Comparison of four methods for quantification of biofilms in biphasic cultures

C. Joannis; M.L. Delia*; J.P. Riba
Laboratoire de Génie Chimique, CNRS UMR 5503, INP-ENSIGC 18 chemin de la loge, 31078 Toulouse, Cedex4, France. Email : MarieLine.Delia@ensigct.fr; Fax: 33 (0)5 62 25 23 79

Running tittle: Comparison of methods for biofilm quantification

Summary

Three methods for determining the total biofilm amount in biphasic cultures have been compared: dry weight by filtration after solvent treatment, optical density with a biomass probe and protein content. The activity of the biofilm was estimated through mineral nitrogen consumption. Calculation of the coefficients of variation shows that these parameters could be used to characterise such a biofilm. The optical density by biomass probe was the most reliable one (repeatability <0.5%) to quantify total biofilm and a linear relation was verified against dry weight.

Key words: biofilm, biphasic system, analytical methods, biomass probe.

Introduction

Since the 1980s, there has been considerable interest in 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 (Song *et al.*, 1989) and in the field under conditions of full-scale site bioremediation (Sandvick *et al.*, 1986). Since information is required on microorganisms able to use hydrocarbon substrates, investigations tend to be carried out on liquid biphasic media prior to begin soil studies. We investigated here 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 to the presence of extracellular polymers. Thus, conventional methods for the determination of the biomass (cell counting, measurement of dry weight by filtration, spectrophotometric determination of optical density) or cell activity (kinetics of degradation of carbonated substrate), can not directly be used to quantify the total biofilm amount {cells + extracellular polymer} in these systems. Several authors, studying biphasic cultures, had to cope with difficulties derived from the heterogeneity of the system and the adherence of cells to the organic phase (Hugh and Fietcher, 1973; Rosenberg and Rosenberg, 1985; Goswami and Singh, 1991; El Aalam *et al.*, 1993; Ascon-Cabrera and Lebeault, 1993). They have proposed modifications for dry weight, protein, carbohydrate, lipid and fatty acid content determination. However, these authors have never mentioned the presence of biofilm and only microorganisms were taken into account in biphasic systems. Thus, we lack information about methods for biofilm quantification in biphasic systems and about their accuracy.

This paper describes four methods for the quantification of the biofilm amount in biphasic systems: two direct methods (measurement of dry weight and optical density), and two indirect methods (determination of protein content and mineral nitrogen consumption). For each method, the experimental error was estimated and was compared to the experimental error of the same method used on free cell monophasic cultures run with the same bacteria.

Materials and Methods

Microorganisms. *Pseudomonas aeruginosa* ATCC 15522 was grown on a medium which composition was digested soy peptone: 5 g, meat extract: 1 g, yeast extract: 2 g, NaCl: 5 g, agar: 15g in 1 L of osmosed water. The pH was adjusted to 7.2 with 1M NaOH.

Reactor. The reactor was a cylindrical, glass stirred-tank (200 mm diameter, 300 mm high, 4l working volume). The tank was fitted with four baffles. The impeller was a stainless steel, disc-turbine with 6 flat blades (75 mm diameter overall) located half way between the tank bottom and the liquid surface. Temperature and stirring rate were set at 30°C and 250 rpm, respectively. Samples of a constant volume (12.35 ml) were removed using a piston sampler.

Culture conditions. *Monophasic culture*. The same medium as above but without agar was used. Cells from a preculture incubated at 30°C for 12 h in a 300 ml shake flask (100 mL working volume) were used as inoculum in order to obtain an initial cell concentration in the reactor of around 3.10^6 cell.ml^{-1}.

Biphasic culture. A mineral salt medium was used as the aqueous phase. The mineral salt medium composition was: $(NH_4)_2SO_4$: 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 in 1 l of osmosed water. The pH was adjusted to 7 using 1M NaOH and the medium was autoclaved for 20 min at 121°C. The sole carbon source was n-dodecane (2% v/v) (Fluka). A preculture was inoculated from agar slants in shake flask (100 ml working volume). It has grown at 30°C for five days and 1 ml of its aqueous phase was inoculated in a culture in shake flasks (100 ml working volume). This last culture has grown at 30°C for 2 days. 10 ml of its aqueous phase was used as inoculum for the biphasic system.

Analytical methods

Dry weight. Samples (12.35 ml) of monophasic culture were filtered through pre-dried cellulose acetate filters (0.2 µm pore size) in a vacuum filtration apparatus. The filters were dried to constant weight in an vacuum oven at 70°C. This method could not, however, be applied to samples containing biofilms due to problems with filter clogging. Samples from biphasic cultures were centrifuged for 10 min at 20,000 rpm and room 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 removed by suction. The pellet and the top layer were mixed with 15 ml of a solvent mixture (acetone, petroleum ether, 3:1 v/v). A preliminary study showed that this solvent was the most suitable for the recovery of our biofilm. The resulting preparation was mixed for 10s and centrifuged. The pellet was suspended in osmosed water and treated in the same way as for the monophasic cultures.

Optical density. The optical density (OD_{620}) of monophasic samples was measured at 620 nm against water as blank. Samples were diluted to bring the optical density into the linear range between 0 and 0.8 Absorbance Units. As the emulsion of the biphasic culture is unstable, it is impossible to determine its optical density with a spectrophotometer. Thus, a biomass probe (Model 652 cell growth monitor, Wedgewood Technology, Inc., San Carlos) was used. The probe looks like an oxygen sensor and possesses an optical cell (2cm pathlenght) at the end which allows to measure optical density of the emulsion at 600 nm (OD_{600}) directly in the reactor. The blank was done with medium culture before inoculation and after the stabilisation of the emulsion in order to take into account its absorbance.

Proteins. For monophasic cultures, 0.5 ml were centrifuged for 10 min at 11,000 rpm in order to remove culture medium containing free proteins. The pellet was washed with sterile water and centrifuged. The resulting pellet was suspended in 0.5 ml of sterile water and was used for assay. For biphasic cultures, 0.5 ml samples were taken without treatment. A preliminary study showed that there was no significant protein amount in the aqueous phase. Only proteins contained in the biofilm were determined. The samples were incubated in a boiling water bath after addition of 0.5 ml of 0.25 M H_3PO_4. Different durations of hydrolysis were examined. After hydrolysis, samples were centrifuged for 10 min at 11,000 rpm and 0.5 ml of the supernatant were removed for

determination of proteins. Protein concentration was estimated by Lowry's method using solutions of BSA containing an equal volume of H_3PO_4 as reference.

Mineral nitrogen. To 25 ml of aqueous phase (NH_4 concentration below 6 $mg.l^{-1}$), 1 ml of Nessler reagent, prepared from Charlot (1961), was added and the absorbance was measured 10 min later at 420 nm in a spectrophotometer.

Statistics. The coefficient of variation (e%) was used to estimate the experimental error. e% was calculated as: $e\% = \frac{s}{\overline{m}} \times 100$, where $\overline{m}$ was the arithmetic mean of n values and s the standard deviation of these n values. Five measurements were carried out for each method (n=5).

Results and discussion

The arithmetic mean and the coefficient of variation were calculated for different concentrations of biomass and biofilm in both monophasic and biphasic cultures.

Dry weight

Figure 1 shows plots of the coefficient of variation against the corresponding arithmetic mean for monophasic and biphasic cultures. From the relationship between coefficient of variation and measured concentration, we established a model of the following type: $e\% = \frac{s}{\overline{m}} \times 100 = \alpha \ \overline{m}^{\beta}$. The model parameters α and β were calculated by a least square method. The relationships between the arithmetic mean and the coefficient of variation are presented in Table 1. It can be seen by comparison of Figures 1a and 1b that the coefficient of variation for the biphasic cultures was less than that for the monophasic cultures, indicating that we employed a suitable method for recovery of the biofilm. No more than 10% error was observed at concentrations above 0.64 $g.l^{-1}$ for biomass and above 0.61 $g.l^{-1}$ for biofilm. In order to improve these results, larger sampling volumes would be taken as, in theory, the coefficient of variation is inversely proportional to sampling volume. However, a comparison with literature shows that the coefficients of variation listed in Table 1 are lower than those calculated for an other complex system: penicillin fermentation (Stone *et al*., 1992).

Table 1 Comparison of the four methods in monophasic and biphasic cultures

		Free cell in monophasic cultures	Biofilm in biphasic cultures
Dry weight (DW)	e%	$e\% = 4.82\ DW^{-1.65}$	$e\% = 6.06\ DW^{-1.05}$
Optical density (OD_{600})		$DW = 0.97\ OD_{600} + 0.12$ (r^2=0.893)	$DW = 1.24\ OD_{600} + 0.03$ (r^2=0.902)
Proteins (P)	e%	$e\% = 1.45\ P^{-1.00}$	$e\% = 1.20\ P^{-0.92}$
	P/ DW	53%	82%
Mineral nitrogen (N)	e%	$e\% = 0.41\ N^{-2.26}$	
	N/ DW	14.6%*	17.1%

e%: coefficient of variation as an estimation of experimental error
*given by literature (Characklis, 1990)

Figure 1. Coefficient of variation as a function of biomass concentration in monophasic culture (a) and biofilm quantity in biphasic culture (b).

Optical density

Up to 1.5 $g.l^{-1}$, a linear correlation is found between optical density and dry weight. We used a cell growth probe to validate this result in monophasic cultures. The OD_{600} determined by the probe was found to be proportional to the OD_{620} measured spectrophotometrically. A linear correlation between dry weight and OD_{600} was thus obtained (Table 1). We then measured OD_{600} in the biphasic cultures using the probe. The plot of optical density against biofilm dry weight is shown in Figure 2. As the corresponding equation is linear (Table 1), the biomass probe could be used to determine dry weight *in-situ*. The repeatability of the cell growth probe is excellent (< 0.5% from manufacturer's data). Moreover, this technique fastly and directly provides the result without any added treatment of samples. Thus, this method is well suited for the quantification of biofilms in biphasic cultures.

Figure 2. Dry weight of biofilm growing in biphasic culture as a function of optical density given by the biomass probe

Proteins

We used the Lowry method as it was the simplest and most sensitive method for our range of protein content (0-1.5 $g.l^{-1}$). Generally, the same reagent is used for protein extraction and quantification. For instance, NaOH is often used in the Lowry method and H_3PO_4 with the Coomassie Blue method (Cordova-Lopez et al., 1996). In a preliminary study, we observed a yellow coloration on adding NaOH to the biphasic cultures. This was attributed to the presence of extracellular polymers and it led to inaccurate results with the Lowry method. We therefore used H_3PO_4 to extract proteins. We tested this method on a suspension of *Saccharomyces cerevisiae* EC-1118 (Lallemand Inc., Canada) in sterile water with a known protein content. The time required for maximal protein extraction was determined by measuring protein concentration after different durations of hydrolysis (Figure 3 a). After 25 min hydrolysis, all the proteins were recovered. During the same experiment on the biphasic culture (Figure 3 b), maximal protein extraction was also observed after 25 min hydrolysis.

Figure 3. Effect of hydrolysis time on protein extraction of *Saccharomyces cerevisiae* suspended in sterile water (a) and *Pseudomonas aeruginosa* embedded in a biofilm in biphasic cultures (b).

Figure 4 presents the plots of the coefficients of variation against the corresponding arithmetic means for the monophasic and biphasic cultures, respectively. It can be seen that the values for monophasic cultures were higher than those for the biofilm cultures. This was attributed to losses of biomass during centrifugation and washing. A coefficient of variation under 10% was observed for protein concentrations above 0.13 $g.l^{-1}$ for the monophasic cultures, and 0.09 $g.l^{-1}$ for the biphasic cultures (for concentrations ranging from 0 to 1.5 $g.l^{-1}$). Protein contents (P in $g.l^{-1}$) were plotted against corresponding biomass or biofilm concentration expressed as dry weight (DW in $g.l^{-1}$). A linear relationship was obtained for both monophasic and biphasic cultures. They are presented in Table 1 expressed in percentage. For *Pseudomonas aeruginosa*, we found a protein percentage of 53% with respect to dry weight, which is in the range (34-83%) reported in the literature (Larpent-Gourgaud and Sanglier, 1992). Protein percentage is higher in biofilms, indicating either a change in bacterial metabolism or an accumulation of proteins in the extracellular polymer of the biofilm.

Figure 4. Coefficient of variation as a function of protein concentration in monophasic (a) and biphasic (b) culture.

Mineral nitrogen

Determination of the consumption of the carbonate substrate is difficult due to the heterogeneity of the system. Thus, for the estimation of biofilm activity, consumption of mineral nitrogen was studied. Since the sole source of nitrogen in the aqueous phase of the biphasic medium is ammonium sulphate, nitrogen content could be estimated by subtraction of the amount remaining in the aqueous phase of the biphasic media after centrifugation, from the initial amount of the ammonium sulphate. The coefficient of variation was therefore the same in the biphasic and monophasic cultures. It can be seen from the plot of coefficient of variation against arithmetic mean (Figure 5) that e% was remarkably low (< 6%) over the range of concentration of interest (0.3-0.7 $g.l^{-1}$). For the biphasic cultures, the amount of nitrogen consumed (N) by cells was plotted against dry weight biofilm (DW). We found a percentage of nitrogen in biofilm of 17.1% (Table 1), which is above that reported in the literature for free cells of *Pseudomonas aeruginosa* (Characklis, 1990). It indicates that there was an accumulation of proteins in extracellular polymers of the biofilm, which has been reported by other authors (Flemming, 1993).

Figure 5. Coefficient of variation as a function of ammonia concentration in aqueous phase

The cell growth probe appears to be a rapid and accurate method for determination of the dry weight of biofilms in biphasic systems. However, to determine biofilm dry weight in soil systems the method using solvent will be required. Further information can be obtained by measuring protein concentration and mineral nitrogen consumption, which are adapted to soil systems.

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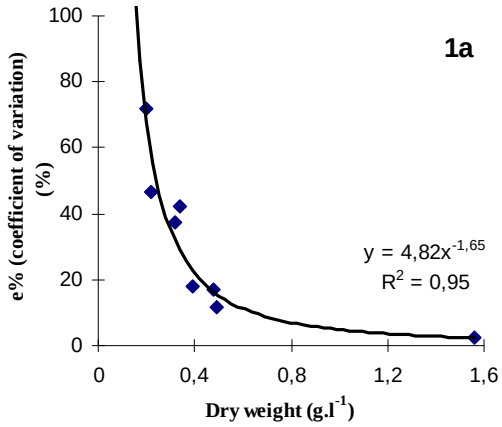
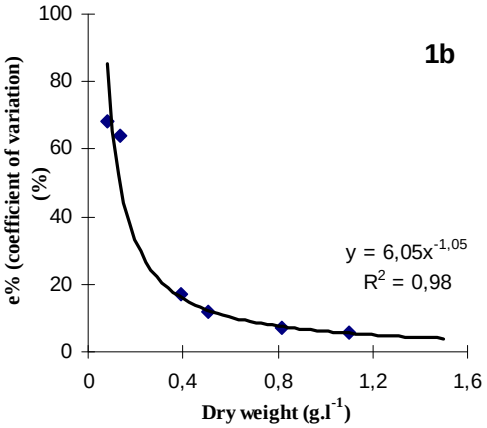
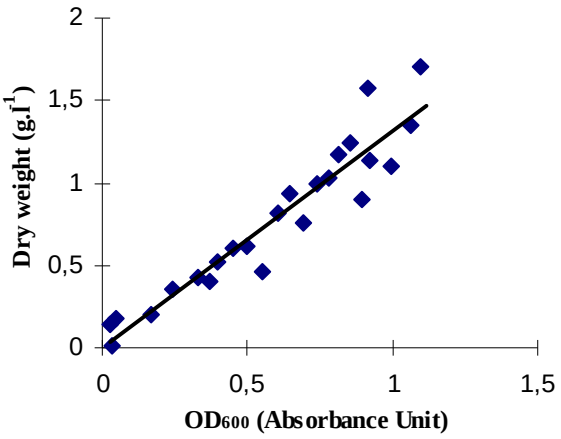
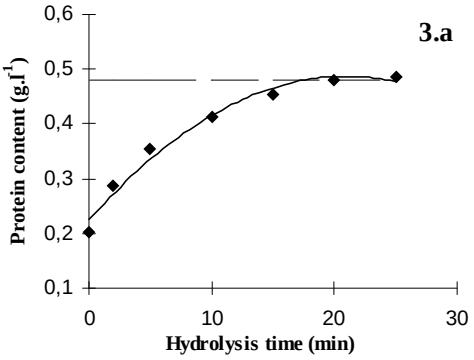

1a
e% (coefficient of variation) (%)
100
80
60
40
20
0
0
0,4
0,8
1,2
1,6
Dry weight (g.l-1)
y = 4,82x-1,65
R2 = 0,95


1b
e% (coefficient of variation) (%)
100
80
60
40
20
0
0
0,4
0,8
1,2
1,6
Dry weight (g.l-1)
y = 6,05x-1,05
R2 = 0,98


2
1,5
1
0,5
0
0
0,5
1
1,5
Dry weight (g.l-1)
OD600 (Absorbance Unit)


3.a
Protein content (g.l-1)
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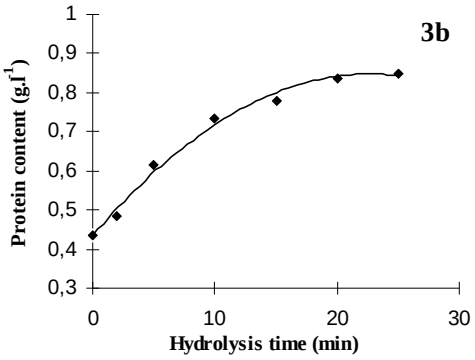
0,5
0,4
0,3
0,2
0,1
0
10
20
30
Hydrolysis time (min)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Protein content (g.l-1)
1
0,9
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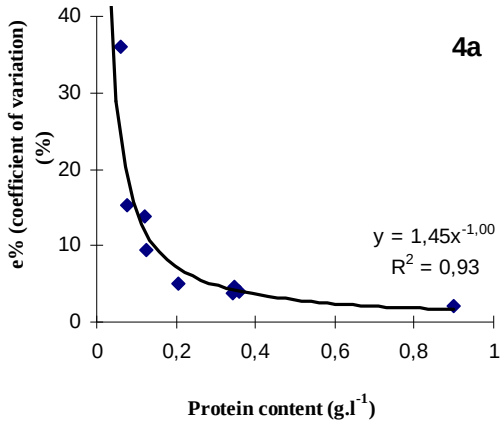
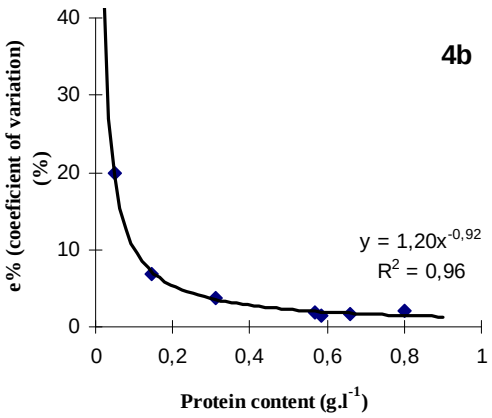
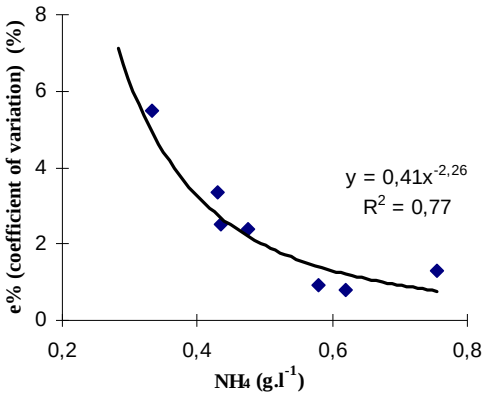
0,7
0,6
0,5
0,4
0,3
0
10
20
30
Hydrolysis time (min)


4a
e% (coefficient of variation) (%)
40
30
20
10
0
0
0,2
0,4
0,6
0,8
1
y = 1,45x-1,00
R2 = 0,93
Protein content (g.l-1)


4b
e% (coefficient of variation) (%)
40
30
20
10
0
0
0,2
0,4
0,6
0,8
1
y = 1,20x-0,92
R2 = 0,96
Protein content (g.l-1)


8
6
4
2
0
0,2
0,4
0,6
0,8
e% (coefficient of variation) (%)
NH4 (g.l-1)
y = 0,41x-2,26
R2 = 0,77