THE ROLE OF EXOMETABOLITES ISOLATED FROM AQUATIC MACROPHYTES IN THE ACTIVITY OF OIL-OXIDIZING MICROORGANISMS (*Pseudomonas melochlora*)

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ABSTRACT

Oil hydrocarbons are known to be biodegradable, but the process of oil biodegradation depends on many factors. This article is intended to reveal the role exometabolites isolated from aquatic macrophytes in the activity of oil-oxidizing microorganism *Pseudomonas melochlora.* Amino acids, carbohydrates and organic acids influence differently the oil-oxidizing activity of *P. melochlora*. Addition of amino acids to growth medium resulted in increasing growth rates of bacterium and harvest. Exogenous carbohydrates also stimulated both the growth of *P. melochlora* and oil biodegradation. Organic acids did not show any consistent patterns with regard to growth rate of the bacterium used, its respiration activity, and the ability to degrade the oil.

KEYWORDS:
oil, biodegradation, macrophytes, exometabolites.

INTRODUCTION

Oil hydrocarbons have been known for decades to be biodegradable [1, 2], and microorganisms of various genera (both anaerobic and aerobic) were reported to be useful for biodegradation of oil and other xenobiotics [3, 4]. The process of oil biodegradation depends on many factors (temperature, salinity, etc) and many efforts were undertaken to improve its efficacy [5, 6]. It is especially difficult to control the process of oil biodegradation in the nature because of the complex interactions between participants (microbes, fungi, plants and animals) in various biocenosia. To perform a fundamental analysis of the possible cooperative action of biodegraders and other organisms, additional studies are needed. This article is intended to reveal the role of exometabolites isolated from aquatic macrophytes in the activity of oil-oxidizing microorganism *Pseudomonas melochlora.*

MATERIALS AND METHODS

Exometabolites of the following aquatic macrophytes were taken for this research: rush (*Phragmites australis*), various species of reed mace (*Typha angustifolia* and *Typha latifolia* L.), bulrush (*Scirpus lacustris* L.), and elodea (*Elodea canadensis* R.). To obtain root excreta (exometabolites) of the macrophytes, each of the plants was carefully washed with water, parched and weighted. After that, plants were treated with 3% peroxide, washed twice with sterile water, put into a 2-L flask with sterile distilled water (400 ml), and incubated in this flask for 24 h at natural lighting. Solutions with macrophyte excreta were concentrated in a rotary evaporator at 40-50 °C.

Pseudomonas melochlora (Winslow, Broadhurst, Buchanon, Krumwiede, 1917, 555) was obtained from Kazan Institute of Epidemiology and Microbiology (Kazan, Russia). It is an aerobic bacterium with bacillus-like morphology having a blue-green fluorescent pigment, and is able to oxidize oil and its products.

P. melochlora was grown in Muntz growth medium with oil at 28 °C for 2-6 days till stationary growth phase. Growth medium (pH 7.0–7.2) contained (g/L water): NH_4NO_3 - 0.8, $NH_4H_2PO_4$ - 1.0, KH_2PO_4 - 0.2, $MgSO_4 \cdot 7H_2O$ - 0.1, NaCl - 0.1, and desalinized oil (Romashkinskoe oil field in Tatarstan, Russia) - 600 mg/L. For cultivation of bacteria, 2 ml of bacterial subculture was inoculated into 400-ml flasks (after that the concentration of microorganisms was about $0.2\text{-}0.3 \times 10^6$ cells/ml) with amino acids, carbohydrates and organic acids isolated from aquatic macrophytes. 10 amino compounds (glutamic acid, alanine, aminoisovaleric acid, phenylalanine, arginine, asparaginic acid, leucine, serine, methionine and glutamine) correspond to only 3 concentrations (1.5×10^{-4}M; 7.5×10^{-5}M; 1.5×10^{-5}). Carbohydrates (glucose, saccharose, maltose, mannitol, lactose, inositol, dulcitol (galactitol), arabinose, rhamnose, sorbitol) as well as organic acids (α-ketoglutaric acid, succinic acid,

fumaric acid, oxaloacetic acid, malic acid and pyruvic acid) correspond to 3 concentrations too ($5x10^{-5}$M; $1x10^{-5}$M; $5x10^{-6}$M).

Growth rate of *P. melochlora* was evaluated in Petri dishes with agar as well as with or without (control) the above supplements. The amount of the used oil in growth medium was checked at the end of the experiment. The extraction of un-oxidized oil was performed with the use of carbon tetrachloride (in proportion 1:20). To eliminate the mortified bacteria, oil extract was centrifuged at 8,000 *g*. Then, optical density of the solution was analyzed using an infrared spectrophotometer at 3350 nm.

The respiration of *P. melochlora* in a mixture of oil-oxidizing cultures was analyzed by Warburg method [7]. The analysis was performed during 3 h at 28 °C with half-hour periodicity. The initial optical density of *P. melochlora* at 400 nm was about 0.6 (approximately $590x10^3$ cells/ml), and oil concentration 2.59 g/L (one drop) or 5.18 g/L (two drops).

RESULTS AND DISCUSSION

We found that exometabolites isolated from various aquatic macrophytes are utilized by *P. melochlora* during its living activity. The influence of the compounds on growth, respiration and oil oxidation depends on the chemical nature of the exometabolite. It was detected that amino acids variously influence the growth of *P. melochlora* (in order of attenuation of stimulation): glutamic acid > alanine > amino-isovaleric acid > phenylalanine ($1.5x10^{-5}$M) (Fig. 1). Similarly, concerning the oil-oxidizing activity, we detected the following order of attenuation of stimulation: glutamic acid > phenylalanine > methionine > amino-isovaleric acid > leucine > asparaginic acid > serine > alanine > arginine at $7.5x10^{-6}$M, and glutamic acid, amino-isovaleric acid > alanine > phenylalanine > arginine at $1.5x10^{-5}$M and $1.5x10^{-4}$M. (Fig. 1). Addition of amino acids to growth medium resulted in increase of bacterial growth rates and harvest of the culture after 4 days of cultivation (Fig. 1). By the end of the 3rd day, 100% glutamic acid, 98.3% of alanine and 89.4% of valine have been used. Phenylalanine did not change the growth rate and harvest of the bacterium.

The prolonged adaptation period of *P. melochlora* to substrate in control is probably connected to alteration of the enzymatic system of bacterial cells during their activity in oil oxidation. The role of amino acids is likely to be linked with the biostimulation of the process. The increase of the growth rate after introduction of some amino acids may favor this suggestion. For example, the addition of glutamic acid to the growth medium led to 2.6-fold increase of the bacterial number while the intensity of oil consumption was elevated by 20% (Fig. 2). Moreover, it is know that hydrocarbon-oxidizing microorganisms use amino acids very rarely as a source of carbon [8].

It is already known that the basic part of the oil obtained from the Romashkinskoe oil field is exposed to biodegradation during the first 5 days [9]. For our experiments, this process lasted 2-3 days and 6 days (in experimental variants and control, respectively). Due to accumulation of metabolic products, this process was slowed, and the remaining oil fractions (as well as asphaltenes and polyester resins) were not amenable to bacterial biodegradation.

The consumption of oxygen for respiration is one of the most important parameters to evaluate the intensity of microbiological processes in water. We found that the addition of amino acids to growth medium with oil resulted in a. complex increase of oxygen consumption by *P. melochlora* in comparison with control: glutamic acid > amino-isovaleric acid > alanine > arginine >methionine > phenylalanine (Fig. 3)

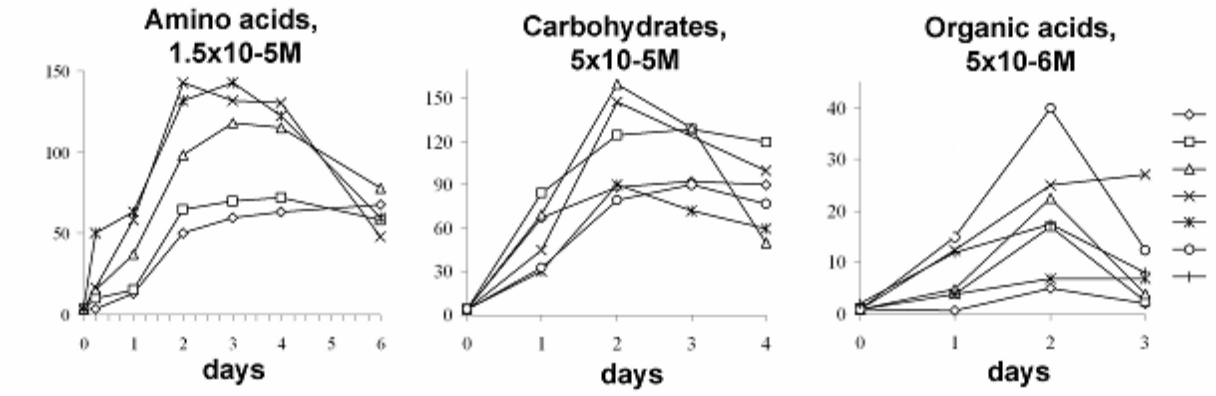

FIGURE 1 - Dynamics of *P. melochlora* growth in Muntz medium with the supplement of oil, amino acids, carbohydrates and organic acids. Y-axis shows a number of *P. melochlora* cells (millions per ml), and X-axis – days of cultivation. Symbols: (amino acids) ◊ – control, □ – phenylalanine, Δ – amino-isovaleric acid, × – alanine, ∗ - glutamic acid; (carbohydrates) ◊ – control, □ – glucose, Δ – mannitol, × – maltose, ∗ – saccharose, ○ – lactose; (organic acids) ◊ – control, □ – α-ketoglutaric acid, Δ - succinic acid, × - malic acid, ∗ – fumaric acid, ○ – oxaloacetic acid, + - pyruvic acid.

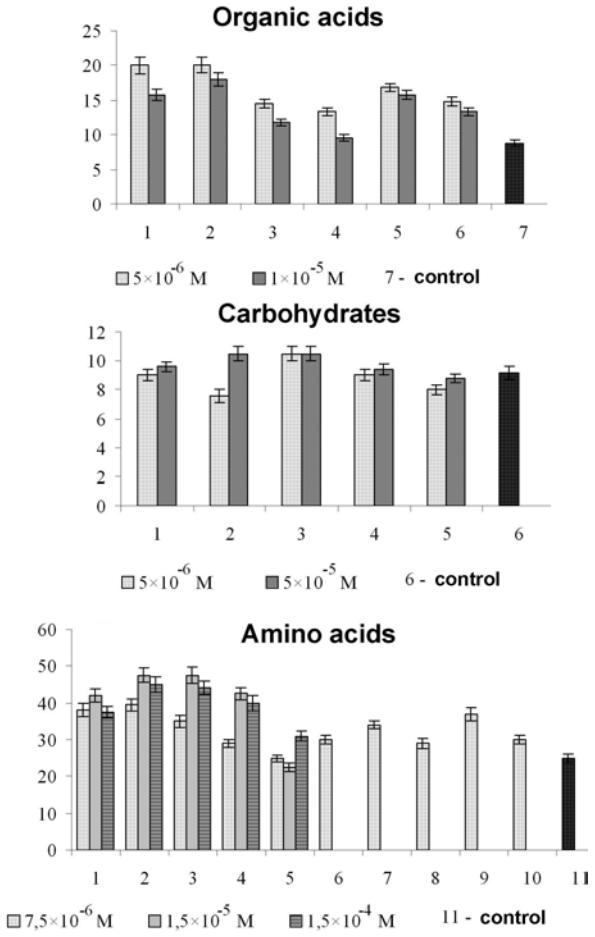

FIGURE 2 - The influence of amino acids, organic acids and carbohydrates on the oil consumption rate by *P. melochlora.* Y-axis shows the oil consumption by *P. melochlora* cells (in %). Characters: (amino acids) 1 – phenylalanine, 2 - glutamic acid, 3 - amino isovaleric acid, 4 – alanine, 5 – arginine, 6 – asparaginic acid, 7 – leucine, 8 – serine, 9 – methyonin, 10 – glutamine, 11 – control; (carbohydrates) 1 – glucose, 2 – mannitol, 3 – maltose, 4 – saccharose, 5 – lactose, 6 - control; (organic acids) 1 - α-ketoglutaric acid, 2 - succinic acid, 3 – malic acid, 4 - fumaric acid, 5 - oxaloacetic acid, 6 - pyruvic acid, 7 – control.

So, the addition of amino acids (isolated from aquatic macrophytes) to growth medium with oil resulted in shortening of the lag to log phase transition, increase of harvest (except for arginine at $1.5x10^{-5}$M), and elevation of the respiration activity. The most prominent action was detected with glutamic acid.

Exogenous carbohydrates also stimulated growth of *P. melochlora* and oil biodegradation (Figs. 1 and 2): maltose, mannitol>glucose>saccharose>lactose (at $5x10^{-5}$M), but maltose> glucose>saccharose>lactose>mannitol (at $5x10^{-6}$M).

Unlike the amino acids, carbohydrates did not influence the lag to log phase transition in *P. melochlora.* After 2 days, the difference between control and experimental variants was evident with mannitol or maltose (Fig. 1). The addition of maltose or mannitol resulted in 10.5% and 24% increase (after 4 and 10 days, respectively) of oxidized oil. It is very likely that carbohydrates serve as easy-to-reach carbon source. A slight increase of oil utilization in the presence of maltose and mannitol ($5x10^{-6}$M), and even inhibition of its biodegradation in variants with lactose ($5x10^{-6}$M and $5x10^{-5}$M) and saccharose ($5x10^{-6}$M) (Fig. 2) due to utilization of these carbohydrates may confirm our suggestion.

As for respiration activity, we detected the following dependence at $1x10^{-5}$M (Fig. 3): maltose >mannitol >saccharose>arabinose>sorbitol, lactose >rhamnose>inositol> glucose>dulcitol (galactitol).

In comparison with amino acids and carbohydrates, the addition of organic acids did not show any consistent patterns with respect to growth rate of the bacterium used, its respiration activity and the ability to degrade the oil. We found that organic acids variously influence the growth of *P. melochlora* (in order of attenuation of stimulation at $5x10^{-6}$M): oxaloacetic acid>malic acid>succinic acid>pyruvic acid>α-ketoglutaric acid>fumaric acid (Fig. 1). Concerning the oil-oxidizing activity, we detected the following order of attenuation of stimulation (at $5x10^{-6}$M,

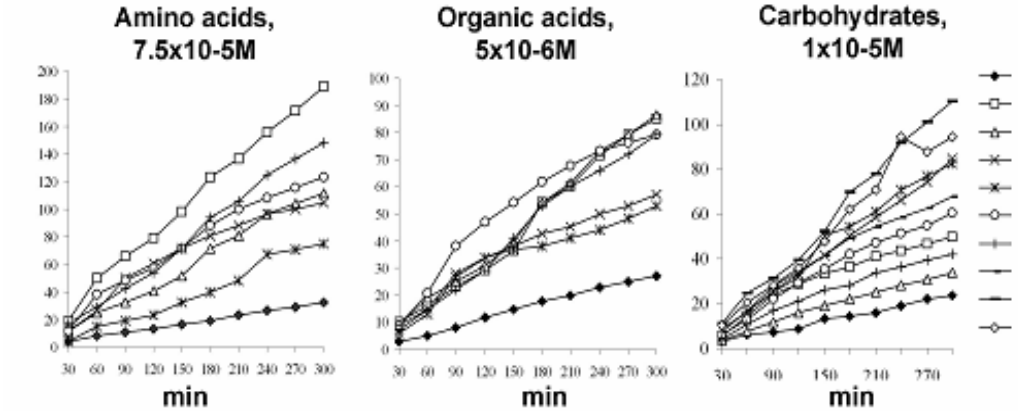

FIGURE 3 - The influence of amino acids, organic acids and carbohydrates on *P. melochlora* respiration activity (pH = 7.0; t = 28 °C; oil initial concentration = 2.6 g/L). Y-axis shows the amount of the consumed oxygen (in μl). Characters: (amino acids) ◆ – control, □ – glutamic acid, Δ – arginine, × - methionine, ∗ – phenylalanine, ○ – alanine, ┼ – amino-isovaleric acid; (organic acids) ◆ – control, □ - α-ketoglutaric acid, Δ - succinic acid, × - malic acid, ∗ – fumaric acid, ○ – oxaloacetic acid, ┼ - pyruvic acid; (carbohydrates) ◆ – control, □ – inositol, Δ – dulcitol, × – saccharose, ∗ – arabinose, ○ – rhamnose, ┼ – glucose, ▪ – sorbitol, ▬ – maltose, ◊ – mannitol.

$1x10^{-5}$M): α-ketoglutaric acid, succinic acid>oxaloacetic acid>pyruvic acid, malic acid>fumaric acid (Fig. 2). At $5x10^{-6}$M, the respiration of *P. melochlora* was influenced as follows: succinic acid>α-ketoglutaric acid>pyruvic acid > oxaloacetic acid>fumaric acid>malic acid (Fig. 3).

We detected that *P. melochlora* used organic acids mainly during its active growth phase. For example, during the first day of cultivation, the consumption of α-ketoglutaric acid was 20-fold, that of oxaloacetic acid 17-fold and pyruvic acid 10-fold. So, the addition of organic acids to growth medium resulted in activation of physiologic processes in *P. melochlora* that, in turn, led to increased biodegradation of oil.

Thus, amino acids, carbohydrates and organic acids influence differently the oil-oxidizing activity of *P. melochlora*. The bioactive compounds arose from accretion of aquatic macrophytes. As was reported previously, the process of exometabolism is especially active during the vegetation of the plants [10], and in the periphytic area the processes of bacterial destruction of xenobiotics take place. The increase of bacterioplankton in water is one of the reasons for restoration of biological features of natural waters [11]. The symbiotic relations between aquatic macrophytes and the oil-oxidizing microflora seem to be very important in biodegradation of oil and other pollutants.

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