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EXPERIMENTAL ARTICLES

# Effect of Perfluorodecalin, Carbogal, and Perfluoromethyldecalin on Growth and Ice-Forming Activity of Bacteria

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**Abstract**—The isolates of pseudomonads (56) and of *Plesiomonas shigelloides* (7) exhibiting ice-forming activity were obtained from plant leaves and rhizosphere. The theoretical possibility of the application of perfluorocarbons (PFC) with a gas-transporting function (perfluorodecalin, carbogal, and perfluoromethyldecalin) for the cultivation of bacteria with different levels of ice-forming activity (IFA) in order to enhance their growth rates, biomass yields, and IFA was demonstrated. Introduction of 5% perfluorodecalin, carbogal, or perfluoromethyldecalin resulted for two strains in a 1.7–3.1-fold increase in biomass and a 3.2–24.5-fold increase in ice-forming activity compared to the control (without PFC).

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*Key words*: bacteria, perfluorocarbons, submerged culture, ice-forming activity.

The ability to initiate the process of crystallization of cooled water (–1°C and lower) was found in *Pseudomonas fluorescens, P. syringae, P. fragi, P. cepacia, P. pseudoalcaligenes*, and in other widespread saprophytic and phytopathogenic bacteria [1, 2]. The ice-forming activity of these bacteria is important both for meteorology and for agriculture. They are a natural source of atmospheric ice-forming particles and participate in plant damage caused by frost. The ice-forming activity (IFA) of these microorganisms is caused by the presence of specific proteins in their cell walls, which initiate the crystallization of supercooled water [3].

Intensive processes of aerobic metabolism are characteristic for pseudomonads and other bacteria exhibiting IFA [4]. Submerged culture is known to result in the highest yield of the products of microbiological synthesis [5]. Aeration and improved conditions of oxygen transfer to the cells are required for the normal aerobic growth in deep layers of liquid medium. The application of liquid perfluorocarbons (PFC) for this purpose is of interest. They have many practically important features, including high biological and chemical stability and the absence of toxicity to living organisms. They are also able to dissolve gases (up to 50 vol % of oxygen and up to 200 vol % of $CO_2$) and to modify the cell membranes to facilitate transport [6].

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We have recently been investigating the potential of PFC for submerged cultivation of microorganisms of various groups for the subsequent application of these results in the biotechnological industry [7–9].

The goal of the present work was the experimental verification of the possibility of the application of gas-transporting perfluorocarbons (perfluorodecalin, carbogal, and perfluoromethyldecalin) for the cultivation of ice-forming bacteria of the genera *Pseudomonas* and *Plesiomonas* in order to intensify their growth, biomass production, and IFA.

## MATERIALS AND METHODS

Perfluorodecalin, carbogal, and perfluoromethyldecalin were produced by OAO Konstantinov Kirovo–Chepetsk chemical plant. Perfluorodecalin is manufactured as a component (7 vol %) of the certified Perftoran blood substitute (TU 95.1233-92, with modification 1, batch no. 24). It is a colorless, odorless, transparent liquid, insoluble in water and in traditional organic solvents, highly inert chemically, resistant to strong acids and bases, nonflammable, explosion-proof, stable up to 400°C, and is considered nontoxic. Its density at 20°C is 1945 kg/m$^3$, boiling temperature, 142°C. The solubility (vol %) of oxygen in perfluorodecalin is 51; of nitrogen, 32; and of $CO_2$, 193. Carbogal and perfluoromethyldecalin are cheaper PFC; they are transparent liquids manufactured in experimental series for

**Table 1.** Ice-forming activity of the *Pseudomonas* and *Plesiomonas* bacteria

| Species | Total number of isolates studied | Number of isolates: | | |
|---|---|---|---|---|
| | | with high IFA* | with low IFA** | without IFA*** |
| *Pseudomonas putida* | 5 | 0 | 1 | 4 |
| *P. aeruginosa* | 7 | 0 | 2 | 5 |
| *P. fluorescens* | 21 | 4 | 5 | 12 |
| *P. stutzeri* | 3 | 0 | 0 | 3 |
| *P. mendocina* | 2 | 0 | 0 | 2 |
| *P. alcaligenes* | 4 | 0 | 0 | 4 |
| *P. pseudoalcaligenes* | 14 | 2 | 3 | 9 |
| *Plesiomonas shigelloides* | 7 | 1 | 1 | 5 |

* Freezing temperature of over 90% droplets not below –6°C.
** Freezing temperature of over 90% droplets not below –10°C.
*** Freezing temperature of over 90% droplets the same as in control (double-distilled water).

research purposes. They are identical to perfluorodecalin in most respects.

For the enrichment culture of pseudomonads, the following synthetic medium was used (g/l): $MgSO_4 \cdot 7H_2O$, 0.2; $K_2HPO_4$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.05; $CaCl_2$, 0.02; $MnCl_2 \cdot 4H_2O$, 0.002; $NaMoO_4 \cdot 2H_2O$, 0.001; $NH_4Cl$, 1.0; ethanol, 4.0. For the isolation of pure cultures by the Drigalskii method, 15.0 g/l agar was added [9]. The incubation was performed at 26°C in the dark.

For the determination of the bacterial species, the MICRO-LA-TEST (PLIVA-Lachema, Czech Republic) identification test systems, tables, and codebooks were used [10].

The pure cultures of the identified bacterial isolates were used for the experiments on submerged culture in nutrient broth (NB) [11]. The 750 ml Erlenmeyer flasks with 145 ml medium were inoculated with 5 ml of the cells washed off the medium with physiological saline. The inoculum contained $3 \times 10^9$ cells/ml according to the optical turbidity standard (OSO 42-28-85-02). The cell concentration after inoculation was $1 \times 10^8$ cells/ml, according to OSO 42-28-85-02. One of the PFC was introduced into the experimental flasks in concentrations of 0.2, 1.0, 5.0, or 25.0 vol %; nutrient broth without PFC was used as control.

The cultivation was performed at (26 ± 2)°C with shaking at 230 rpm. After 24 h of cultivation, 10 ml samples were taken to determine the number of viable cells by plating tenfold dilutions of the cell suspension in the physiological saline on nutrient agar.

The ice-forming nuclei were determined in the cell suspension and in its dilutions in double-distilled water. Their concentration was obtained as the number of droplets frozen due to the presence of crystallization centers according to the modified equation [2]:

$$C = \ln \frac{N_0}{N} \times \frac{10^R}{V},$$

where $C$ is the concentration of ice-forming nuclei; $N_0$, the total number of droplets; $N$, the number of unfrozen droplets; $R$, the number of tenfold dilutions; and $V$, the droplet volume, 0.01 ml.

The hydrophobicity of aluminum foil cuvettes was assured prior to the experiment. The inner surface of a cuvette was treated with 3% paraffin solution in chloroform, and the cuvette was incubated in the flow of warm sterile air for four min, to complete evaporation of the solvent. Fifty 0.01 ml drops of the dilution of the culture ($10^8$ or $10^6$ cells/ml) were then placed in the cuvette with a micropipette.

The distance between the droplets was not less than 5 mm. The cuvette was inserted into the MK-2 mini cryostat chamber (Biomashpribor, Ioshkar-Ola, Russia), on the surface cooled to the required temperature. The number of the frozen droplets was determined visually, through the inspection window of the cryostat.

In the analysis of the results of the ice-forming activity of bacterial cultures, the freezing temperature of double-distilled water was considered. This temperature was determined in every experiment.

## RESULTS AND DISCUSSION

The IFA of the 56 isolates of seven *Pseudomonas* species and of seven isolates of *Plesiomonas shigelloides* obtained from the leaves and rhizosphere of Kirov oblast plants was determined according to the modified method [2] (Table 1).

In 44 cases (69.8% of the isolates tested), the droplets of bacterial suspension in double-distilled water ($10^8$ cells/ml) commenced freezing at –11…–13°C; complete freezing of all the droplets occurred only at –19…–20°C. Since the freezing pattern of double-distilled water was the same, these strains were considered to have no IFA. Twelve isolates (19.1%), which caused freezing of over 90% of the droplets at the temperature not below –10°C exhibited low IFA. Seven strains

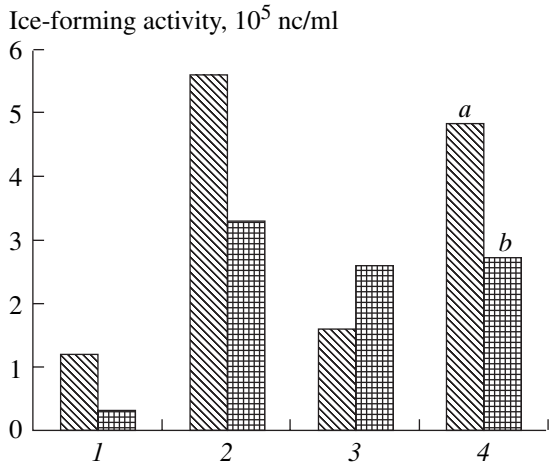
**Table 2.** Freezing dynamics of the droplets of *Pseudomonas* and *Plesiomonas* cell suspensions ($10^6$ cell/ml) in the mini cryostat chamber

| Strains | Fraction of frozen droplets at different temperatures, % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | –3°C | –5°C | –7°C | –9°C | –11°C | –13°C | –15°C | –17°C | –19°C |
| *P. fluorescens VU* 547 | | | 8 | 34 | 62 | 88 | 100 | | |
| *P. fluoresceris VU* 548 | | 6 | 38 | 100 | | | | | |
| *P. fluorescens VU* 549 | | | 22 | 34 | 82 | 100 | | | |
| *P. fluorescens VU* 550 | | | 4 | 32 | 64 | 82 | 100 | | |
| *P. pseudoalcaligenes VU* 551 | | 2 | 12 | 56 | 76 | 92 | 100 | | |
| *P. pseudoalcaligenes VU* 552 | | | 8 | 24 | 38 | 74 | 100 | | |
| *Plesiomonas shigelloides VU* 553 | | 2 | 18 | 52 | 100 | | | | |
| Double-distilled water | | | | | 10 | 32 | 52 | 90 | 100 |

(11.1%) exhibited pronounced IFA with over 90% of the droplets frozen at temperatures not below –6°C.

The highest IFA among the investigated isolates was observed in *P. fluorescens*. Among the 21 identified isolates of this species, four (19.0%) exhibited pronounced, and five (23.8%), weak IFA. Isolates with both weak and pronounced IFA were also found among *P. pseudoalcaligenes* and *Plesiomonas shigelloides*. Only weak IFA was found in the isolates of *P. putida* and *P. aeruginosa*. None of the analyzed cultures of *P. stutzeri, P. mendocina*, and *P. alcaligenes* exhibited IFA.

The characteristics and the freezing dynamics of the isolates of *P. fluorescens, P. pseudoalcaligenes*, and *Plesiomonas shigelloides* with the highest IFA were studied, and these isolates were stored in the microbial cultures collection of the Vyatka State University (Table 2).




Number of nucleation centers (nc) per $10^9$ microbial cells at –9°C in the cultures of *P. fluorescens* VU 548 (*a*) and *Plesiomonas shigelloides* VU 553 (*b*) grown in the media with perfluorocarbons (*1*, without PFC; *2*, with 5.0 vol % perfluorodecalin; *3*, with 5.0 vol % carbogal; *4*, with 5.0 vol % perfluoromethyldecalin).

A 100-fold decrease in the microbial cell load resulted in the active ice formation in the suspensions of the organisms with high IFA at –9°C and lower temperatures (Table 2). Strain *P. fluorescens* VU 548 was the most active ice-former; all the droplets of its suspension froze at –9°C. Strain *Plesiomonas shigelloides* VU 553 had a somewhat weaker IFA (all the droplets froze at –11°C).

Strains *P. fluorescens* VU 548 and *Plesiomonas shigelloides* VU 553 were chosen for the subsequent experimental investigation of the application of the gas-transporting PFC for the cultivation of ice-forming bacteria of the genera *Pseudomonas* and *Plesiomonas* in order to enhance their growth rate, biomass yield, and IFA.

The cultures were grown in nutrient broth with varying concentrations of PFC as described above; the number of living cells and IFA (as the number of ice-forming centers per 1 ml at –9°C) were determined (Tables 3, 4).

The results presented in Tables 3, 4 indicate intensification of growth and of the number of nucleation centers in the medium with PFC. Among the four concentrations tested (0.2, 1.0, 5.0, and 25.0 vol %), 5.0 vol % of perfluorodecalin, carbogal, or perfluoromethyldecalin was the optimal PFC concentration for the increase in both bacterial numbers and the number of the nucleation centers.

Perfluorodecalin caused the most efficient increase in biomass and the number of nucleation centers. The introduction of 5.0 vol % of perfluorodecalin resulted in a 2.2–3.1-fold biomass increase and a 14.9–24.5-fold increase in the number of nucleation centers compared to the control. Carbogal (5.0 vol %) caused a 1.7–2.4-fold increase in biomass and a 3.2–14.5-fold, in the number of nucleation centers; 5.0 vol % perfluoromethyldecalin caused a 1.9–2.7-fold and 11.1–17.7-fold increase, respectively.

Since the introduction of 5.0 vol % PFC into the liquid media for the cultivation of *Pseudomonas* and *Plesiomonas* bacteria caused the simultaneous increase in

**Table 3.** Concentrations of viable microbial cells and of ice-forming nuclei at –9°C by *P. fluorescens* VU 548 grown in liquid cultures with perfluorocarbons

| Perfluorocarbons | Perfluorocarbons concentration, vol % | Number per 1 ml of culture ($\bar{X} \pm I_{95}$) | |
|---|---|---|---|
| | | living cells | nucleation centers |
| Perfluorodecalin | 0.2 | $(3.5 \pm 0.5) \times 10^9$ | $(4.6 \pm 0.5) \times 10^5$ |
| | 1.0 | $(7.1 \pm 0.6) \times 10^9$ | $(1.7 \pm 0.2) \times 10^6$ |
| | 5.0 | $(9.8 \pm 0.8) \times 10^9$ | $(5.5 \pm 0.6) \times 10^6$ |
| | 25.0 | $(9.6 \pm 0.8) \times 10^9$ | $(5.3 \pm 0.6) \times 10^6$ |
| Carbogal | 0.2 | $(3.4 \pm 0.6) \times 10^9$ | $(3.8 \pm 0.5) \times 10^5$ |
| | 1.0 | $(5.5 \pm 0.7) \times 10^9$ | $(6.9 \pm 0.6) \times 10^5$ |
| | 5.0 | $(7.7 \pm 0.8) \times 10^9$ | $(1.2 \pm 0.2) \times 10^6$ |
| | 25.0 | $(6.7 \pm 0.8) \times 10^9$ | $(7.2 \pm 0.7) \times 10^5$ |
| Perfluoromethyldecalin | 0.2 | $(3.6 \pm 0.6) \times 10^9$ | $(3.9 \pm 0.4) \times 10^5$ |
| | 1.0 | $(7.3 \pm 0.7) \times 10^9$ | $(8.2 \pm 0.7) \times 10^5$ |
| | 5.0 | $(8.5 \pm 0.7) \times 10^9$ | $(4.1 \pm 0.8) \times 10^6$ |
| | 25.0 | $(8.1 \pm 0.7) \times 10^9$ | $(3.4 \pm 0.5) \times 10^6$ |
| Control | 0 | $(3.2 \pm 0.4) \times 10^9$ | $(3.7 \pm 0.4) \times 10^5$ |

Note: The values represent the average of six repeats.

**Table 4.** Concentrations of viable microbial cells and of ice-forming nuclei at –9°C by *Plesiomonas shigelloines* VU 553 grown in liquid cultures with perfluorocarbons

| Perfluorocarbons | Perfluorocarbons concentration, vol % | Number per 1 ml of culture ($\bar{X} \pm I_{95}$) | |
|---|---|---|---|
| | | living cells | nucleation centers |
| Perfluorodecalin | 0.2 | $(1.9 \pm 0.2) \times 10^9$ | $(1.6 \pm 0.2) \times 10^5$ |
| | 1.0 | $(2.6 \pm 0.2) \times 10^9$ | $(3.8 \pm 0.5) \times 10^5$ |
| | 5.0 | $(3.9 \pm 0.4) \times 10^9$ | $(1.3 \pm 0.2) \times 10^6$ |
| | 25.0 | $(3.6 \pm 0.4) \times 10^9$ | $(8.2 \pm 0.8) \times 10^5$ |
| Carbogal | 0.2 | $(1.8 \pm 0.2) \times 10^9$ | $(8.9 \pm 0.8) \times 10^4$ |
| | 1.0 | $(2.3 \pm 0.3) \times 10^9$ | $(2.5 \pm 0.3) \times 10^5$ |
| | 5.0 | $(3.0 \pm 0.3) \times 10^9$ | $(7.7 \pm 0.8) \times 10^5$ |
| | 25.0 | $(2.5 \pm 0.3) \times 10^9$ | $(4.2 \pm 0.5) \times 10^5$ |
| Perfluoromethyldecalin | 0.2 | $(2.0 \pm 0.2) \times 10^9$ | $(1.9 \pm 0.2) \times 10^5$ |
| | 1.0 | $(2.5 \pm 0.3) \times 10^9$ | $(4.0 \pm 0.7) \times 10^5$ |
| | 5.0 | $(3.5 \pm 0.4) \times 10^9$ | $(9.4 \pm 0.9) \times 10^5$ |
| | 25.0 | $(2.8 \pm 0.3) \times 10^9$ | $(8.1 \pm 0.8) \times 10^5$ |
| Control | 0 | $(1.8 \pm 0.2) \times 10^9$ | $(5.3 \pm 0.4) \times 10^4$ |

Note: The values represent the average of six repeats.

numbers of both the cells and the nucleation centers, the relative IFA increase per $10^9$ cells was determined. The results of this analysis presented in the figure demonstrate that *P. fluorescens* VU 548 exhibited the highest increase in relative IFA. The culture of *Plesiomonas shigelloides* VU 553 also exhibited a significant increase in relative IFA.

These results demonstrate the prospects for further work on the application of PFC for submerged culture of ice-forming bacteria in order to enhance the biomass yield and to increase the IFA of environmental strains. The highest increase in the biomass yield and the highest IFA were observed on the introduction of 5.0 vol % perfluorodecalin into the medium.

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