

# Biodegradation of Fluorene at Low Temperature by a Psychrotrophic *Sphingomonas* sp. L-138\*

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The ability of a psychrotrophic *Sphingomonas* sp. L-138 to assimilate fluorene was assessed and characterized in a liquid minimal medium at low temperature (12 and 20°C). Total fluorene mineralization was accomplished at both temperatures and the maximal rate of fluorene degradation was determined to be 4.8 mg dm<sup>-3</sup> d<sup>-1</sup> at 20°C and 3.2 mg dm<sup>-3</sup> d<sup>-1</sup> at 12°C. It was confirmed that this strain transforms the fluorene through the so-called “five-membered attack” and the expected metabolites of this pathway, 9-fluorenol, 9-fluorenone, phthalic acid, and protocatechuic acid, were detected in extracts of the culture broth. The specific growth rate of *Sphingomonas* sp. L-138 depends upon the initial fluorene concentration and it seems to be limited by the specific surface area of fluorene crystals. It was shown that the expression of the fluorene degradation pathway was found constitutive for this strain and did not depend on the substrate (glucose or fluorene) used in a pre-culture stage.

Polycyclic aromatic hydrocarbons (PAH) are widely distributed environmental pollutants with detrimental biological effects including acute and chronic toxicity, mutagenicity, and carcinogenicity. Because of their genotoxic potential, many PAH have been included into the U.S. Environmental Protection Agency list of priority pollutants. PAH are very persistent in the environment because of their low water solubility and hydrophobic properties. The main sources of PAH contamination are gas manufacturing plants, coking plants, and petrochemical industry. The physicochemical detoxification of PAH-polluted sites lasts several decades, is very expensive and often incomplete. On the other hand, bioremediation processes, such as biostimulation or bioaugmentation, enable the total degradation of PAH at reasonable costs. A key factor governing the microbial activity of microorganisms used in the bioremediation is the environment temperature. The rate of biological degradation of the recalcitrant pollutants is often decreased during the cold periods of the year because of the temperature-limited growth of microorganisms.

The goal of this research is the selection and characterization of psychrophilic and psychrotrophic bacteria isolated from cold environments that possess the ability to degrade efficiently PAH or hydrocarbons derived from petroleum. As the temperature range of cold-adapting bacteria growth is wide (4–30°C), their application in polluted soils and wastewater de-

contamination is a promising approach. Several bacteria of the genera *Arthrobacter* and *Pseudomonas* have been isolated that utilize fluorene as the only carbon and energy source under aerobic conditions at 25–30°C [1–3]. On the other hand, there are the bacterial genera (for example *Alcaligenes denitrificans*) that are able only to cometabolize the fluorene in the presence of other carbon and energy sources [4] at the same temperatures. In this article we described the degradation of fluorene under aerobic conditions in the liquid medium at 12 and 20°C using *Sphingomonas* sp. L-138. The kinetics of fluorene degradation at low temperatures has not yet been studied to our knowledge.

## EXPERIMENTAL

*Sphingomonas* sp. L-138 is a GFP-labelled derivative of *Sphingomonas* sp. LB126, that was isolated from a historically contaminated soil using conventional shaken enrichment liquid medium in which the PAH were supplied as crystals. The strain was identified by the method of the 16S rRNA gene sequencing [5], showing ability to grow on fluorene as the sole carbon and energy source. *Sphingomonas* sp. L-138 bacteria were maintained on the Tris minimal medium agar plates containing 2 g dm<sup>-3</sup> glucose. The Tris minimal medium composition (per dm<sup>3</sup> of distilled water): 6.06 g Tris-HCl, 4.68 g NaCl, 1.49 g KCl, 1.07 g

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$\text{NH}_4\text{Cl}$ , 0.43 g  $\text{Na}_2\text{SO}_4$ , 0.2 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 40 mg  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 30 mg  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ ,  $10 \text{ cm}^3$  of ferric ammonium citrate solution ( $480 \text{ mg dm}^{-3}$ ), and  $1 \text{ cm}^3$  of trace elements solution (in  $1 \text{ dm}^3$  of distilled water:  $1.3 \text{ cm}^3$   $\text{HCl}$  ( $\varphi_r = 25\%$ ), 144 mg  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 100 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 62 mg  $\text{H}_3\text{BO}_3$ , 190 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 17 mg  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 24 mg  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 36 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ). To keep the degradation ability, the bacteria were routinely grown on the Petri dishes containing the Tris minimal agar medium with fluorene crystals as the sole carbon and energy substrate.

The cells grown at  $20^\circ\text{C}$  on the Tris minimal medium agar plates containing  $2 \text{ g dm}^{-3}$  glucose were harvested and resuspended in the Tris minimal medium without carbon source. The cell concentration was determined in terms of colony-forming units (CFU) as  $1.3 \times 10^8$  cells per  $\text{cm}^3$ .  $0.5 \text{ cm}^3$  of this suspension were used as inoculum for the study of fluorene degradation kinetics which was performed in the liquid Tris minimal medium (pH 7) at  $12^\circ\text{C}$  and  $20^\circ\text{C}$ .

The cultivations were performed in sterile glass tubes with screw caps. As the solubility of fluorene in aqueous solutions is very low, it was supplied from a liquid solution in benzene ( $20 \text{ g dm}^{-3}$ ) using glass syringes to obtain the initial fluorene concentration of  $60 \text{ mg dm}^{-3}$ .  $4.5 \text{ cm}^3$  of sterile medium were added after solvent evaporation and tubes were inoculated with  $0.5 \text{ cm}^3$  of seed culture. All tubes were shaken in the dark at  $12^\circ\text{C}$  or  $20^\circ\text{C}$  at the frequency  $180 \text{ min}^{-1}$ . At each sampling point, three tubes were taken for analyses. One inoculated and one control (noninoculated) tube were used for the fluorene analysis. Another inoculated tube was used for the bacterial growth monitoring *via* optical density measurement at 600 nm, and CFU determination.

$5 \text{ cm}^3$  of cyclohexane were used to extract the residual fluorene and hydrophobic metabolites from the culture broth, previously acidified (pH 2) with 5 M-HCl. The extraction was performed in the culture tubes by vigorous mixing (vortexing) for 5 min. After phase separation, triplicates of  $1 \text{ cm}^3$  of the organic phase were placed into small tubes and the solvent was evaporated under a gentle stream of nitrogen gas. The residue was redissolved in acetonitrile, diluted and used for an HPLC analysis.

Fluorene and the metabolites were separated by reverse-phase HPLC analysis (Waters, city in MA, U.S.A.) using a Platinum EPS C18 Alltech column and UV-PDA detector at 254 nm. This diode array detector allowed us to measure the full spectrum of each peak and then to compare it with authentic spectra of different metabolites. The mobile phase at  $1 \text{ cm}^3 \text{ min}^{-1}$  was composed of acetonitrile and  $\text{K}_2\text{HPO}_4$  ( $c = 10 \text{ mmol dm}^{-3}$ , pH 3.2) ( $\varphi_r = 40:60$ ). The fluorene concentration in culture broths was determined with a relative error of 9 %.

The influence of the inoculum type and fluorene

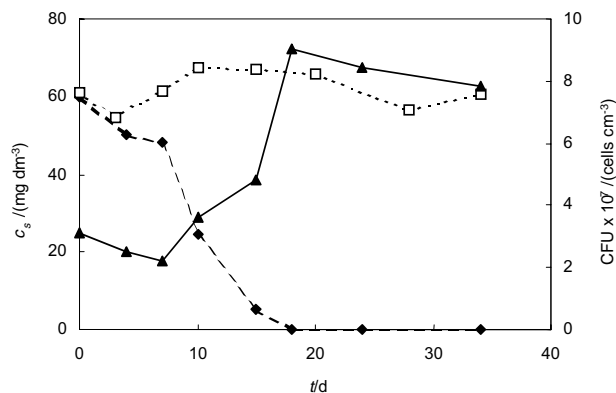
initial concentration on the bacteria growth was studied using *Sphingomonas* sp. L-138 pre-grown at  $12^\circ\text{C}$  in  $100 \text{ cm}^3$  of Tris minimal medium supplemented with  $2 \text{ g dm}^{-3}$  glucose or fluorene. When the absorbance of the culture broth at 600 nm reached the value of 0.4,  $2 \text{ cm}^3$  of the culture were used to inoculate  $100 \text{ cm}^3$  of Tris medium supplemented with fluorene crystals at different initial concentrations ( $\rho/(\text{mg dm}^{-3})$ : 100, 500, 1000, 2000, and 5000). The bacterial growth was monitored by the optical density measurement at 600 nm.

## RESULTS AND DISCUSSION

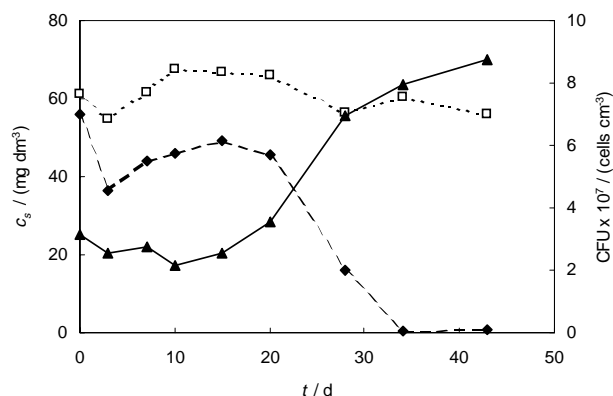
### Degradation of Fluorene

Fluorene degradation by *Sphingomonas* sp. L-138 was studied in a liquid medium at  $12^\circ\text{C}$  and  $20^\circ\text{C}$  in both cases with the same inoculum and the initial cell concentration of  $1.3 \times 10^7$  cells  $\text{cm}^{-3}$ . Fluorene was completely degraded at both temperatures but the rates of degradation were different. At  $20^\circ\text{C}$ ,  $60 \text{ mg dm}^{-3}$  of fluorene was consumed in 18 d (Fig. 1) and the maximum specific rate of degradation was calculated to be  $0.11 \times 10^{-6} \text{ mg d}^{-1} \text{ cell}^{-1}$  that corresponds to  $4.8 \text{ mg dm}^{-3} \text{ d}^{-1}$ . At  $12^\circ\text{C}$ , a twenty days lag phase was observed. Then, an increase in fluorene degradation was detected and the maximum specific rate of fluorene removal reached  $0.08 \text{ mg d}^{-1} 10^{-6} \text{ cells}$  or  $3.2 \text{ mg dm}^{-3} \text{ d}^{-1}$ . After 34 d of cultivation no more residual fluorene was detected in the extract of the inoculated medium (Fig. 2).

As the optimal growth temperature of *Sphingomonas* sp. L-138 is about  $28^\circ\text{C}$ , the lag phase appearance at  $12^\circ\text{C}$  can be attributed to the bacteria adaptation to the low temperature. As shown in Figs. 1 and 2, fluorene removal is associated with bac-



**Fig. 1.** Fluorene degradation by *Sphingomonas* sp. L-138 in liquid minimal medium (Tris-284, pH 7) at  $20^\circ\text{C}$  and shaking frequency of  $180 \text{ min}^{-1}$ . Initial fluorene concentration:  $60 \text{ mg dm}^{-3}$ . Fluorene in control uninoculated ( $\square$ ) and in seed medium ( $\blacklozenge$ ). Bacteria growth ( $\blacktriangle$ ).

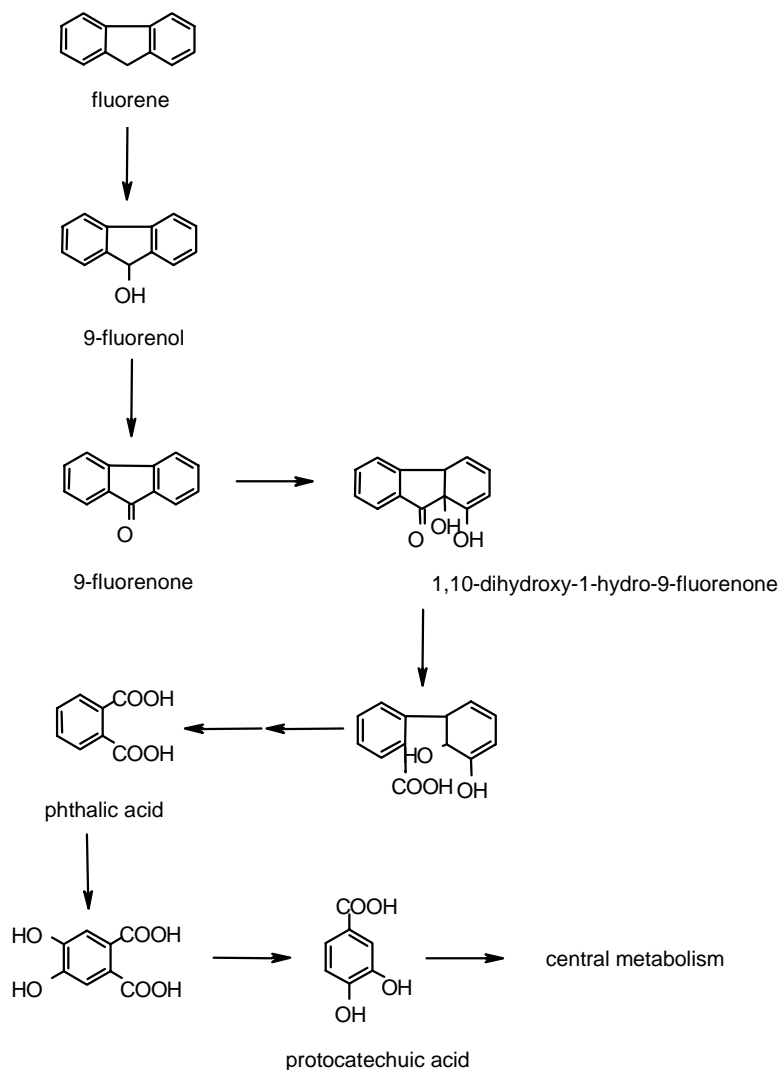


**Fig. 2.** Fluorene degradation by *Sphingomonas* sp. L-138 in liquid minimal medium (pH 7) at 12°C and shaking frequency of 180 min<sup>-1</sup>. Initial fluorene concentration: 60 mg dm<sup>-3</sup>. Fluorene in control uninoculated (□) and in seed medium (◆). Bacteria growth (▲).

terial growth. This indicates that the bacteria are able to develop and assimilate fluorene as their exclusive source of energy and carbon. A modest loss of fluorene in the control flasks was detected at both temperatures. At 20°C about 16 % and at 12°C about 8 % of fluorene was lost by evaporation.

### Metabolites of Fluorene Utilization

Three modes of aerobic attack of fluorene by bacteria have been reported [6]. Two of these pathways are initiated by a dioxygenation of the molecule and the third route is initiated by a monooxygenation at C-9. HPLC analysis of organic extracts from cultures sampled at different incubation times revealed the presence of two metabolites, identified as 9-fluorenol and 9-fluorenone. These observations support the hypothesis that *Sphingomonas* sp. L-138 metabolizes fluorene



*Scheme 1.* Metabolism of fluorene by *Sphingomonas* sp. L-138

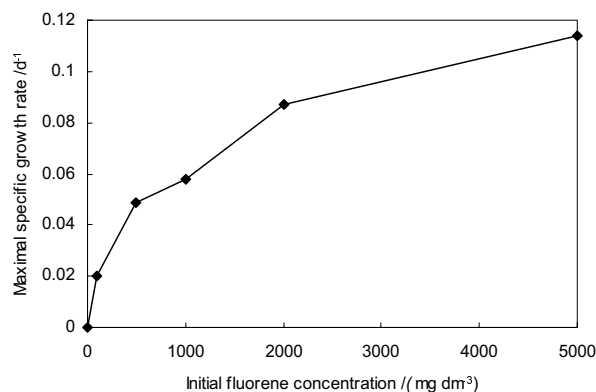
via the so-called "five-membered attack", which consists in the monooxygenation at C-9 of the methylene bridge as shown in Scheme 1 below [6].

The product of this reaction, 9-fluorenol is subsequently dehydrogenated to 9-fluorenone prior to ring cleavage. This kind of initial fluorene oxidation was detected in *Pseudomonas* sp. strain F274 [7] and also in *Brevibacterium* sp. strain DPO 1361 [1]. Both of these strains can utilize fluorene as sole source of carbon and energy. On the other hand, this metabolite route seems to be unproductive for *Arthrobacter* sp. F101 [2, 8] and *Staphylococcus auriculans* DBF63 [9] because it leads to a dead-end product, 4-hydroxy-9-fluorenone.

In order to identify the metabolites of the lower part of the pathway of fluorene assimilation by *Sphingomonas* sp. L-138, the extraction of the liquid medium at pH 2 and also at pH 7 was performed using a more hydrophilic solvent, ethyl acetate. A peak of phthalic acid was identified in the extract of the acidified medium whereas phthalic and protocatechuic acids were extracted from the neutral pH medium. Neither 9-fluorenol or 9-fluorenone accumulation, nor that of other presumed intermediates was observed in medium extracts. This is compatible with the hypothesis that *Sphingomonas* sp. L-138 degrades fluorene completely.

### Influence of Fluorene Initial Concentration on *Sphingomonas* sp. L-138 Growth

At experimental conditions, no fluorene inhibition effect on the microbial growth was observed and the bacteria grew well at all initial fluorene concentrations (100–5000 mg dm<sup>-3</sup>). The maximal specific growth rate of *Sphingomonas* sp. L-138 at 12°C exhibited a saturation dependence on the initial fluorene as shown in Fig. 3.

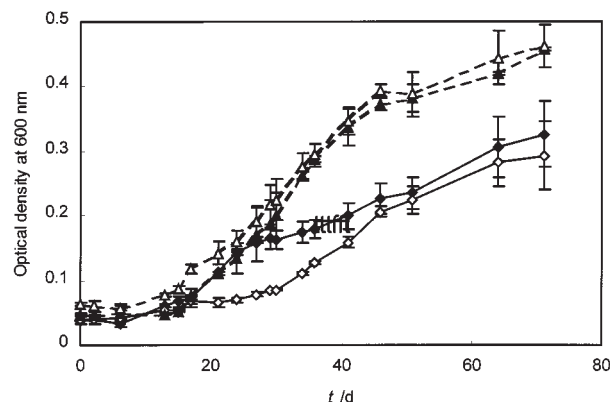


**Fig. 3.** Influence of the initial fluorene concentration on the maximal specific growth rate of *Sphingomonas* sp. L-138. Cultivation performed in Tris minimal medium supplemented with fluorene at 12°C and shaking frequency of 180 min<sup>-1</sup>.

Due to the low water solubility of fluorene, the influence of its initial concentration on the growth rate of bacteria cultivated on PAH used as the sole carbon source, is hard to evaluate. Under experimental conditions applied, the initial fluorene concentration largely exceeded its water solubility (about 2 mg dm<sup>-3</sup> at 30°C). Thus, the major portion of fluorene was available only to the bacteria attached to its crystals. According to microscopic observations in the course of cultivation, *Sphingomonas* sp. L-138 has an increased affinity to hydrophobic surfaces. The fact that the cells were adsorbed on, or accumulated around the fluorene crystals indicates that *Sphingomonas* sp. L-138 is able to consume the solid substrate. This behaviour is related to the particular outer cell wall layer which is composed of glycopospholipids [5]. Based on this observation, the bacteria growth rate apparently depends on the fluorene dissolution rate and on the specific surface area of the fluorene crystals.

### Influence of the Inoculum Type on *Sphingomonas* sp. L-138 Growth

In order to examine the effect of a potential induction of the fluorene degradation pathway, the bacteria were cultivated in aqueous medium supplemented with the fluorene crystals using cells pre-grown on glucose or on fluorene as inoculum. The initial concentration of fluorene used in this experiment was in the range of 100–5000 mg dm<sup>-3</sup>. No significant differences in the bacterial growth onset were observed when bacteria provided from different pre-cultures were growing in the medium supplemented with fluorene at the same initial concentrations. In Fig. 4, the kinetics of *Sphingomonas* sp. L-138 growths at initial



**Fig. 4.** Influence of inoculum preparation on the growth of *Sphingomonas* sp. L-138. Cultivation performed in Tris minimal medium supplemented with fluorene at 12°C and shaking frequency of 180 min<sup>-1</sup>.  $\rho(\text{Fluorene})$  1000 mg dm<sup>-3</sup>:  $\blacklozenge$  pre-culture on glucose,  $\diamond$  pre-culture on fluorene.  $\rho(\text{Fluorene})$  2000 mg dm<sup>-3</sup>:  $\blacktriangle$  pre-culture on glucose,  $\triangle$  pre-culture on fluorene.

fluorene concentrations of 1000 and 2000 mg dm<sup>-3</sup> are shown as examples of this behaviour. The time required for expression of the fluorene degradation pathway lasted about 15 days in both cultures, whether inoculated with bacteria pre-cultured on glucose or with bacteria pre-grown on fluorene.

These results show that the ability of *Sphingomonas* sp. L-138 to utilize fluorene as carbon source does not seem to be inducible and that the expression of fluorene degradation is constitutive, as it does not depend on the substrate (glucose or fluorene) used in the pre-culture.

## CONCLUSION

It was shown that bacteria *Sphingomonas* sp. L-138 are a promising strain to be used in processes of the bioaugmentation because of their ability to degrade fluorene and to grow on solid crystals of the substrate. After an adaptation phase, this strain performs complete fluorene mineralization at 12°C at a maximum specific degradation rate of about 0.08 mg d<sup>-1</sup> 10<sup>-6</sup> cells. The ability of fluorene uptake is a constitutive property of these bacteria and it is not necessary to induce it by a pre-cultivation on fluorene.

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