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EXPERIMENTAL ARTICLES

Fluorene Cometabolism by *Rhodococcus rhodochrous* and *Pseudomonas fluorescens* Cultures

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Abstract—The transformation of fluorene by *Rhodococcus rhodochrous* strain 172 grown on sucrose and *Pseudomonas fluorescens* strain 26K grown on glycerol was studied as a function of the substrate concentration and the growth phase. Under certain cultivation conditions, fluorene was completely consumed from the medium. The specific transformation rate of fluorene was considerably higher when it was transformed in the presence of the cosubstrates than when it served as the sole carbon source. An approach to the evaluation of the specific transformation rate of fluorene during batch cultivations is proposed.

Key words: fluorene transformation, cometabolism, *Rhodococcus rhodochrous*, *Pseudomonas fluorescens.*

The polycyclic aromatic hydrocarbon fluorene is a widespread environmental pollutant. It is a constituent of coal tar, oil, and oil products. Fluorene is formed upon the incineration of some organic compounds [1] and is often detected in the atmosphere and bottom sediments. The microbiological methods of transformation and degradation of toxic polycyclic aromatic hydrocarbons are now considered to be the most promising methods for their detoxification [2], which calls for the search of novel fluorene-transforming microorganisms and the study of metabolic systems involved in fluorene conversion. Research along this line is also important from the standpoint of the microbial production of regio- and stereospecific fluorene derivatives, which are difficult to obtain chemically [3].

Fluorene can be transformed or completely degraded by taxonomically different microorganisms, including gram-positive [1, 4–6] and gram-negative [7, 8] bacteria. Of great interest is the transformation of fluorene in the presence of cosubstrates, since it is known that cometabolism can considerably increase the transformation rate of persistent substrates [9] and that polluted areas are usually contaminated by several compounds rather than by a sole toxicant [10].

The aim of the present work was to investigate the feasibility of transforming fluorene by *Rhodococcus rhodochrous* strain 172 and *Pseudomonas fluorescens* strain 26K during their growth on standard mineral media.

MATERIALS AND METHODS

Rhodococcus rhodochrous strain 172 was selected from the collection of rhodococci at the Laboratory of the Enzymatic Degradation of Organic Compounds, Institute of Biochemistry and Physiology of Microorganisms, for its ability to transform fluorene. The other fluorene-transforming strain, *Pseudomonas fluorescens* 26K, was isolated earlier from the coking industry sewage [11]. These two strains were chosen for investigations because they are taxonomically different and are expected to possess structurally and functionally different metabolic systems involved in fluorene transformation. The choice of growth substrates (cosubstrates) was dictated by the fact that they provided for the most intense growth of the strains chosen for investigations.

The strains were grown at 29°C on a shaker in 750-ml flasks with 100 ml of a mineral medium containing (g/l) NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4$, 0.2; and $CaCl_2$, 0.02. The Fe ion requirement of the strains was satisfied by adding two drops of a saturated $FeCl_3$ solution to the medium. The pH of the medium was about 7.5. Fluorene (Fluka, Switzerland) was added to the medium to a concentration of 100 mg/l in the form of an acetone solution. The acetone added together with fluorene was allowed to evaporate by incubating flasks on the shaker for one day, after which the flasks were inoculated with bacterial cells. Cosubstrates (sucrose and glycerol) were added to the medium to the desired concentrations in the form of sterile concentrated aqueous solutions.

To study the dynamics of fluorene transformation, a few identical flasks were incubated under identical conditions. The flasks were inoculated with bacterial cells to an optical density of 1.0 (the control flasks were not inoculated) and incubated on the shaker. At certain time intervals, one of the flasks was taken for analyses. First, the optical density of the culture was measured at 540 nm

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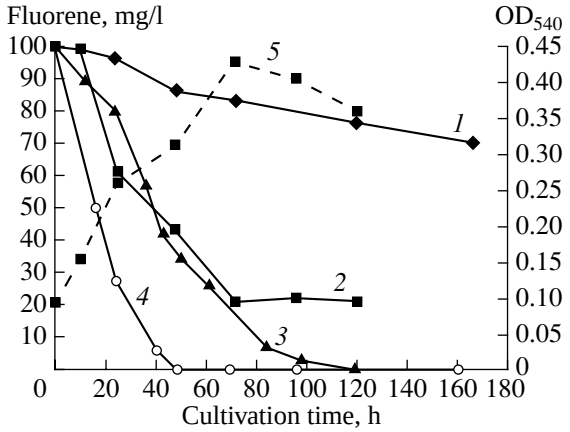

Fig. 1. The dynamics of fluorene in the culture liquid of *R. rhodochrous* 172 grown (*1*) without cosubstrate and in the presence of (*2*) 0.5, (*3*) 1, and (*4*) 5 g/l sucrose as the cosubstrate. Curve (*5*) shows bacterial growth in the presence of 0.5 g/l sucrose.

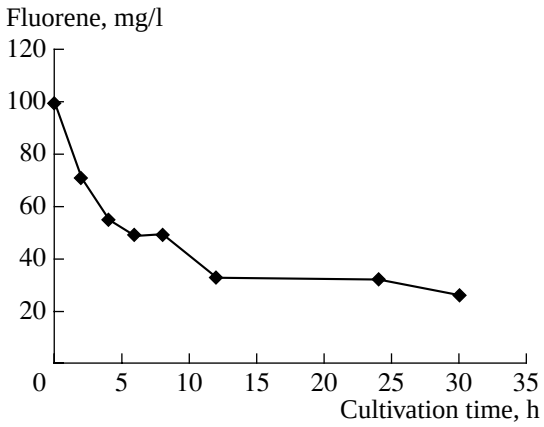

Fig. 2. The transformation of fluorene by washed *R. rhodochrous* 172 cells.

using a KFK colorimeter and a 0.5-cm cuvette. Then, the contents of the flask were thrice extracted with ethylacetate (before the third extraction, the aqueous phase in the flask was acidified to pH 2 with diluted HCl). The extracts were pooled and evaporated using a vacuum rotary evaporator. The dry residue was dissolved in acetone and analyzed qualitatively by thin-layer chromatography (TLC) on Kieselgel 60 F-254 plates (Merck, Germany). The plates were developed using a benzene–dioxane–acetic acid (90 : 10 : 1) solvent mixture. Spots were visualized under UV light. Then the acetone solution was quantitatively analyzed using a Pye Unicam gas–liquid chromatograph equipped with a flame ionization detector and a (1.5 m × 2 mm) column packed with Chromosorb G-AW-DMSC containing 3% SE-30. The injector, column, and detector were kept at 150, 175, and 290°C, respectively.

Experiments on the transformation of fluorene by washed cells were carried out as follows. The strains were grown on the respective growth substrates in the presence of fluorene to the retardation growth phase. Cells were harvested by centrifugation, washed twice with phosphate buffer (pH 7.2), and suspended in the buffer to an optical density of 1.2. This cell suspension was dispensed in equal volumes in flasks. The flasks were supplemented with fluorene and incubated under the conditions described above. The contents of the flasks were analyzed in the same manner as described above for the growth experiments.

RESULTS AND DISCUSSION

When incubated in the mineral medium with fluorene as the sole carbon source, the strains did not exhibit noticeable growth (i.e., changes in the optical density of the cultures were within the error of measurements). In this case, the concentration of fluorene in the medium decreased at a considerably slower rate than in the presence of the respective cosubstrate (Figs. 1, 3). TLC analysis showed that the transformation of fluorene in the presence of the cosubstrates was accompanied by the appearance of some metabolites in the culture liquid, which were not detected when fluorene served as the sole carbon source.

The high rates of fluorene transformation by *R. rhodochrous* 172 were observed for a wide range of initial sucrose concentrations (C^0_{suc} = 0.5–5 g/l) in the cultivation medium (Fig. 1). At C^0_{suc} = 1 g/l, fluorene was completely transformed after 120 h, while after 48 h at C^0_{suc} = 5 g/l.

A comparative analysis of the effects of sucrose on the growth of *R. rhodochrous* 172 and fluorene transformation showed that the acceleration of fluorene

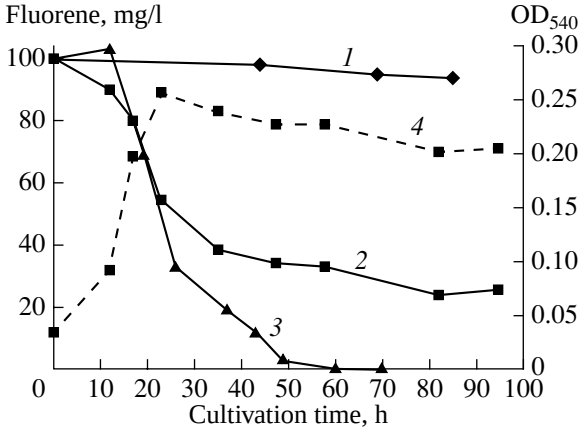

Fig. 3. The dynamics of fluorene in the culture liquid of *P. fluorescens* 26K grown (*1*) without cosubstrate and in the presence of (*2*) 0.6 and (*3*) 1.2 g/l glycerol as the cosubstrate. Curve (*4*) shows bacterial growth in the presence of 0.6 g/l glycerol.

transformation by sucrose was not merely due to its beneficial effect on the biomass. Indeed, in these experiments, the biomass yield *Y* was proportional to the initial concentration of sucrose up to its value C^0_{suc} = 2 g/l. Therefore, the transition of the *R. rhodochrous* 172 culture at C^0_{suc} = 0.5 g/l to the stationary phase was due to the exhaustion of sucrose from the medium. As can be seen from Fig. 1 (curves *2* and *5*), the transformation of fluorene drastically declined when sucrose was exhausted and the culture entered the stationary growth phase. These data strongly suggest that the beneficial effect of sucrose on the transformation of fluorene is due to the cometabolism of sucrose and fluorene, since, by definition, cometabolism is characterized by an increase in the specific (per unit biomass) transformation rate of a substrate in the presence of a cosubstrate [9].

The washed *R. rhodochrous* 172 cells taken from the retardation growth phase and incubated under non-growth conditions could also transform fluorene at a high rate (Fig. 2). These data indicate that some factors responsible for growth limitation may affect fluorene transformation by bacterial cells.

Similar data were obtained with the other strain, *P. fluorescens* 26K. The addition of glycerol enhanced the transformation of fluorene by this strain (Fig. 3). At a sufficiently high initial glycerol concentration (C^0_{gly} = 1.2 g/l), fluorene was completely transformed after 60 h. As in the case with *R. rhodochrous* 172, the transformation rate of fluorene by *P. fluorescens* 26K considerably decreased in the stationary phase, which was caused by the exhaustion of glycerol from the medium (Fig. 3, curves *2* and *4*).

The amount of fluorene transformed in the *P. fluorescens* 26K culture depended not only on the initial concentration of glycerol in the medium, but also on the physiological state of cells. Figure 4a presents some growth curves of *P. fluorescens* 26K cultivated at different initial concentrations of glycerol (these concentrations are indicated alongside the growth curves). The concentrations of fluorene remaining in the culture liquids after 69 h of cultivation are shown in Fig. 4b. A comparison of the biomass yields at different initial glycerol concentrations allowed the inference to be made that, at C^0_{gly} from 0 to 1.2 g/l, the transition of *P. fluorescens* 26K to the stationary growth phase was due to the exhaustion of glycerol from the medium. Within this range of glycerol concentrations, the residual concentration of fluorene in the medium was inversely proportional to the initial concentration of glycerol, so that at C^0_{gly} = 1.2 g/l, fluorene was entirely transformed. Surprisingly, with a further increase in C^0_{gly} to 1.6 g/l, the degree of fluorene transformation decreased (Fig. 4b). Presumably, at C^0_{gly} higher than 1.2 g/l, bacterial transition to the stationary growth phase was caused by a factor other than the exhaustion of glycerol from the medium. This factor, presumably, not only inhibits the growth of *P. fluorescens* 26K but also suppresses the transformation of fluorene.

The data presented in Fig. 3 cannot provide an answer to the question of whether the beneficial effect of glycerol on the transformation of fluorene by *P. fluorescens* 26K cells is due to the effect of glycerol metabolism itself or mediated by the increased biomass accumulated in the presence of glycerol. To answer this question, we employed the following approach.

The specific transformation (or consumption) rate *q* of a substrate is the specific rate of decrease in its concentration *S* per unit biomass *X*, given by [12]:

$$q = -\frac{1}{X}\frac{dS}{dt}. \quad (1)$$

For a batch culture, this rate can be calculated on the basis of the substrate transformation model, as was done by Wang and Loh for the cometabolism of phenol and 4-chlorophenol in the presence of glutamate [10]. Since such a model has not yet been constructed for the transformation of fluorene, let us proceed from empirical data. From formula (1), it follows that the decrease in the substrate concentration during the time lapse (t_1, t_2) is given by the expression

$$-\Delta S = \int_{t_2}^{t_1} q(t)X(t)dt. \quad (2)$$

During batch cultivations, the specific rate *q* varies with time as a result of changes in the medium composition, the biomass concentration, and the physiological state of cells. Let us substitute the function $q(t)$ in expression (2) by a constant q' such that the value of this expression does not change:

$$-\Delta S = \int_{t_2}^{t_1} q(t)X(t)dt = q'\int_{t_2}^{t_1} X(t)dt. \quad (3)$$

The quantity q' is a constant specific rate of substrate transformation which is chosen such that the decrease in the substrate concentration during the time interval (t_1, t_2) is the same as in the case of the actual variable specific rate of substrate transformation. This quantity may be called the effective specific rate of substrate transformation. From equation (3), it follows that

$$q' = \frac{-\Delta S}{\int_{t_2}^{t_1} X(t)dt}. \quad (4)$$

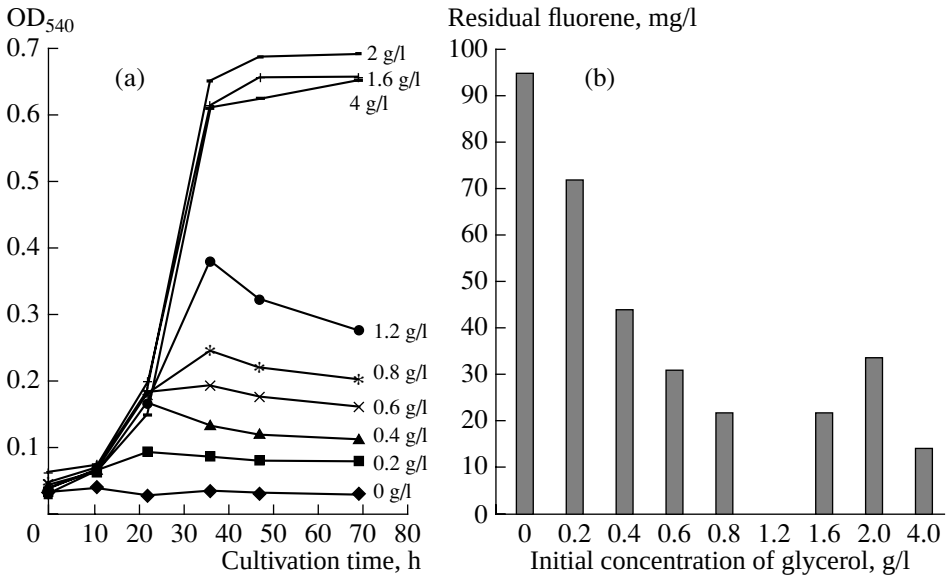

Fig. 4. (a) The growth of *P. fluorescens* 26K at different initial concentrations of glycerol (indicated alongside the growth curves) and (b) the residual concentrations of fluorene in the culture liquids after 69 h of cultivation.

If the concentrations of the biomass and the substrate are measured at the same points, expression (4) transforms into

$$q' = \frac{2(S_1 - S_2)}{(X_1 + X_2)(t_2 - t_1)}. \quad (5)$$

The effective specific rate of substrate transformation given by formulas (4) and (5) can be found by dividing the decrease in the substrate concentration over the time interval (t_1, t_2) by the area below the line showing the time dependence of the biomass (or the optical density of the culture) at this time interval.

Figure 5 shows the results of such calculations for the transformation of fluorene by *P. fluorescens* 26K cells at different initial concentrations of glycerol in the medium. At small cultivation times, the graphs $q'(t)$ for fermentations with glycerol lie above the graph for fermentation without glycerol. However, after about 30 h of cultivation, the specific transformation rates of fluorene in the presence of glycerol fall to values typical of its transformation in the absence of the cosubstrate. Therefore, the transformation of fluorene in the presence of glycerol is enhanced due to the cometabolism of these compounds. Numerically, the specific rate of fluorene transformation by the logarithmic-phase *P. fluorescens* 26K culture grown in the presence of 0.6 g/l glycerol reaches a value higher than 25 mg fluorene per l per h per one optical density unit of cell suspension. This value is higher than that observed in the absence of the cosubstrate (Fig. 5).

Thus, the data obtained in this study show that the *R. rhodochrous* strain 172 and *P. fluorescens* strain 26K grown in standard mineral media in the presence of, respectively, sucrose and glycerol as cosubstrates transform fluorene more efficiently than in the absence of the cosubstrates. The cometabolic transformation of fluorene by these cultures under the given conditions can presumably be used for obtaining valuable products which are difficult to produce by chemical synthesis. The application of these strains to bioremediation purposes requires a search for other cosubstrates that less expensive than sucrose and glycerol.

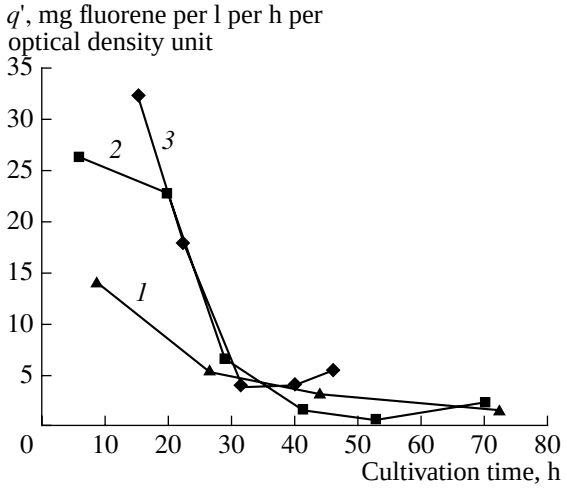

Fig. 5. The specific rates of fluorene transformation by *P. fluorescens* 26K growth (*1*) without a cosubstrate and in the presence of (*2*) 0.6 and (*3*) 1.2 g/l glycerol as the cosubstrate.

ACKNOWLEDGMENT

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