Evaluating the biofilm formation and performance in biphasic organic/water culture

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In this work, two methods for determining the total biofilm amount in biphasic cultures, dodecane/salt solution, have been studied. The first method represents the direct method "classical dry weight method" while the second is indirect method "mineral nitrogen consumption". In dry weight method, a formation rate of 10 mg/l.h can be achieved after 100 hours. This rate has been declined to 5.2 mg/l.h in the next 100 hours. After 220 hours no biofilm accumulation was observed. Because of the heterogeneity of the system, the estimation of biofilm performance was studied by determination of the mineral nitrogen consumption. The average value of consumed nitrogen was 380 mg/l in 168 hours with a rate of 2.24 mg/l.h. This value represents 75% of the initial amount of nitrogen in the biphasic medium. From the obtained results, it can be concluded that the nitrogen is not the limiting factor for the biofilm formation.

لدراسة تكون الفيلم الحيوي علي السطح الفاصل بين سائلين عديمي الامتزاج استخدمت طريقتان أحدهما طريقة مباشرة و هي تعيين الوزن الجاف للفيلم المتكون و الثانية طريقة غير مباشرة و هي دراسة معدل استهلاك النيتروجين المعدني. في الطريقة الأولي تم التوصل الي معدل نمو و قدره ١٠ مج/لتر.ساعة بعد زمن قدره ١٠٠ ساعة ثم انخفض هذا المعدل الي ٥,٢ مج/لتر.ساعة بعد المائة ساعة التالية. و بعد زمن قدره ٢٢٠ ساعة لم يلاحظ أية معدلات للنمو. في الطريقة الثانية تم قياس معدل استهلاك النيتروجين مع الزمن و كان أعلي معدل للاستهلاك ٢,٢٤ مج/لتر.ساعة و هذه القيمة تمثل ٧٥% من النيتروجين الأصلي الموجود في وسط النمو و هذا يعطي انطباعا بأن النيتروجين لا يمثل العامل الذي يحد من نمو الفيلم الحيوي

Keywords: Biofilm, Dodecane, Biodegradation, Pseudomonas aeruginosa, Biphasic media

1. Introduction

A biofilm is a layer-like aggregate of microorganisms naturally attached to aquatic plants, animal, piers, ship hulls, water pipes, Lake Benthos, groundwater aquifers and stream bed. It consists of a sticky rigid structure of polysaccharides and other organic contaminants. This slim layer is anchored firmly to a surface and provides a protective environment in which microorganisms grow [1]. Since 1980s, there has been considerable attention towards the use of petroleum-degrading bacteria for the treatment of soils contaminated with hydrocarbons. A large amount of these bacteria is embedded in biofilms. Some studies have been carried out in both pure and mixed cultures in soil microcosms [2] and in the field under conditions of full-scale site bioremediation [3]. Since information is required on microorganisms able to use hydrocarbon substrates, investigations tend to be carried out on liquid biphasic media prior to soil studies.

This study investigated a biphasic liquid system in which bacteria grow embedded in a biofilm at the emulsion interface. The problems to quantify this biofilm arise from the heterogeneity of the system, the adherence of microorganisms to oil droplets and the presence of extracellular polymers. Thus conventional methods for the determination of the biomass can not directly be used to quantify the total biofilm amount. Several authors, studying biphasic cultures, had to cope with difficulties derived from the heterogeneity of the system and adherence of cells to the organic phase [4-7]. They have proposed modifications for dry weight, protein, carbohydrate, lipid and fatty acid content determination. However, these authors never mentioned the presence of biofilm and only microorganisms were taken into account in biphasic systems. Therefore, we lack informa-

tion about methods of biofilm quantification in biphasic systems and about their accuracy.

This paper describes two methods of the quantification of biofilm in biphasic systems. The first of these two methods is direct (measurement of dry weight), while the second is indirect (mineral nitrogen consumption). For each method, the experimental error was estimated.

2. Materials and methods

2.1. Microorganisms

Pseudomonas aeruginosa ATCC 15522 was chosen for this study as it was isolated from petroleum polluted soil consequently, it was adapted to use petroleum as a source of carbon. This strain was grown on a medium which consists of: digested soy peptone 5g, meat extract 1 g, yeast extract 2 g , NaCl 5 g, and agar 15 g, all in one liter of osmosed water. The pH level was adjusted at 7.4 with 1N NaOH.

2.2. Reactor

As shown in fig. 1, the reactor used in this study was a cylindrical, glass stirred tank (200mm diameter, 300 mm high and 4 liter working volume). The tank was fitted with four baffles. The impeller was stainless steel, disc-turbine with six flat (75 mm diameter overall) located half way between the tank bottom and the liquid surface. The Dimensions of the reactor are tabulated in table 1. The temperature and stirring rate were set at 30oC by means of water bath at 250 rpm. Samples of a constant volume (12.50 ml) were removed using a piston sampler as shown in fig. 1.

2.3. Biphasic culture

A mineral salt medium proposed by [6] was used as the aqueous phase. The mineral salt medium composition was as shown in table 2. 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°C. The sole carbon source was n-dodecane (2% v/v).

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.

Table 1
Reactor dimensions

		Volume 4 liter
Turbine	d_t	43 mm
	d_a	75 mm
	D	190 mm
	d'	15 mm
	t	6 mm
	h	80 mm
Cylinder	D	200 mm
	H	127 mm
	L	300 mm
Baffles	E	15 mm
	e'	10 mm
	E	250 mm
Water bath	A	330 mm
	A'	290 mm

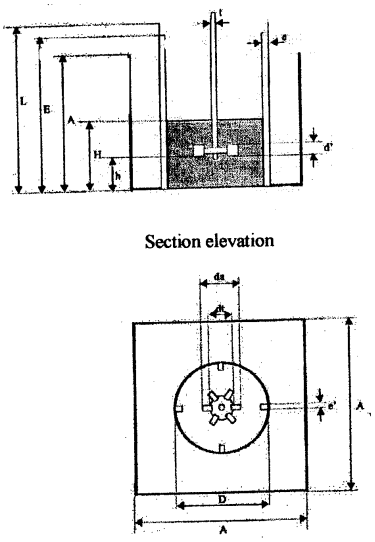

Fig. 1. Schematic diagram of the reactor.

Table 2
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

2.4. Culture activation

A preculture was inoculated from agar slants in a 100 ml working volume shaking flask. It was grown at 30°C for five days and 1ml of its aqueous phase was inoculated in a culture in a 100 ml working volume shaking flask. This last culture was grown at 30°C for two days. 10 ml of its aqueous phase was used as inoculum for the biphasic system. In this study ambient temperature was chosen as the working temperature since most of the wastewater treatment systems operate around 25°C.

3. Analytical methods

3.1. Sampling

In the study of biofilm characteristics, samples should not withdrawn by pipette to avoid the adhesion of biofilm on its sides. Two precautions must be considered: a) the volume of samples must be equal; b) the sample is representative. It is suggested to use a piston sampler to attain the above precautions. The piston sampler with a volume of 12.50 ml as shown in fig. 2 was used in this study.

3.2. Dry weight as direct method

Samples of 12.50 ml of biphasic culture were centrifuged for 10 minute at 20,000 rpm and at ambient temperature. The resulting supernatant was composed of the aqueous phase and a top viscous layer consisting of dodecane, biomass cells and polymers. The aqueous phase was removed by suction. The pellet and the top layer were mixed with 15 ml of a solvent mixture (acetone and petroleum ether 3:1 v/v) to solubilize the hydrocarbon. A preliminary study showed that the selected solvent was the most suitable for the recovery of the biofilm.

Finally the mixture (top layer + solvent) was agitated for 10 s and centrifuged to remove the excess solvent. The pellet was suspended in osmosed water and filtered through pre-dried cellulose acetate filters (0.2 μm pore diameter) in a vacuum filtration apparatus. The filter papers were dried to a constant weight in vacuum oven at 70°C.

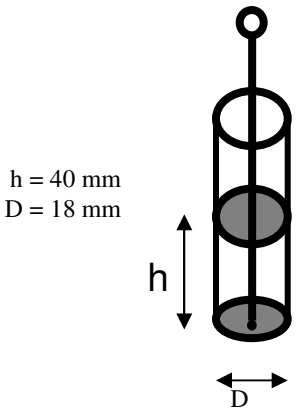

Fig. 2. Piston sampler.

3.3. Mineral nitrogen content as indirect method

Determination of the consumption of carbon substrate is difficult due to the heterogeneity of the system. Thus, for the estimation of the biofilm activity, consumption of mineral nitrogen was studied. Since microorganisms consume nitrogen during their growth as it is one of the essential components (14%) of their cells and the only source of nitrogen in the culture media was $(NH_4)_2SO_4$. The performance of the microorganisms can be observed through the determination of the residual content of this salt. The nitrogen content could be estimated by the subtraction of the amount remaining in the aqueous phase of the biphasic media after centrifugation from the initial amount of the ammonium sulfate. One of the most common techniques for determining the mineral nitrogen content is the Nessler method in accordance with the standard methods [8].

4. Results and discussion

4.1. Formation of biofilm

In order to achieve nearly completely-mixed conditions, agitation was the proper method to obtain a homogenous emulsion. The development of biofilm could be observed on the dodecane droplets (dodecane– water interface) as shown in fig. 3 below.

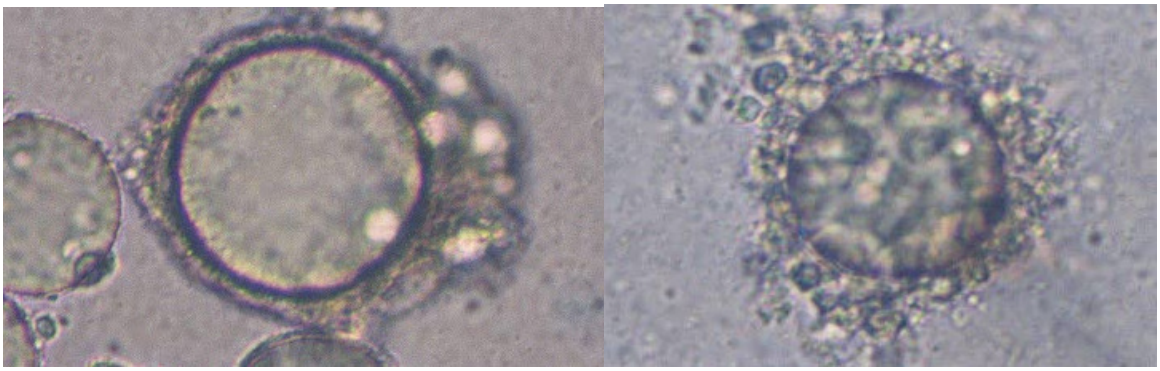

Fig. 3. Formation of biofilm.

4.2. Dry weight

To ensure the accuracy of dry weight method in determination of biofilm formation, three samples were withdrawn and tested in parallel to each other. Fig. 4 shows the typical formation rate of biofilm. After a lag phase of 20 hours, where no increase in biofilm weight was observed. The peak rate of biofilm formation was 10 mg/l.h. after 100 hours and a decline in formation rate to 5.2 mg/l.h. has been achieved after 200 hours. After 220 hours no biofilm accumulation was observed.

The last phase can be intrepretated by the difficulty of oxygen transfer through the biofilm. The overall material balance showed a high residual concentration of mineral nitrogen. It reveals that the nitrogen was not completely consumed. So it can be concluded that the oxygen transfer is the rate limiting factor for the biofilm formation. As the maximum solubility of oxygen at ambient temperature equals 9.8 ppm, this value is not sufficient for the biomass growth.

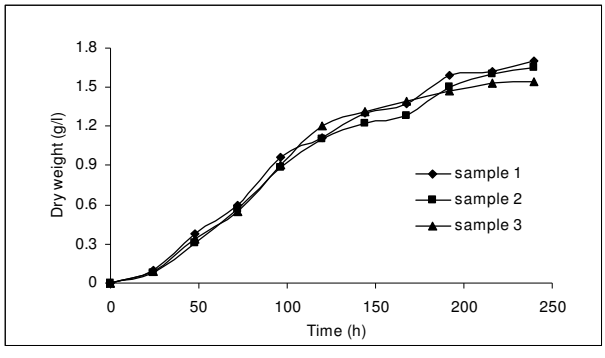

Fig. 4. Variation of dry weight of biofilm as a function of time.

4.3. Mineral nitrogen

As can be seen from fig. 5, the average value of consumed nitrogen was 380 mg/l in 168 hours with a rate of 2.24 mg/l.h. This value represents 75% of the initial amount of nitrogen in the biphasic media. After 168 hours the rate of nitrogen consumption was almost zero, at the same time an excess of nitrogen was measured, this ensures that nitrogen is not the limiting factor. Since the nitrogen substrate was not completely consumed, it gives an impression about a limitation phenomenon. The most probably explanation of these phenomena is the difficulty of mass transfer through the biofilm.

4.4. Relationship between dry weight of biofilm and consumed nitrogen

The amount of nitrogen consumed by cells was plotted against Dry Weight (DW) as shown in fig. 6. According to the best fitting line equation DW = 0.0042N + 0.0171 with degree

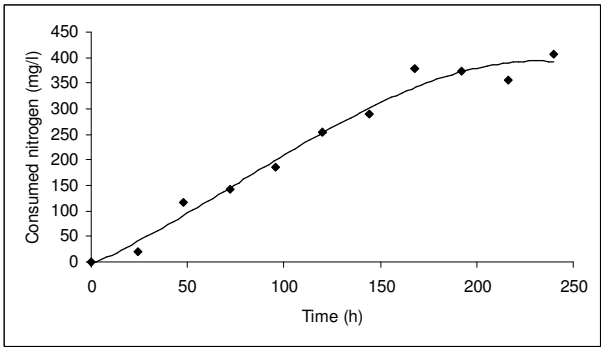

Fig. 5. Consumption of nitrogen as a function of time.

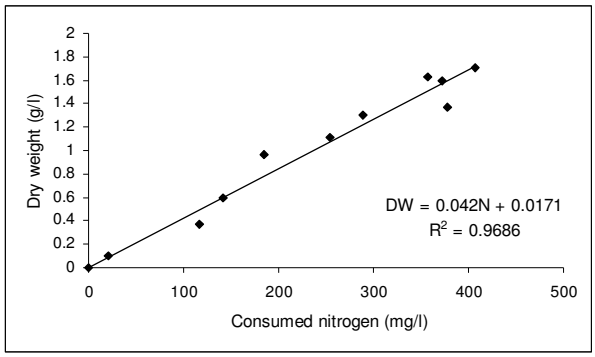

Fig. 6. The relation between dry weight and consumed nitrogen.

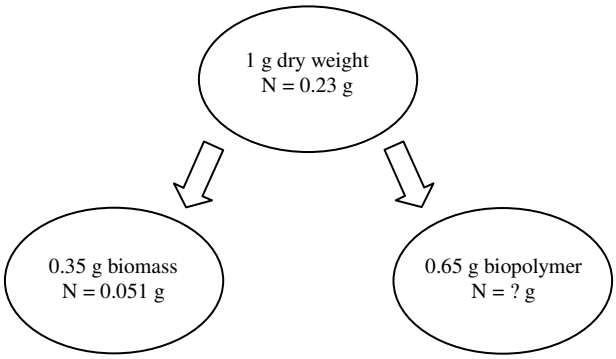

Fig. 7. Nitrogen balance in the biofilm.

of accuracy of 97%, the nitrogen represents 23% of the amount of the biofilm dry weight.

This percentage is higher than that given by literature [9] for the biomass (14.6%), that ensures the presence of nitrogen in the biopolymer which represents 65% of the biofilm dry weight [10].

From overall material balance the percentage of nitrogen in the biopolymer can be easily calculated as follows:

N_2 content in the biofilm = N_2 content in Biomass + N_2 content in Biopolymer,

0.23 g = 0.35 x 0.146 g +??,

?? = 0.179 g,

% N_2 in the biopolymer = (0.179/0.65)x100 = 27%.

4.5. Statistics

The coefficient of variation (%e) was used to estimate the experimental error. %e was calculated as: %e = (s/m)x100, where m was the arithmetic mean of n values and s the standard deviation of these n values. Three measurements were carried out for the dry weight method. The arithmetic mean and the coefficient of variation were calculated for different concentrations of biomass and biofilm.

Fig. 8 shows plots of the coefficient of variation against the corresponding arithmetic mean for biphasic culture. From the above relationship, a model of the following type: %e

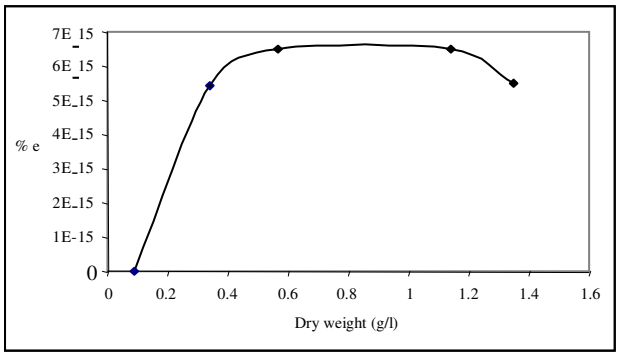

Fig. 8. Correlation between coefficient of variation and dry weight.

= (s/m)x100 = a m^{β} can be established. The model parameter a and β were calculated by the least square method where a = -1E-14m^2. It can be seen from the plot of coefficient of variation against arithmetic mean that %e was remarkably low.

5. Conclusions

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

- In dry weight method, a growth of biofilm with a rate of 10 mg/l.h can be achieved after 100 hours. This rate has been declined to 5.2 mg/l.h in the next 100 hours. After 220 hours no biofilm growth was observed.
- The average value of consumed nitrogen was 380 mg/l in 168 hours with a rate of 2.24mg/l.h. this value represents 75% of the initial amount of nitrogen in the biphasic medium.

• The results indicate that the extracellular polymer contain nitrogen in its constitution which represents 27%. It gives a good method for characterization of the extracellular polymer.
• Since the nitrogen substrate was not completely consumed, it gives an impression about a limitation phenomenon. The most probably explanation of this phenomena is the difficulty of mass transfer through the biofilm.
• As the maximum solubility of oxygen at ambient temperature equals 9.8 ppm, this value is not sufficient for the biomass growth, so the mass transfer of oxygen is the limiting factor in the process.

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Received September 8, 2003
Accepted October 26, 2003