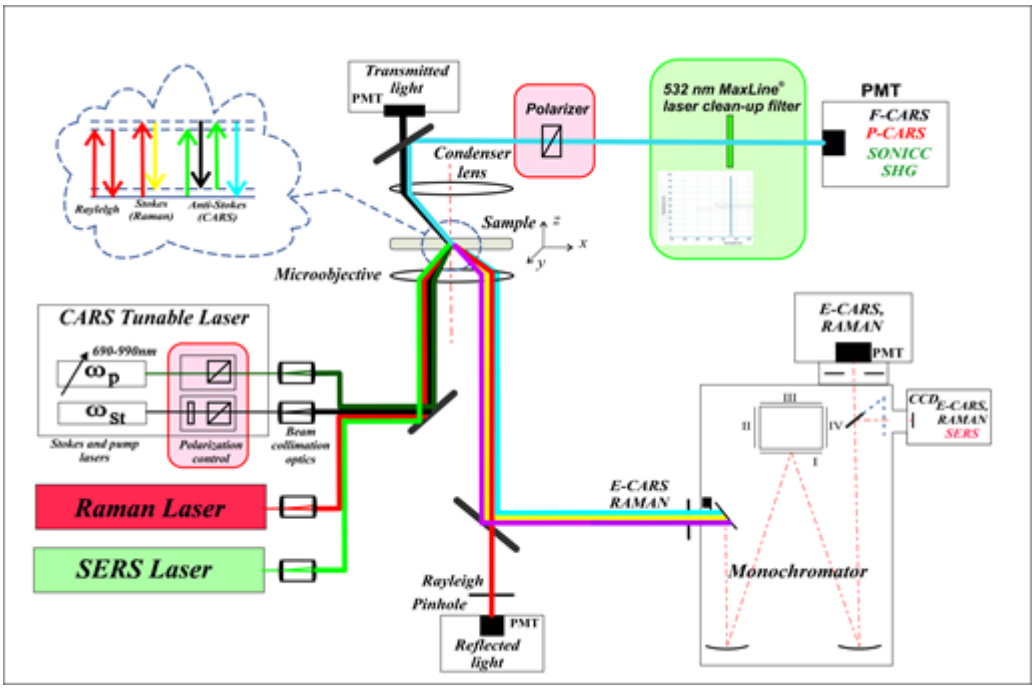


Fig (3): layout of CARS microscope.

3. Results and discussion

3.1. Characterization of sulphate reducing bacteria (SRB) biofilm

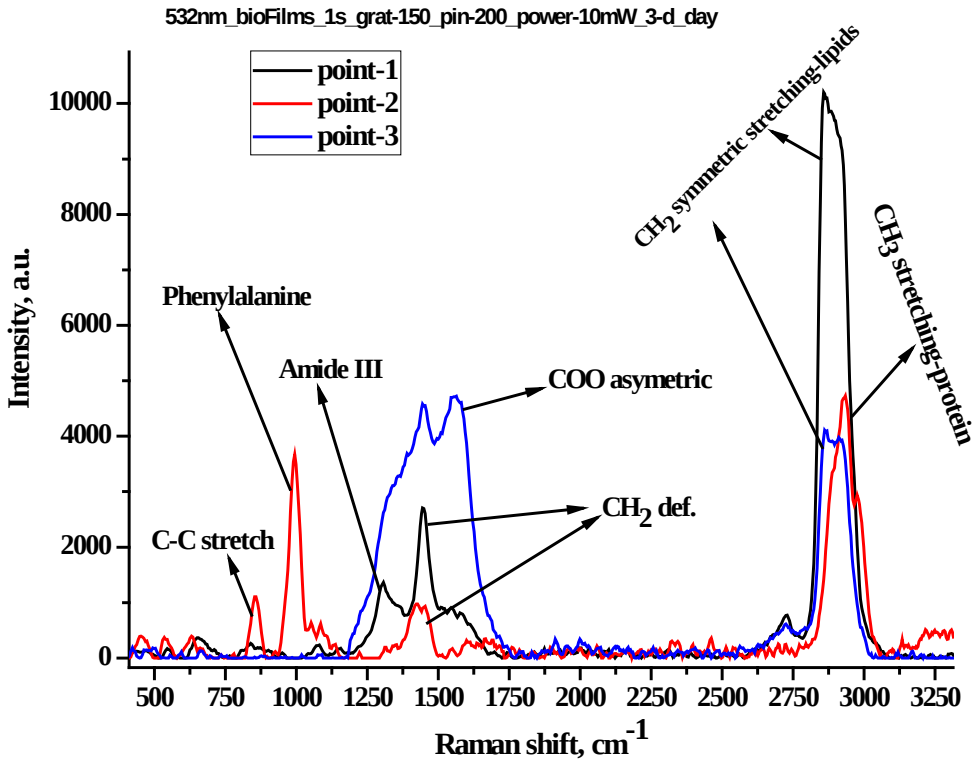


Fig (6): Raman spectrum of SRB biofilm after 3rd days (scratch the surface of the steal)

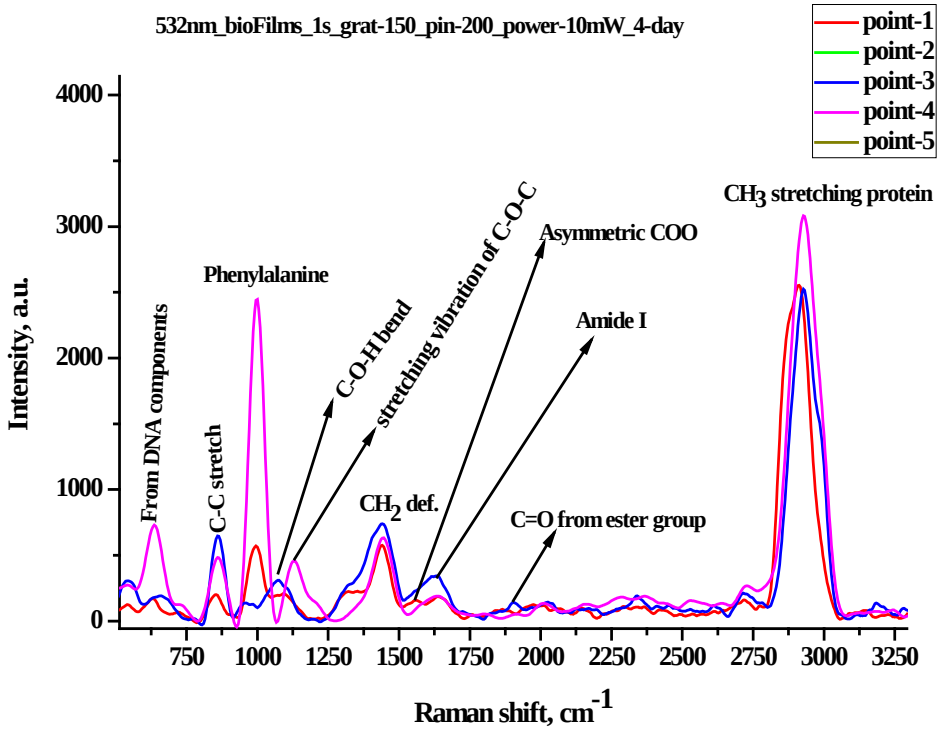


Fig (7): Raman spectrum of SRB biofilm after 4th days (scratch the surface of the steal)

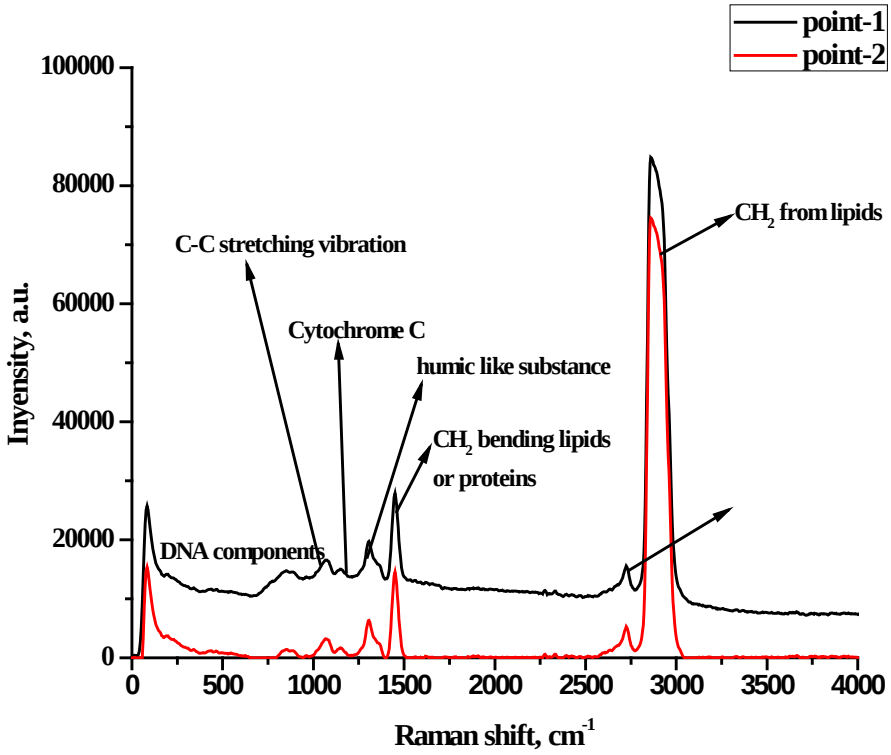


Fig (8): Raman spectrum of SRB biofilm after 7th days (scratch the surface of the steal)

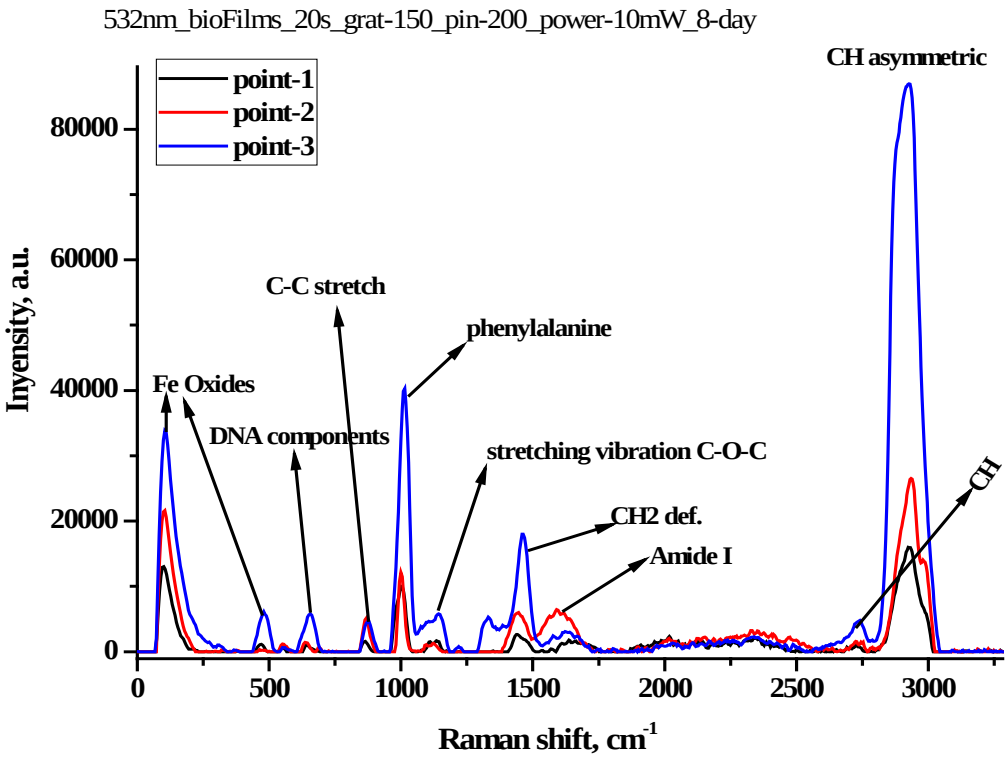


Fig (9): Raman spectrum of SRB biofilm after 8th days (scratch the surface of the steal)

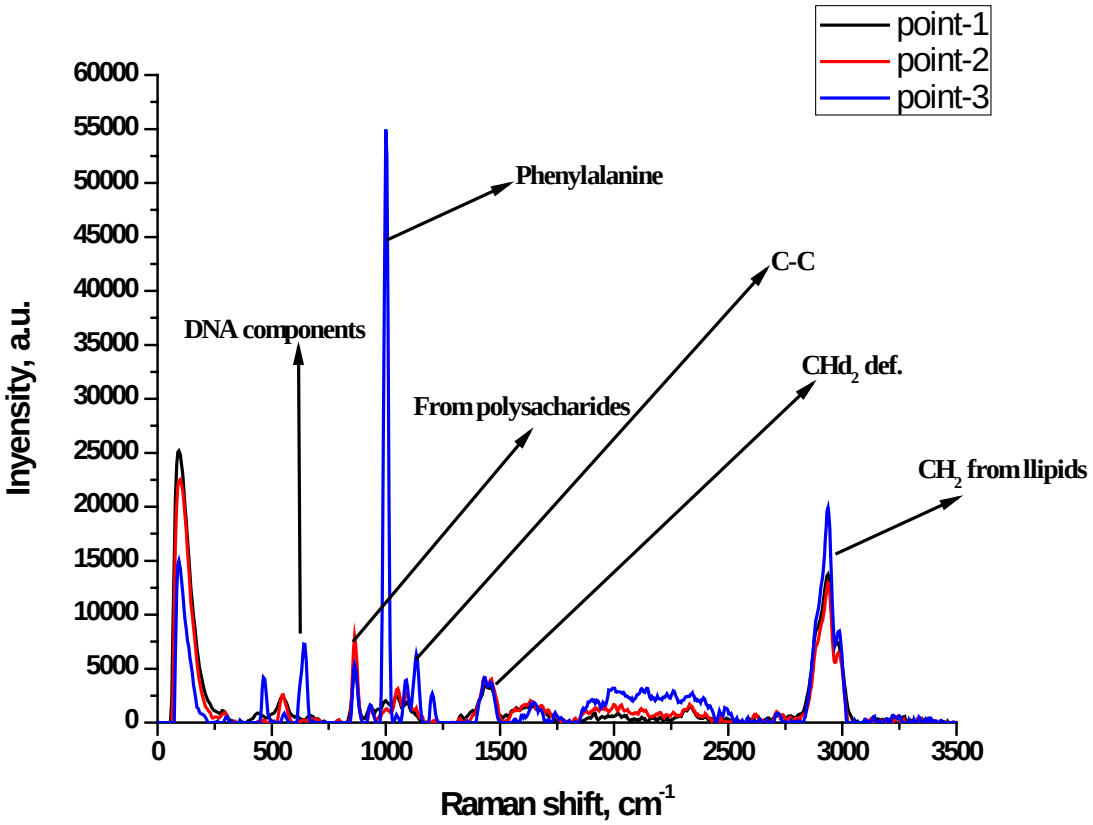


Fig (10): Raman spectrum of SRB biofilm after 10th days (scratch the surface of the steal)

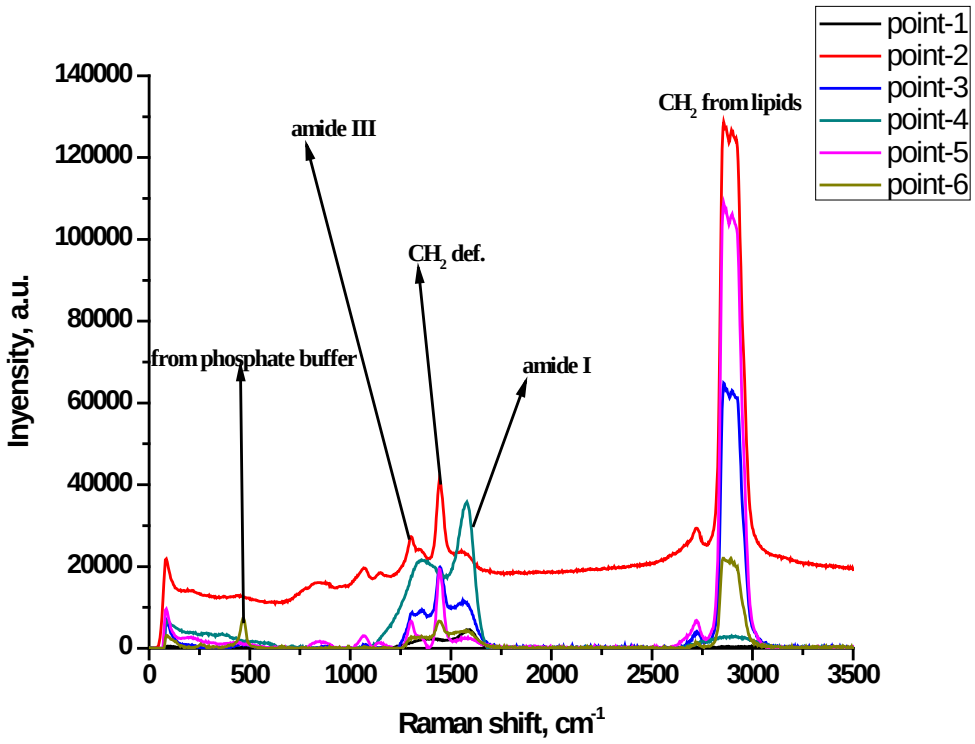


Fig (11): Raman spectrum of SRB biofilm after 12th days (steal substrate)

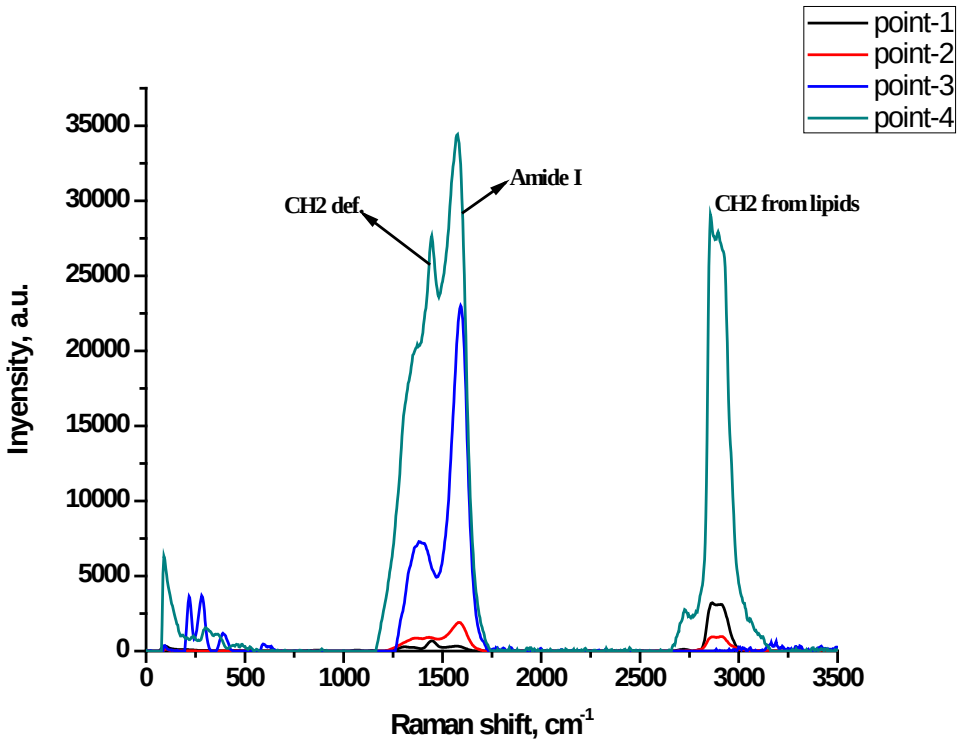


Fig (12): Raman spectrum of SRB biofilm after 15th days (steal substrate)

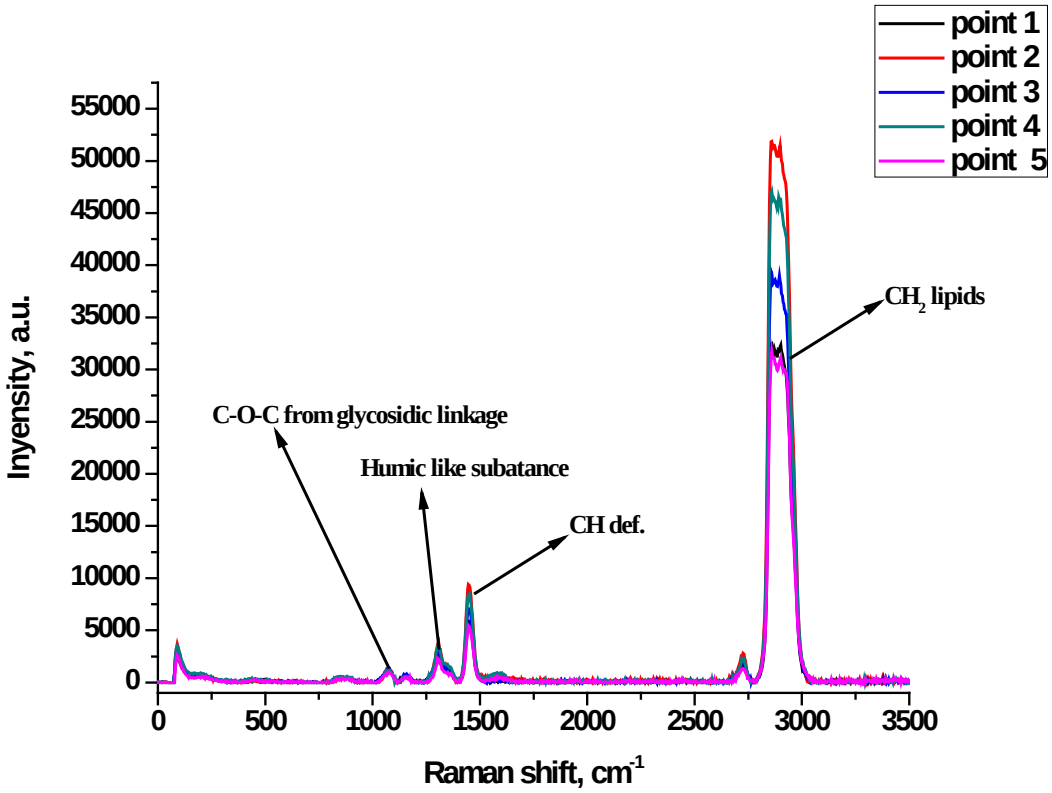


Fig (13): Raman spectrum of SRB biofilm after 22nd days (steal substrate)

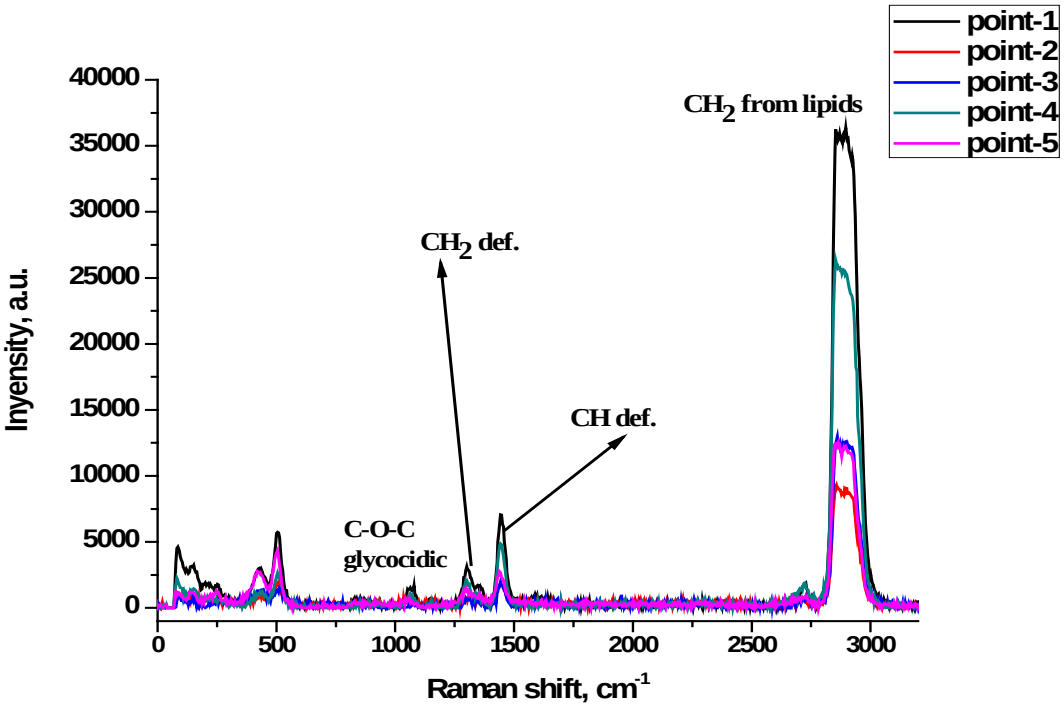


Fig (14): Raman spectrum of SRB biofilm after 25th days (steal substrate)

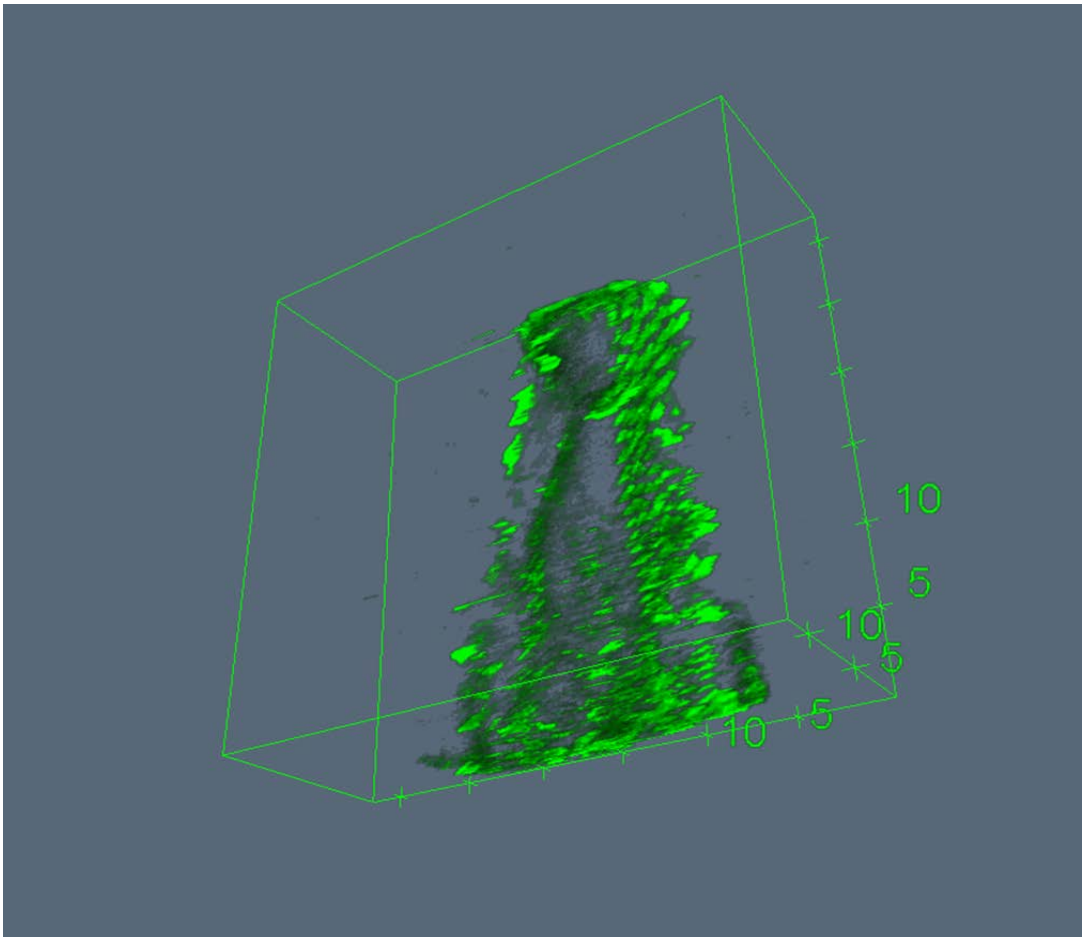


Fig (15): CARS imaging of SRB biofilm after three days (steal substrate)

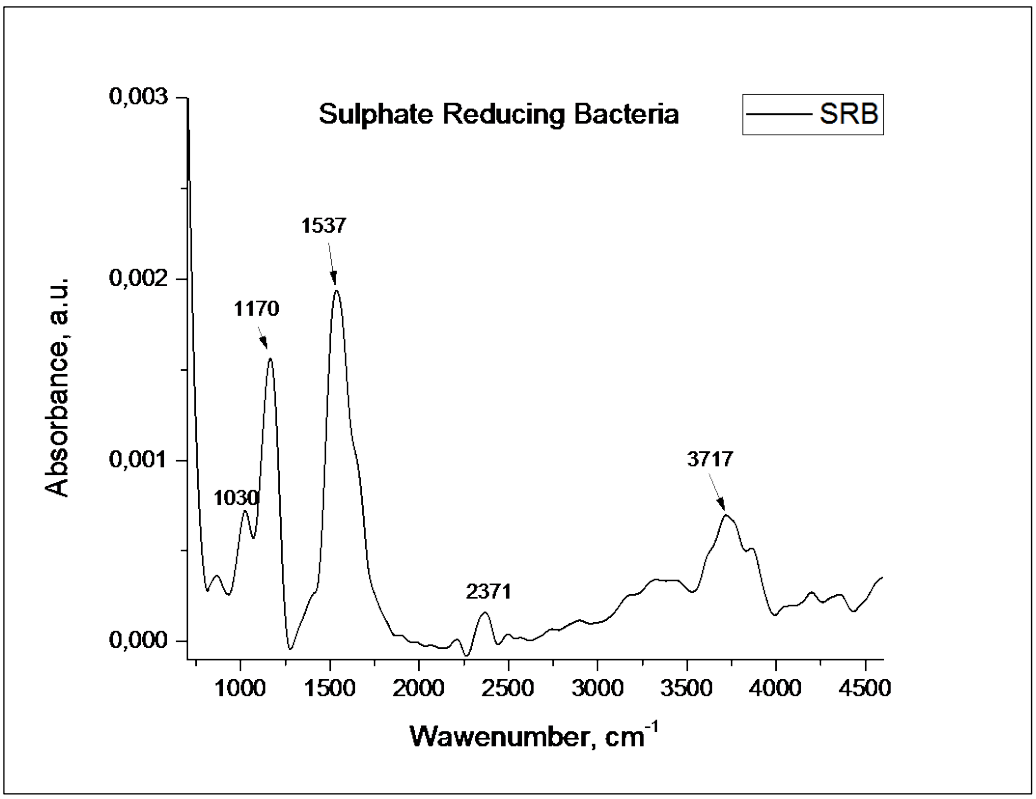


Fig (15): FT-IR of SRB biofilm (steal substrate)

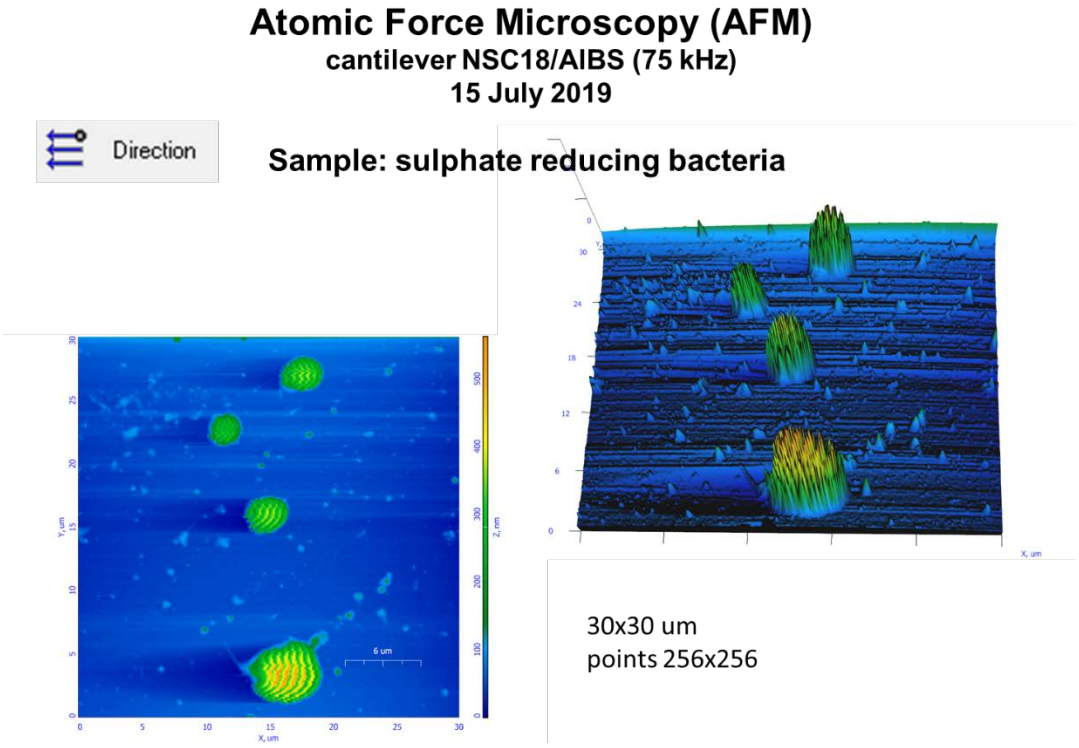
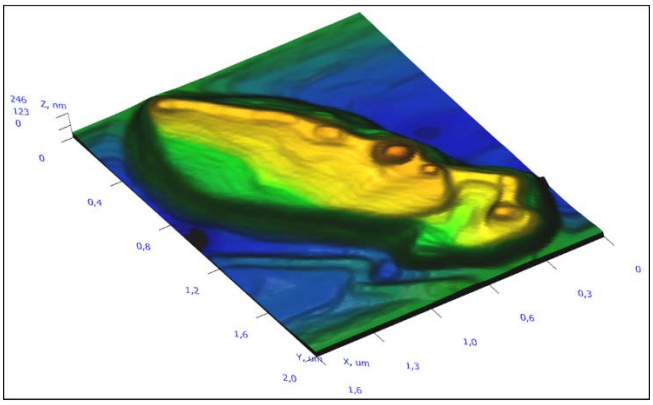


Fig (16): Atomic force microscope imaging of SRB biofilm after 7th days (steal substrate)

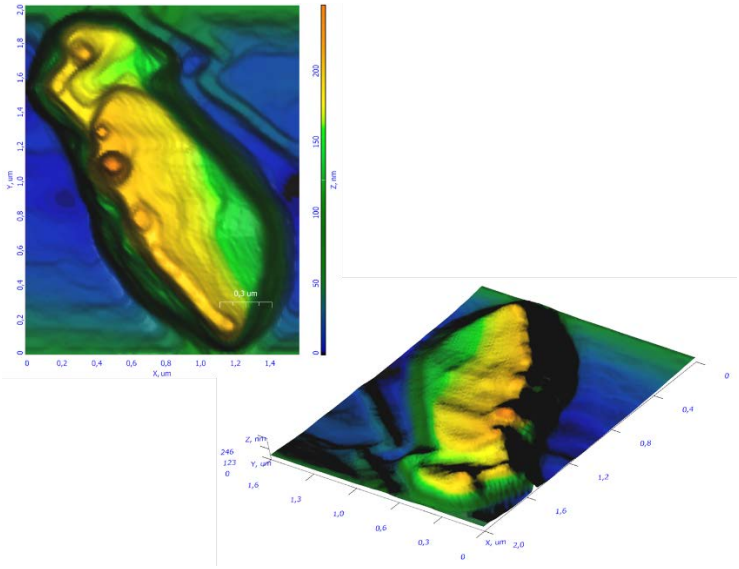
Atomic Force Microscopy (AFM)

cantilever NSC18/AIBS (75 kHz)

Sample: sulphate reducing bacteria



scan area: 2x1.6um
resolution: 256x256



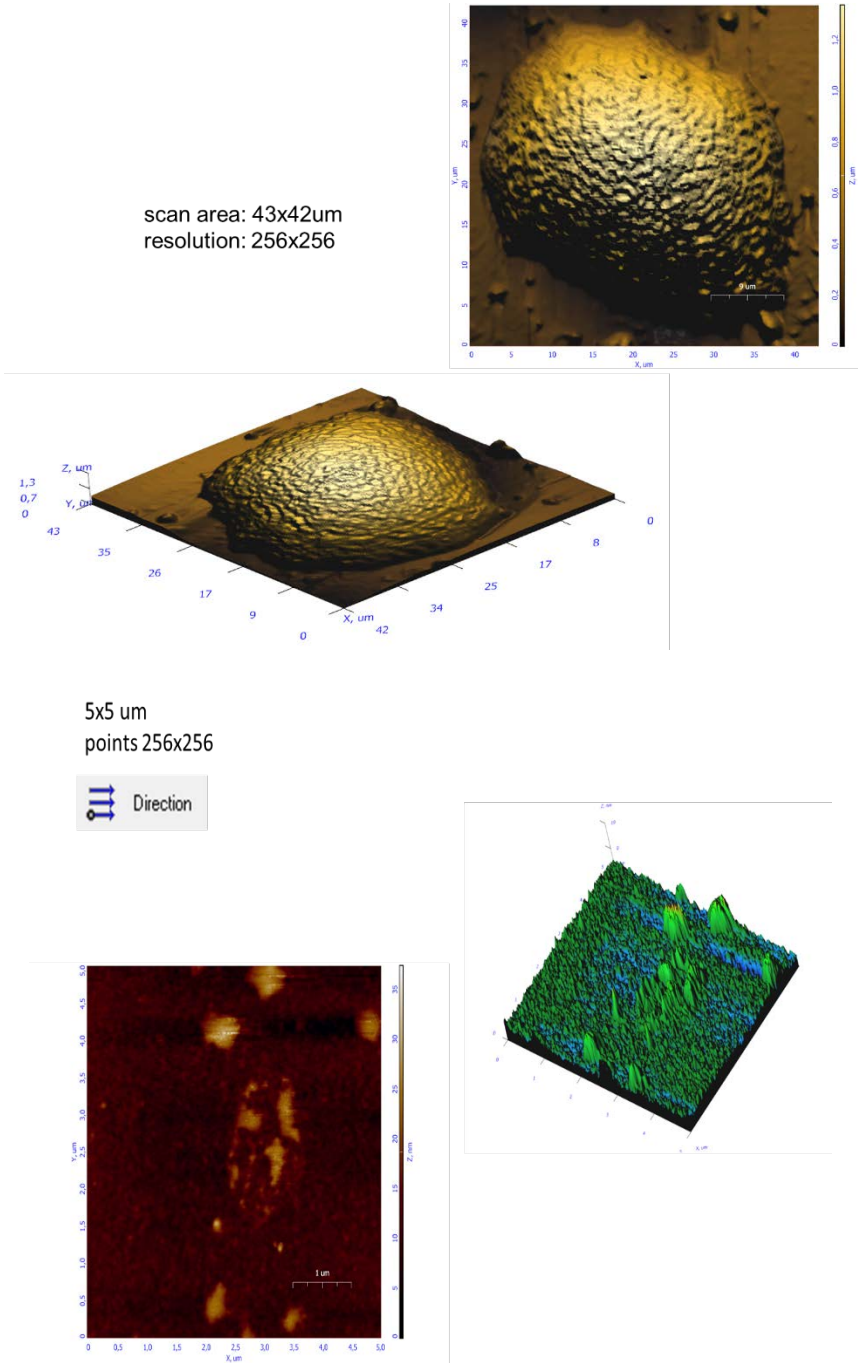


Fig (17): AFM imaging of SRB biofilm after 10th days (steal substrate)

Discussion.

Raman spectra of biofilm that were shown above stated the appearance of nucleic acids, polysaccharides, proteins and lipids peaks as well. It was noticed that in the first 10th days some protein peaks were formed as phenylalanine around 1000 cm^{-1} band**.** Appearance of amide I and amide III was observed at nearly 1655, and 1270 cm^{-1}, respectively. In the first 8th days peak of 2930 cm^{-1} that indicated CH_3 stretching mode of protein was recorded. After the 8th days the

peak was shifted to a round 2800 cm^{-1} that indicated formation of symmetric and a symmetric CH_2 of lipids as shown in figures. This results were in agreement with previous research where it was stated that formation of proteins in the initial phase is more than lipids due to contribution of protein in the initial adhesion and stability of biofilm matrix [16]. Further, peaks were appeared in 1145 cm^{-1} that indicated C-C, 1448 cm^{-1} that referred to CH_2 def. from lipids and proteins, all other peaks were assigned on the spectra. Additionally, CARS imaging, IR spectra and atomic force microscope imaging were performed as a complementary data for further characterization and identification of biofilm.

3.2. Characterization of algae and algal biofilm

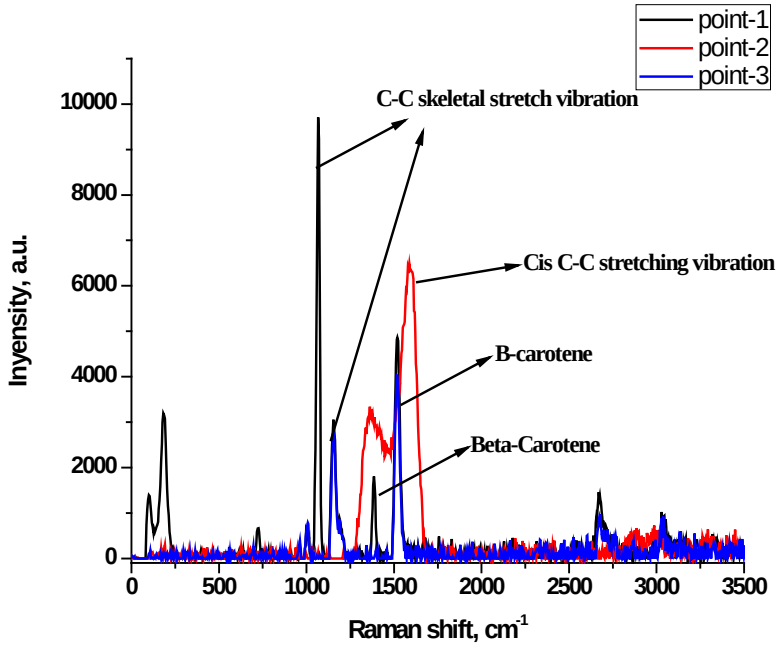


Fig (18): Raman spectra of algae (*Anabaena* sp.).

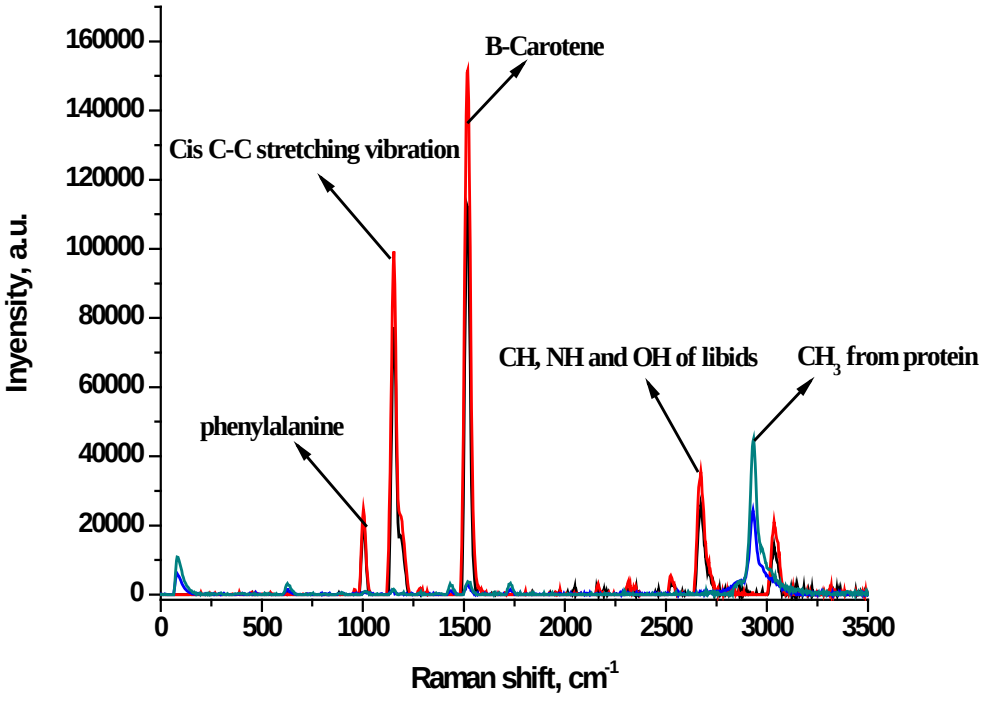
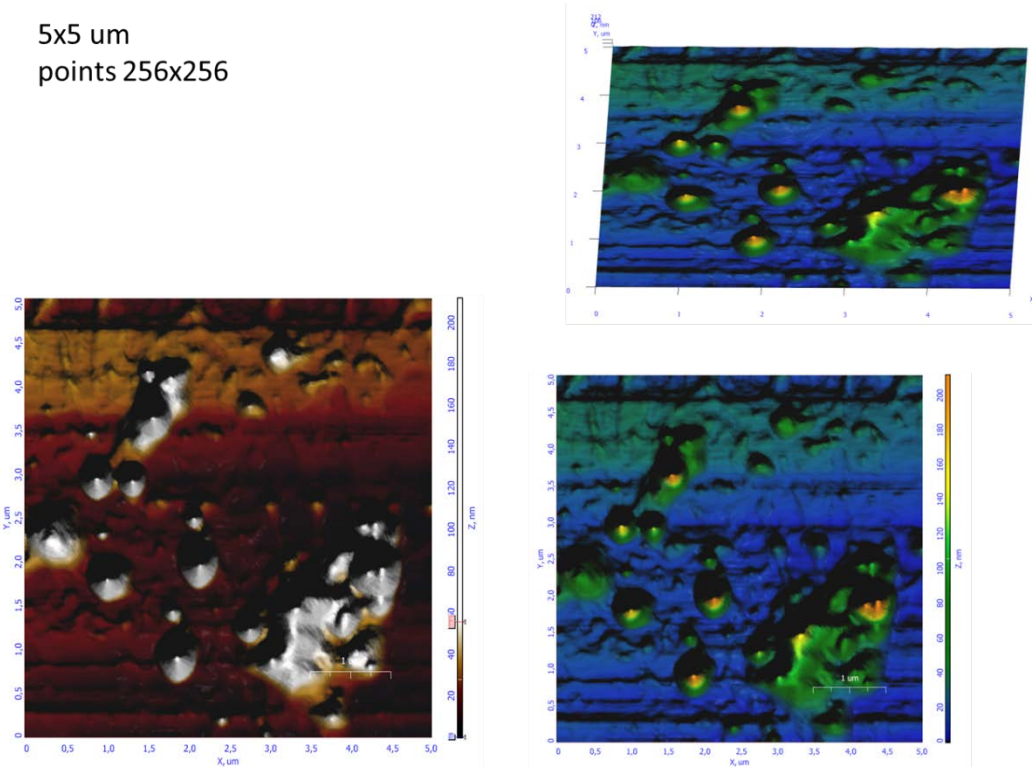


Fig (19): Raman spectra of algal biofilm.



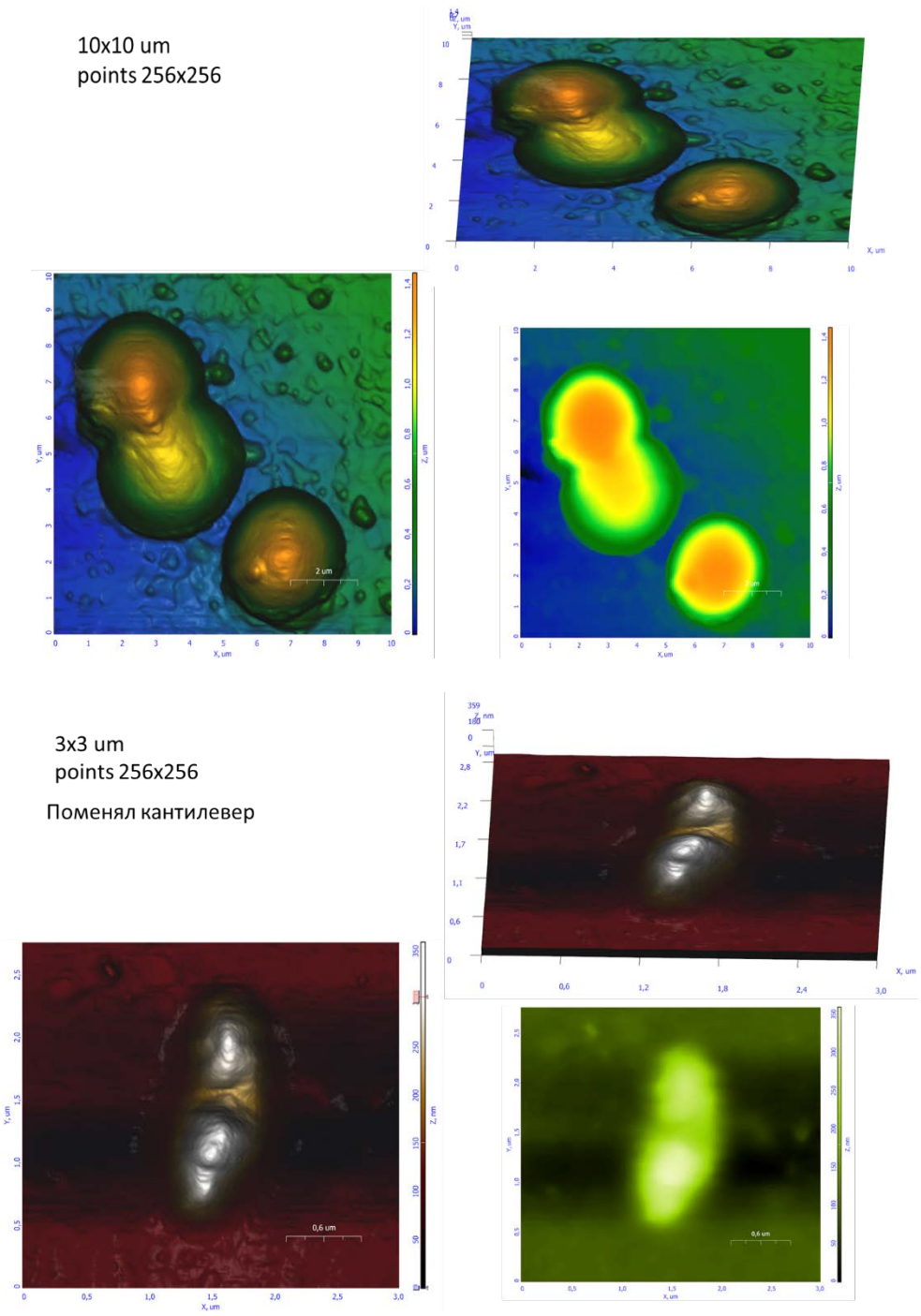


Fig (20): Atomic force microscope of algae.

4. Conclusion

For Raman studies of biofilms we used spectrometer equipped with blue laser source at 473nm and green light laser source operating at 532 nm. The obtained results clearly demonstrate that spontaneous Raman scattering modality is good enough to measure the raman spectra of biofilms. Though, we intend to implement also the SERS modality for these measurements in the future.

Results showed the appearance in spectra of proteins and lipids bands such as phenylalanine around 1000 cm^{-1}, CH_3 from proteins at 2930 cm^{-1}, CH_2 def. from lipids and proteins at nearly 1450 cm^{-1} and some other spectra that illustrated above. Appearance of these raman bands indicated formation of lipids, proteins that refers to biofilm matrix formation. Also, CARS imaging showed formation of sublayers of SRB biofilm. From the obtained results, it can be concluded that Raman spectroscopy is a promising technique for biofilm characterization and identification of its chemical composition and structure. Identification of these specific spectra featured to biofilm composition and structure might be of a proper contribution to the industry and medicine. For instance, it could widen the use of these techniques in different areas such as in clinics, food, marine industries, etc. In this study, identification of SRB bacterial biofilm will help in its inhibition of corrosion formation at steal surface in some industries such as petroleum one. On the other hand, identification of algal biofilm composition will help to increase the amount of biomass that will help in biodiesel and biofuel formation.

5. References

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