QUANTIFICATION OF BIOFILM FORMED AT A PLAN LIQUID-LIQUID INTERFACE

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ABSTRACT

This study describes an in situ technique allows measuring thickness of biofilm growing at liquid-liquid interface. Two probes (100µm) were used to measure the electrical conductance, the first probe penetrates the first liquid (hydrophobic liquid) then the upper surface of the formed biofilm and the later penetrates the second liquid (salt solution), then the lower surface of the biofilm. The hydrophobic liquid-biofilm and biofilm-salt solution interfaces can be identified from the change in conductance values between the three phases. Results of this work showed that rate of biofilm formation were 15µm/day for non-aerated system and 20µm/day for aerated system. The relation between biofilm thickness and its dry weight for aerated and non-aerated systems can be linearly correlated according to the following formulas: **Th. = 0.462DW + 0.063** and **Th. = 0.472DW+0.0429** respectively.

Keywords:
Biofilm, Dodecane, Biphasic media, *Pseudomonas alkanolytica,* Biofilm thickness, Dry weight.

INTRODUCTION

Biofilms have been successfully used in many areas of biotechnology. A large number of research projects are currently being conducted on biofilm reactors for the production of bioactive substances for plant and animal cell cultures, wastewater treatment and biodegradation of soils contaminated with hydrocarbons. Biofilms develop and grow wherever there is a gaseous/liquid interface [1], solid/liquid interface [2] or a liquid/liquid interface [3].

Biofilm is a complex and variable gel matrix consisting of exo-polymeric substances (EPS) which are produced by microorganisms embedded within. The ratios between the number of bacteria and biofilm thickness as well as between EPS and biofilm thickness are not constant and vary according to the nature of bacteria, their physiology and the quality of the environment which they are in contact. Biomass quantity and microbial activity are the parameters mostly used to estimate the amount of biofilm [4].

Different methods have been suggested for direct quantification of biofilm which include the determination of its thickness and mass. Biofilm thickness is an important parameter in both theoretical analysis and practical application of biofilms due to its role in mass transfer.

Biofilm thickness has been determined by using various techniques. Light microscopy is a simple and rapid method which is limited to biofilms with a flat homogeneous surface [5]. Another technique based on measuring the optical density was applied to estimate thickness of biofilms growing on transparent layer using a microprobe made of optical fiber which penetrates the biofilm and the interface water-biofilm is determined from the optical density profile [6]. This technique was used successfully to locate biofilm-fluid and biofilm-substrate interfaces in a tubular reactor by [7].. Biofilm thickness can also be indirectly determined by measuring thermal resistance of the fixed biomass [8] or on the basis of dry weight by calculating bio-volume with the help of conversion factors [9].

All methods suggested for estimation of biofilm thickness are concentrated on biofilms growing on solid surface, none of which can be applied to biofilm developing at liquid-liquid interface. Such system of biofilm is widely used for the purpose of biodegradation of hydrocarbons.

Experimental Work

This study describes an in situ technique allows measuring thickness of biofilm growing at liquid-liquid interface. Two probes (100µm) were used to measure the electrical conductance, the first probe penetrates the first liquid (hydrophobic liquid) then the biofilm and the later penetrates the second liquid (salt solution), then the biofilm. The interfaces hydrophobic liquid-biofilm and biofilm-salt solution can be identified from the change in conductance values between the three phases

MATERIALS AND METHODS

1- Apparatus:

An open cylindrical tank used in this study for developing the biofilm was made of Pyrex glass. It was 22 cm diameter and 10 cm depth with a working volume of 3.6 litters (Figure 1).

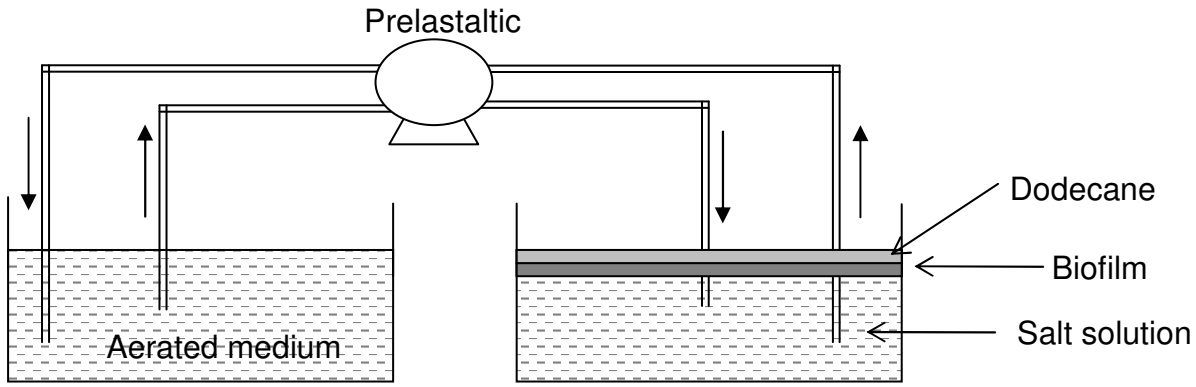


Figure (1) Schematic diagram of the apparatus

The biofilm reactor was fed with 3500 ml of the medium culture and dodecane was added in a (2 %v/v). After inoculation, the biofilm was allowed to grow for during a period of 10 days.

The same procedures were carried out with pre-aerated the culture medium. For that purpose, two open reactor tanks with the same dimensions were used. The biofilm was allowed to grow in the first reactor while the other was used for aerating the culture medium which is recycled to the reactors at a rate of 1ml/s by a peristaltic pump to achieve a hydraulic residence time of 1 hr. The same pump was used to maintain the effluent flow at the same flow rate. From the resulting data of dodecane layer measurements, substrate (dodecane) consumption rate could be predicted. n-Dodecane commercialized by Fluka was used as the only carbon source.

2- Microorganisms:

The strain of *Pseudomonas alkanolytica* used in this study was obtained from the American Type Culture Collection (ATCC 21034). This strain was chosen because of its ability of adherence to hydrophobic liquids.

3- Biphasic culture:

A mineral salt medium proposed by [10] was used as the aqueous phase. The mineral salt medium composition was as shown in table (1). All salts were dissolved in one liter osmosed water. The pH level was adjusted to 7.4 using 1M NaOH and the medium was autoclaved for 20 minutes at 121^{o}C. The sole carbon source was n-dodecane (2% v/v).

Table (1) Composition of culture media

Compound	Quantity
$(NH_4)_2SO_4$	2 g
Na_2HPO_4	3.61 g
KH_2PO_4	1.75 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2$	50 mg
$FeSO_4.7H_2O$	1 mg
$CuSO_4.5H_2O$	50 mg
H_3BO_3	10 mg
$MnSO_4.5H_2O$	10 mg
$ZnSO_4.7H_2O$	70 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 mg

The choice of dodecane as a sole source of carbon was to simplify the study, as the assimilation of a mixture of hydrocarbon is difficult with respect to a pure hydrocarbon. Moreover, dodecane represents 42% of the petroleum pollutants.

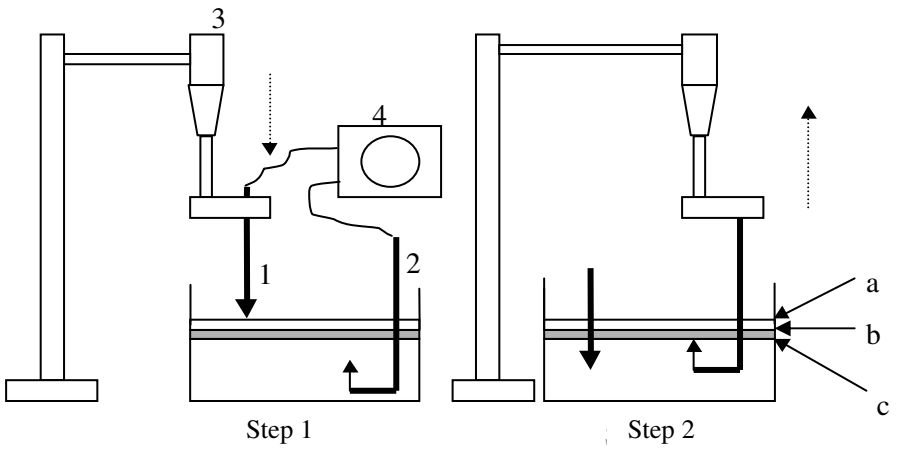
4- Measurements

4.a- Biofilm thickness

The proposed system used to measure biofilm thickness is illustrated in (figure 2). The technique of measurement was carried out in two successive steps:

In the first step, the probe (1) was mounted on a manual micromanipulator; it was introduced from the top of the reactor perpendicular to the biofilm. Location of the probe tip in the layer of hydrocarbon was monitored with the help of binocular microscope. Electrical conductance was measured through the dodecane layer while the probe (2) is immersed in the medium salt.

A sudden change in the value of electrical conductance is observed when the probe (1) touches the biofilm (point "b"), then the interface dodecane-biofilm can be identified and the thickness of the hydrocarbon layer can be determined (Step 1).



1, 2- Electrodes

3- Micrometer

4- Conductivity meter

a- Air – Dodecane interface

b- Dodecane – Biofilm interface

c- Dodecane – Salt solution interface

Figure (2). Schematic diagram of the apparatus

In the second step, the probe (1) is immersed in the medium salt and probe (2) was mounted on the micromanipulator and introduced from the medium salt perpendicular to the bottom of the biofilm. An abrupt decrease in the value of electrical conductance is observed when the probe (2) reaches the medium salt-biofilm interface (point "c"), which was slowly moved up into the biofilm and then the dodecane layer till the dodecane-air interface which can be identified with the help of a binocular microscope (Step 2). The first step determines thickness of hydrocarbon layer and the second determines thickness of both biofilm and hydrocarbon layer, then biofilm thickness can be calculated as the differences between the two values.

4.b- Dry weight:

Total amount of biofilm was measured at different ages in terms of dry weight. For that purpose, a series of experiments were carried out in which biofilm was allowed to grow for a certain age (1, 2, 3,…13 days). Dry weight of biofilm was determined according to the following technique:

Samples were centrifuged for 10 min at 20,000 rpm at ambient temperature. The resulting supernatant was composed of the aqueous phase and a top viscous layer consisting of dodecane, cells and polymer. The aqueous phase was separated while the pellet and the top layer were treated with 15 ml of a solvent mixture (acetone & petroleum ether; 3:1 v/v). The pellet was suspended in osmosed water and filtered through pre-dried cellulose acetate filters (0.2 μm pore size) in a vacuum filtration apparatus. The filters were dried to a constant weight in a vacuum oven at 70^{o}C.

RESULTS AND DISCUSSION

Figure (3) shows the formation of biofilm at dodecane – salt solution interface. It can be noticed that the surface of the formed biofilm is non homogeneous, so the determined thickness represents the average value.

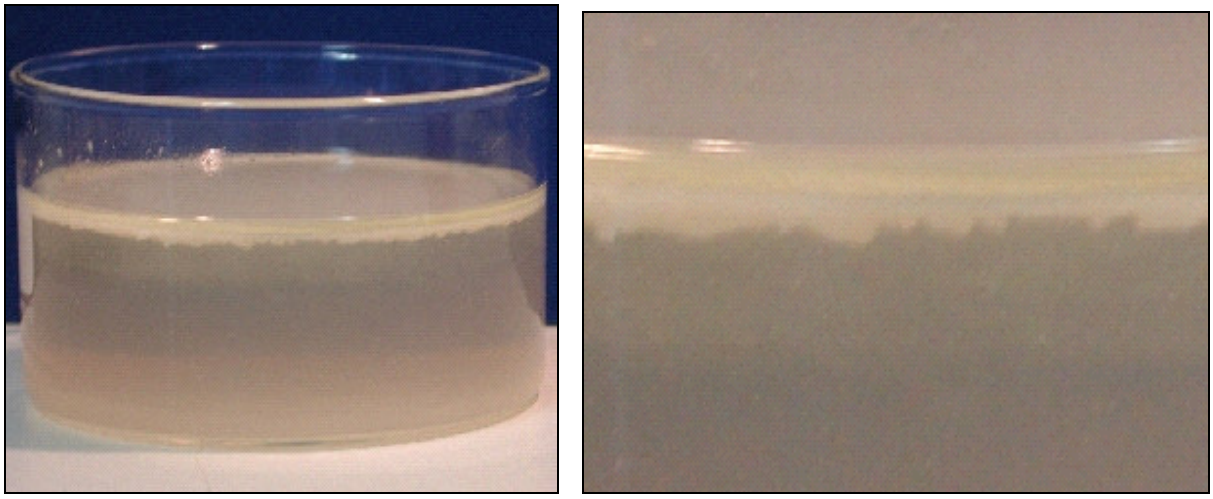


Figure (3). Formation of biofilm at liquid – liquid interface

Results of measurements, interpreted and calculated according to the above procedures, are presented in figure (4) for both formatted biofilms either non-aerated medium or aerated medium.

Dodecane-biofilm interface is marked as sudden change in electrical conductance during movement of the probe (1) down, while the biofilm-medium salt interface is marked as a sudden decrease when the probe (2) reaches the biofilm. Calculated values of biofilm thickness for both biofilms (aerated & non-aerated) during 13 days are illustrated in figure (3). These values represent an average of 5 measurements ($\pm$ 10 μm) at different location as the thickness of biofilm is non homogeneous.

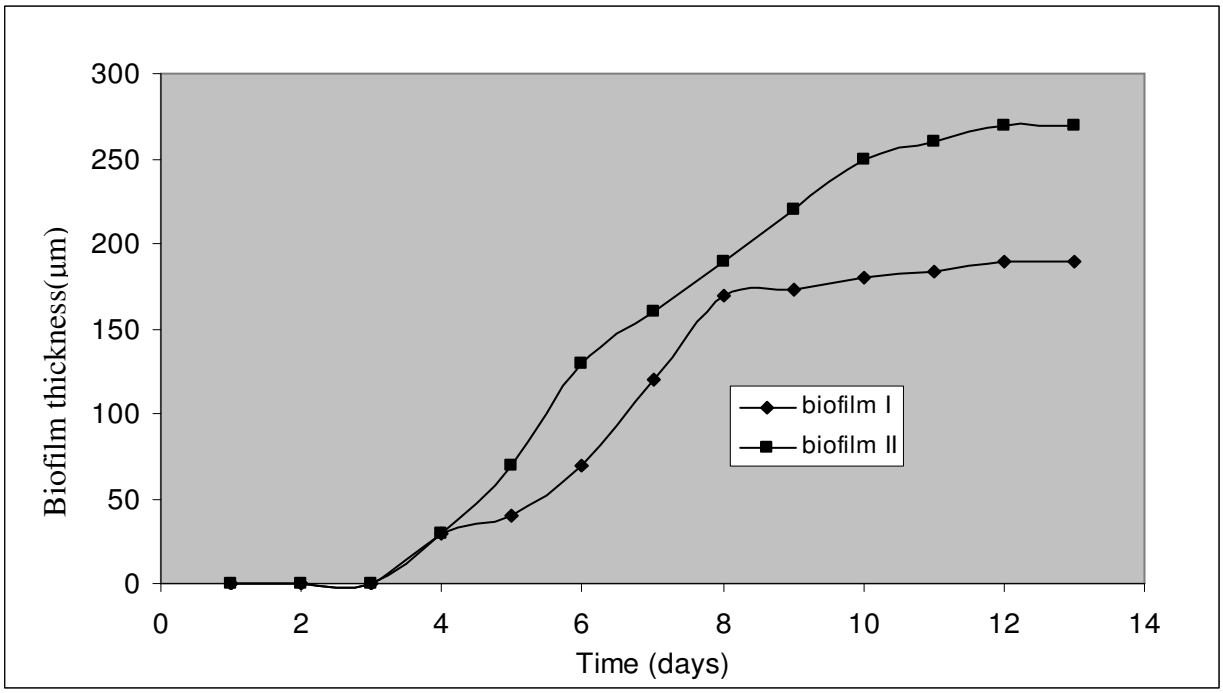


Figure (4). Time course for biofilm thickness

To avoid difficulties of biofilm penetration by the probe as biofilm is growing on liquid substrate, two movable sensors were used and biofilm thickness was determined indirectly.

The values of electrical conductance through the hydrocarbon layer were the same as that of air, while a remarked increase in this value was observed when the sensor touches the biofilm and it is still lower than that of the medium salt. This can be explained by the high percentage water content of biofilm (>90%) [4].

A critical value of biofilm thickness was observed in the two cases in a period of 8 days, it can be explained by the limitation of substrate transfer through the biofilm which leads to stopping of microorganisms growth and hence formation of extracellular polymer. Such critical value of biofilm thickness was higher in case of pre-aerating medium as in the other case, another parameter controls the growth rate, and this parameter is the limited quantity of oxygen in the medium culture.

A good correlation was observed between biofilm thickness and specific substrate consumption for both biofilms as ml/cm^2 is illustrated in figure (5).

For the same thickness of biofilm, we can observe that the consumed amount of substrate in the case of pre-aerated biofilm was higher than that for non-aerated system, hence it can be concluded that aerated system results in increasing of reaction rate in addition to increasing of critical thickness of biofilm. It can be explained by the fact that, limitation of oxygen in the second case leads to stopping of micro-organism growing.

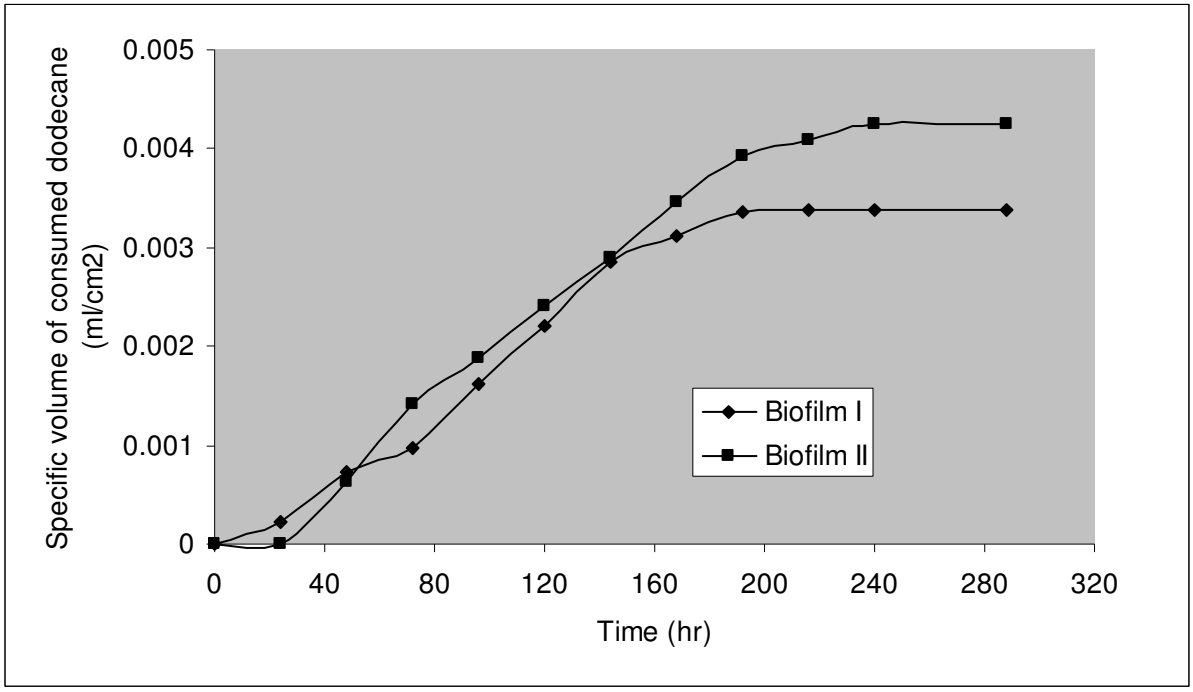


Figure (5). Time course for substrate consumption

To ensure the accuracy of dry weight method in determination of a biofilm formation, three samples were withdrawn and tested in parallel to each other. Figure (6) shows the typical formation rate of biofilm. After a lag phase of 24 hours, were no increase in biofilm weight was observed. The peak value of biofilm formation was 4 mg/cm^2 for aerated medium and 3 mg/cm^2 for non-aerated medium. After 240 hours, no biofilm accumulation was observed.

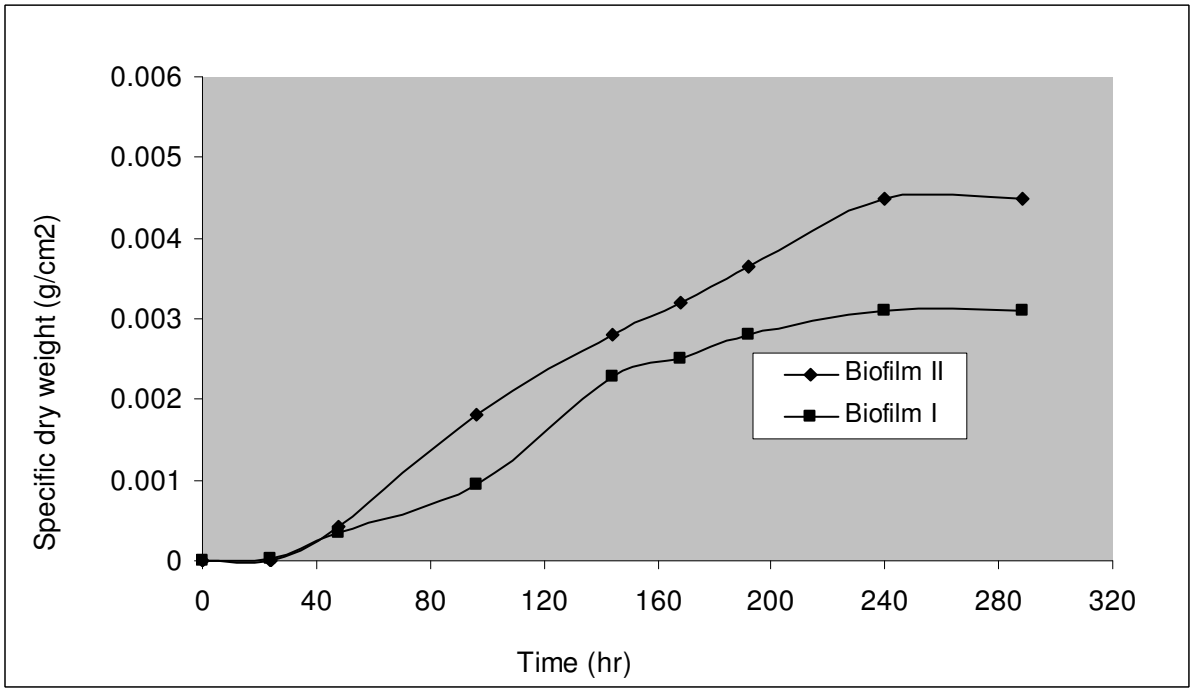


Figure (6). Time course biofilm dry weight

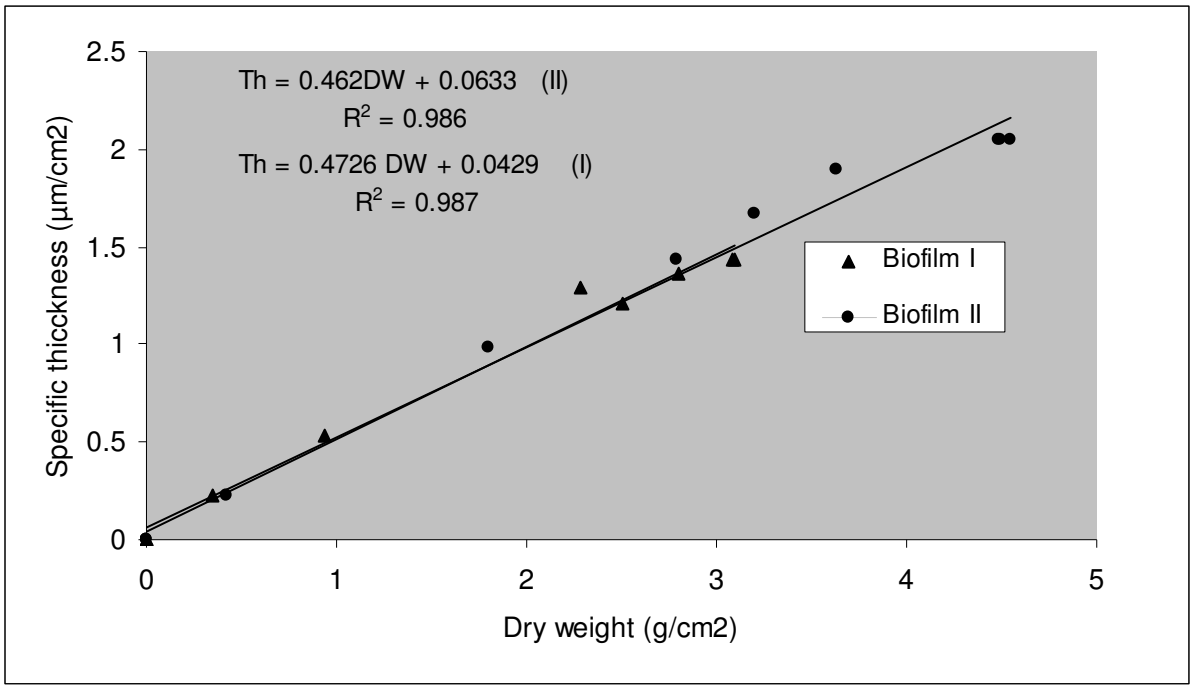


Figure (7). Relationship between specific dry weight and specific thickness

A relationship between biofilm dry weight and its thickness for aerated and non-aerated system is illustrated in figure (7). Dry weight was found to be linearly correlated with biofilm thickness according to the following formulas: **Th. = 0.462DW + 0.063** and **Th. = 0.472DW+0.0429** respectively.

CONCLUSIONS

From the results of the present laboratory scale investigations, the following conclusions can be drawn:

- The proposed technique allows a simple method to in-situ characterize plan biofilms , hence, it may be of great useful to assess biofilm developed in an emulsion system when good correlation can be achieved.
- Under the conditions of such experiments, results of this work showed that rate of formation of biofilm w 15µm/day for non-aerated system and 20µm/day for aerated system.
- Experimental error for this method was found to be little than 10%.
- Dry weight was found to be linearly correlated with biofilm thickness according to the following formulas: **Th. = 0.462DW + 0.063** for aerated system and **Th. = 0.472DW + 0.0429** for non-aerated system.
- Probes with small cross sections reduce the possibility of physical damages of the biofilm structure during penetration, but damage can occur unavoidably while withdrawing the probe from the biofilm.

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