Laboratory technologies regarding the obtaining of some biofilms under different working conditions

Received for publication, April 25, 2007
Accepted, June 15, 2007

STELA POPOVICI*, VERONICA STOIAN, DIANA PĂSĂRIN***

**National Research & Development Institute for Chemistry and Petrochemistry – ICECHIM, 202 Splaiul Independenţei, sector 6, 060021 Bucharest, Romania*
e-mail: dianapasarin11@yahoo.com

***Department of Genetics, Faculty of Biology, University of Bucharest, 1-3 Aleea Portocalilor, sector 5, 77206 Bucharest, Romania*

Abstract

Present study refers to the formation way of different biofilm types with lipolythic properties.

To accomplish a functional biofilm for the clearing of oily, greasy waters, there can be used closed reactors with stable parameters or open plants with wide variation of work conditions [6]. Those from the first category need complex plants; they enter faster in the work program, the biofilms resulted in these conditions are sensible and are easily broken by accidental environmental conditions. Instead, they can be controlled and programmed quantitatively and qualitatively.[1] Open plants with spontaneous biofilms, on plates, allow the development of some resistant biofilms at the variations, which appear in content of waste waters (variations of oil content, presence of detergents, temperature shocks and pH, etc.).[3]

In present paper, experiments were conducted in open plants with biofilms resulted in aeration-recirculation conditions. The biofilms in these conditions enable content change of these in terms of situation.

Keywords: microbial biofilms, waste waters, biotechnology, *Pseudomonas*

Introduction

For the study of biofilms obtaining conditions, a series of laboratory experiments have been initiated and they include both experiments involving selected microorganisms from *Pseudomonas* kind and spontaneous microorganisms, which self-selected themselves based on content of immersing medium (oily wastewaters). These experiments were conceived to obtain some information regarding the formation way of biofilms and development of spontaneous microorganisms in a medium, which has the oil as unique source of carbon.

To determine the changes produced in medium with the help of biofilms, COD initial analyses (chemical oxygen demanded) were conducted, after 30 days and 60 days with the help of C99 HANNA Multiparameter Bench Photometer and HANNA C9800 reactor. [2]

Bioremediation of greases wastewaters with the help of fixed microorganisms as biofilm has functional efficiency, because this is more active than suspension cells (due to expression of some genetic factors, which phenotypically modify the cell). Once the biofilm with lipolythic characteristics was formed, this has a long life in oligotrophycal nutrition conditions.[4] The method has as effect almost the removing of organic and mineral compounds from waste waters, and it is not the generator of additional pollution.

Materials and Methods

Four ways of biofilms formation were experimented at laboratory level:

1. by direct cultivation with a laboratory pure growth *Pseudomonas putida* through direct spread on plates;
2. by cultivation of spontaneous developed growth (self-selection);
3. by cultivation of pure and wild growth, spread on plates and dissemination in medium;
4. reconstruction of biofilm starting from dehydrated (dry) fragments of biofilm.

In first case, pure culture of *Pseudomonas putida* was reactivated through growth on Luria Bertani (LB) specific medium (7329A - Acumedia Manufacturers). Culture obtained after 48 hours was spread on immersed

plates. In this case, a detachment of the layer from the plate is observed, adhering islands remaining from place to place, which later develop by population of the free spaces.

In the second case, a uniform biofilm was obtained in a longer period of time. This is formed of very different microorganisms as morphologic aspect. Noticed at microscope, microorganisms composing this type of biofilm vary in morphology cocobacillary forms to rod-shaped, elongated forms with different mobility degrees.

In the third case, the biofilm is composed both of pure *Pseudomonas putida* culture and of wild cultures, which belong to "pseudomonads" group, and which were sampled and preserved on specific medium (7329A - Acumedia Manufacturers) for "pseudomonads". This kind of biofilm proved to be the most active and efficient during the functioning.

In the fourth case, reactivation of a de-hydrated biofilm was experimented, this remaining un-dislocated on the plant's walls. This biofilm had an interesting evolution because re-hydration produced at the beginning slowly enough in the same time with the detachment in good part of adhering layers and new reconstruction of biofilm through population of the free spaces. Microscope samples show after 96 hours that the most part of cells are dead cells and just a small part are viable, with low enough motility. After 30 days, the biofilm is reconstructed and matches the qualities of an active and well developed biofilm. This experiment proves that the biofilm functions like "integral unit" and it can protect in its interior viable cells even after it is visible dry (dehydrated). These experiments supply us useful data for practical applications and offer us solutions for different situations. The four types of biofilms formation in time were watched also from the viewpoint of functioning efficiency by determination of chemical oxygen demanded. Thus, all the four types of biofilms had an efficient functionality finally, and for industrial applications all the four modalities can be used depending on existent needs and conditions.

Worth mentioning that, the introduction of phosphates and nitrates as stimulant in biofilms formation and functioning was avoided, because this would have increased the load degree of wastewater to be cleaned. Our preoccupations focus on punctual cleaning technologies at the end of a technological manufacture process for oils of different seeds, which also contain other elements than oily greases (proteins, sugars, salts, etc.), and which however complete the medium.

Utilizations of some biological methods for waste waters cleaning at the place where they are generated would lead to the decrease of environment costs and to achieving some technologies without wastes.

Results and Discussions

Frequent laboratory experiments highlighted the fact that biofilms obtained traced the formation and functioning stages assuming the following: colonization, reversible and irreversible adherence, maturation and dissemination in free spaces, described in literature of specialty. The adherence of microorganisms is a slow phenomenon in comparison with adsorption of soluble molecules presented in liquid, on an inert and dipping surface. [5]

The four types of constitution in time of biofilms were also studied from the functioning efficiency view point by determination of chemical oxygen demanded. Thus all four types of biofilm had an efficient final functionality and for industrial applications all the four modalities can be used depending on existent needs and conditions.

Experimental results regarding the activity of the four biofilm types are shown in tabel 1, 2, 3 and 4.

Table 1. Determination of chemical oxygen demanded (COD) for biofilm formed from laboratory pure culture of *Pseudomonas putida*

Nr. crt	Sample	Initial medium mg O_2/dm^3	Medium – 30 days mg O_2/dm^3	Medium – 60 days mg O_2/dm^3
		Reagent HR	Reagent HR	Reagent MR
1	1	13.800	920	361
2	2	14.000	930	368

3	3	13.920	910	396
Average		13.906	920	375

Biofilm formed only from laboratory pure culture had some disadvantages as regards the fixation. The spread culture was dislocated partially, and it was laid-down on the walls of recipient and the free spaces were specifically completed in time through the expansion (colonization) of biofilm-maturated fragments. Obtained results were slightly diminished against the type of biofilm that comprises wild and pure cultures. (table 3)

Table 2. Determination of chemical oxygen demanded (COD) in the case of biofilms formed through spontaneous growth by "pseumonads" kind wild cultures

Nr. crt	**Sample**	**Initial medium mg O_2/dm^3**	**Medium – 30 days mg O_2/dm^3**	**Medium – 60 days mg O_2/dm^3**
		Reagent HR	**Reagent HR**	**Reagent MR**
1	1	14.200	880	511
2	2	13.900	900	508
3	3	14.000	870	421
Average		14.033	883	480

A resistant biofilm resulted from experiments carried out with spontaneous cultures, but which formed more slowly and had a lower activity than those which formed from pure culture and pure culture + wild culture (tables 1 and 3). This culture was fixed slowly in thin layer with different sizes and shapes of cells. From this naturally dried biofilm another experiment was resumed (table 4).

Table 3. Determination of chemical oxygen demanded (COD) in the case of biofilms formed from *Pseudomonas putida* pure and wild culture through spreading on plates and dissemination in medium

Nr. crt	**Sample**	**Initial medium mg O_2/dm^3**	**Medium – 30 days mg O_2/dm^3**	**Medium – 60 days mg O_2/dm^3**
		Reagent HR	**Reagent HR**	**Reagent MR**
1	1	14.120	950	316
2	2	13.910	990	273
3	3	13.990	930	291
Average		14.006	956	293

This type of biofilms provided the best results in laboratory conditions in 60 days period. It had a linear evolution and the results were as expected. Small variations at the three sampled probes are inevitable because the sample can comprise the material itself from different places of the plant.

Table 4. Determination of chemical oxygen demanded (COD) for the biofilms formed starting from dehidrated fragments (apparently dried)

Nr. crt	**Sample**	**Initial medium mg O_2/dm^3**	**Medium – 30 days mg O_2/dm^3**	**Medium – 60 days mg O_2/dm^3**
		Reagent HR	**Reagent HR**	**Reagent MR**
1	1	14.310	1.130	615

2	2	14.350	1.200	591
3	3	14.500	1.190	600
Average		14.386	1.173	602

Biofilm, resulted by reactivation of biofilm formed of wild cultures (table 2) and let without medium to dehydrate, had an interesting evolution, because most cells were dead. Reconstruction was done based on a small number of cells, which can be evaluated at 2-3%. This phenomenon proves the capacity of the biofilm to be able to reactivate itself in austere conditions. Reconstruction in a longer period justifies the results obtained.

We considered necessary to begin from different experimental types to find what happens in different practical situations.

Biofilm formed in the first case, only from *Pseudomonas putida* laboratory pure culture, had a fast fixation although irregular, through dislocating of spread cells creating spaces, which were then occupied through maturation and expansion of initial formations. The results obtained in the first case were good enough, having achieved decreases of COD from 13.800 to 361mg O_2/dm^3 (table 1).

In the second case, the experiment comprised biofilms formed by spontaneous spreading from natural medium. This was regular, as general aspect, but it was formed more slowly and had a shorter activity. As activity, it reduced the load of medium from 14.200 to 421 mg O_2/dm^3 COD (table 2).

The third experiment, when the biofilms were formed from *Pseudomonas putida* pure and wild culture by spreading and dissemination in medium, they had the best results both from time and from activity viewpoint, with cleaning effect of grease waters. The decreasing of COD made it from 14.120 to 273 mg O_2/dm^3 (table 3).

Another experiment was conducted using dry biofilm after one-month inactivity period. This biofim, after re-hydration, had 2-3% viable cells and its reconstruction was achieved in a long period, fact that also influenced the activity of polluting load decreasing. This was achieved from 14.500 to 615 mg O_2/dm^3 COD.

This last experiment proved functional resistance of the biofilms in stress conditions (desiccation) and simulated accidental situations in the technological functioning.

Conclusions

For a complete valorization of the wastes from liquid media, the microbial biofilms can be successfully used. These can use completely the carbon sources from medium due to their high physiological adapting capacity.

Bibliography

1. ANWAR H., STRAP J. L., COSTERTON W. J.– Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. Antimicrobial agents and chemotherapy, **36**: 1347-1351, 1992
2. CHEN J. – ATP bioluminiscence: A rapid indicator for environmental hygiene and microbial quality of meats. Dairy, Food Environ. Sanit., **20**: 617-620, 2000
3. COLON PAUL – La chimie verte. Editions Tec&Doc, Lavoisier, Biodégradabilité, **17**, pp. 487-509, 2006
4. HYDE FW., ALBERG M., SMITH K. – Comparison of fluorinated polymers against stainless steel, glass and polypropylene in microbial biofilm adherence and removal. Journal Ind. Microbiol. Biotechnol, **19**(2):142-9, 1997
5. LAZĂR V.– Aderenţa microbiană, Ed. Academiei Române, 2003
6. WIESMANN UDO, CHOI IN SU, DOMBROWSKI E. – Fundamentals of Biological Wastewater Treatment, Ed. John Wiley & Sons Inc., **7**: 151-167, 2007