Microbiology, Vol. 73, No. 3, 2004, pp. 300–304. Translated from Mikrobiologiya, Vol. 73, No. 3, 2004, pp. 358–363.

EXPERIMENTAL ARTICLES

Microbial Degradation of Mustard Gas Reaction Masses: Isolation and Selection of Degradative Microorganisms, Analysis of Organic Components of Reaction Masses, and Their Biodegradation

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Received February 25, 2003; in final form, April 28, 2003

Abstract—Bacterial strains growing in medium with mustard gas reaction masses (RM) as the carbon source were obtained. Growth cessation in the above medium was caused by the exhaustion of bioutilizable substrates, first of all monoethanolamine (MEA) and ethyleneglycol (EG), rather than by the accumulation of toxic metabolites in the culture liquid or in the cells. The main RM components, 1,4-perhydrothiazines (PHT), formed in the course of chemical detoxication of mustard gas, were identified and analyzed. The predominant component of the PHT mixture was *N*-(2-hydroxyethyl)-2-methyl-1,4-perhydrothiazine hydrochloride. Concentrations of all the PHT decreased by 50% in the course of culture growth; their destruction was a result of microbial metabolism.

Key words: mustard gas, reaction masses, microbial degradation, 1,4-perhydrothiazines.

The technology of the destruction of chemical warfare agents (CW), such as vesicants (mustard gas, lewisite, and mustard gas–lewisite mixtures), developed in Russia is based on chemical detoxication of CW with the formation of reaction masses (RM) of low toxicity; the latter are subjected to incineration or bitumination with the burying of bitumen blocks at special polygons. However, both methods of RM detoxication are not environmentally safe since RM incineration results in the production of volatile toxic aerosols and since there are still unresolved problems regarding the bitumen block stability during storage for a long time and the toxicity of the compounds present in these blocks.

The application of biotechnological methods based on the degradative activity of microorganisms that mineralize the toxicants may ensure sufficient ecological safety of the destruction of CW.

A biotechnological method of mustard gas degradation involving its alkaline hydrolysis to thiodiglycol (TDG) with subsequent bioutilization of the latter was developed [1]; the mechanism and conditions of TDG destruction by the selected bacterial strain *Alcaligenes xylosoxydans* subsp. *denitrificans* TD2 were studied [2, 3]. Chemical–biotechnological methods of the destruction of RM produced from mustard gas–lewisite mixtures were developed, which include utilization of organic compounds at the terminal stage by an association of microorganisms adapted to these carbon sources [4].

The capacity of microorganisms for the destruction of a wide range of xenobiotics may be important for the decontamination of territories polluted with CW or the products of their transformation as a result of possible leakage, accidental ejection, etc., during their storage, transportation, and destruction.

A method of phytoremediation of soils contaminated by mustard gas RM was developed; this method uses plants able to accumulate sulfur-containing compounds and thus to lower the concentrations of these compounds in soils 3- to 5-fold under standard conditions of plant growth or 20- to 25-fold under plant treatment with phytohormones [5]. The introduction of microorganisms capable of bioutilization or transformation of mustard gas detoxication products into such soils may improve soil decontamination manyfold and help achieve complete mineralization of CW or their transformation to less toxic compounds.

The technology of mustard gas detoxication developed and accepted in Russia includes the use of a

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decontamination mixture of monoethanolamine (MEA) and ethyleneglycol (EG) (9 : 1), which allows the process to proceed without gas evolution. Mustard gas RM are composed of moderately toxic compounds of toxicity classes III and IV (LD_{50} = 7000 mg/kg). The main components of the detoxication products, 1,4-perhydrothiazines (PHT), are formed as a result of mustard gas interaction with MEA. The contents of *N*-(2-hydroxyethyl)-perhydrothiazine hydrochloride and *N*-(2-hydroxyethyl)-2,6-dimethylperhydrothiazine in RM were determined to be 31.2 and 18%, respectively. In addition, mustard gas RM contain an oligomer, ethyl-enechlorhydrine, formed as a result of mustard gas interaction with EG, as well as residual amounts of MEA and EG [6].

The aim of this work was the isolation and selection of microorganisms capable of bioutilization and transformation of organic components of the RM formed during chemical detoxication of mustard gas, analysis of these components, and the study of the degradative activity of microorganisms towards these compounds.

MATERIALS AND METHODS

The study was carried out with several strains of *Pseudomonas putida* growing with mustard gas RM as the carbon source, two strains of *Alcaligenes xylosoxydans* subsp. *denitrificans* (TDG degraders) and eight strains of *Pseudomonas putida* degrading polycyclic hydrocarbons.

Bacteria were cultivated in MS mineral medium [2] on a shaker (220 rpm) at 30°C. Carbon substrates were RM (toxicity class IV) produced during mustard gas detoxication with the use of a mixture of MEA and EG (90 and 10%, respectively); it was obtained from the State Institute of Technology of Organic Synthesis, Shikhany, Saratov oblast, Russia. The addition of RM (1 vol %) to the mineral medium was performed immediately before medium inoculation. The following carbon substrates were also used: a mixture of MEA and EG at concentrations corresponding to their contents in RM (0.25 and 0.025%, respectively); each of these compounds separately (0.25%); and glutamate (1%). Inoculum was grown on agarized MS medium with 1% mustard gas RM.

Cell growth was monitored by measuring the optical density of the culture on a Specol spectrophotometer at 560 nm and by determining the number of colony-forming units (CFU) through plating serial culture dilutions on agarized M7 medium (an analogue of nutrient agar).

Organic components of mustard gas RM were analyzed in the course of cultivation of bacteria by chromatography and chromatography–mass spectrometry. Supernatant of the culture liquid was extracted with chloroform, benzene, heptane, or diethyl ester; the extracts were dried in a flow of argon and analyzed on a Biokhrom-1 gas–liquid chromatograph equipped with a flame-ionization microdetector and a quartz capillary column (QCC) packed with an SE-54 stationary phase. The temperature was programmed to change from 100 to 270°C at a rate of 8°C/min; the injector and detector temperatures were 200 and 270°C, respectively.

Subsequent identification of individual components was carried out on a Hewlett-Packard HP 5890 chromatograph combined with a mass spectrometer. A QCC packed with 5% diphenyl and 95% dimethylpolysiloxane was used under the temperature regime described above. Mass spectra were analyzed using the Wiley 275.L database.

RESULTS AND DISCUSSION

Bacterial strains capable of utilizing organic components of mustard gas RM were isolated from soils contaminated by products of vesicant detoxication (Shikhany, Saratov oblast). Soil samples were placed into a glass column with branch pipes for air supply, through which a dilute solution of mustard gas RM (1 : 100) was periodically added. After the first stage of selection, which extended for several months, a microbial association was isolated that grew actively in liquid mineral medium with the above carbon sources.

At the second stage of selection, the isolated microbial association was grown in liquid medium with mustard gas RM for 3 months; exponential-phase cells were regularly transferred to fresh medium. Selected bacterial strains grew in the above medium at a specific growth rate ranging from 0.02 to 0.04 h^{-1} and were identified according to Bergey's Manual [7] as *Pseudomonas putida*.

When one of the isolates, strain *P. putida* SH, was grown in the medium with mustard gas RM, the transition of the culture from the exponential growth phase to the stationary phase was accompanied by a sharp decrease in both the optical density of the culture and the number of viable cells (to 80%) (Fig. 1, curves *1* and *2*); this may be due to either the accumulation of toxic substances in the culture liquid or in the cells or to strain peculiarities.

The third stage of the isolate selection was carried out by subculturing of cells that remained viable in the late stationary phase (for a weeks). After several passages, strain *P. putida* SH1 (biovar A) was selected, which was fairly resistant to cell lysis under preset conditions (Fig. 1, curve *3*).

The concentration of RM in the medium was shown to have an effect on the growth characteristics of the selected strain SH1. An increase in the RM concentration from 0.5 to 3.0% resulted in an increase in the culture turbidity (from 0.8 to 2.4 OD units), while the specific growth rate decreased from 0.07 to 0.01 h^{-1} and the lag phase was extended from 4 to 120 h (Fig. 2). Under these conditions, the pH lowered; the optimum pH value for cell growth (7.5–8.0) was maintained by the addition of 10% NaOH.

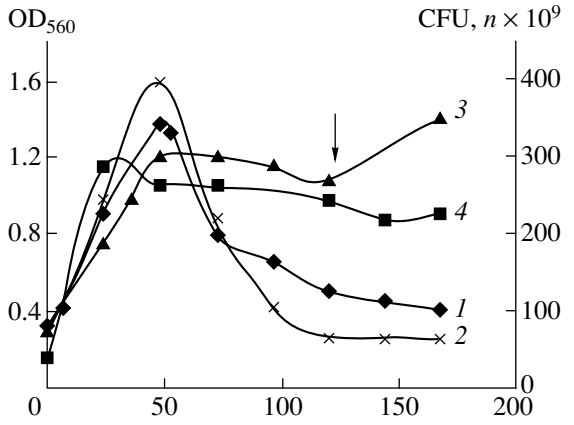

Fig. 1. Time courses of the growth of strains *P. putida* SH and SH1: (*1*) OD_{560} and (*2*) CFU during growth of strain SH in medium with mustard gas RM; (*3*, *4*) OD_{560} during growth of strain SH1 (*3*) in medium containing mustard gas RM and (*4*) in medium containing a mixture of MEA and EG. The arrow indicates the addition of MEA and EG to medium with mustard gas RM.

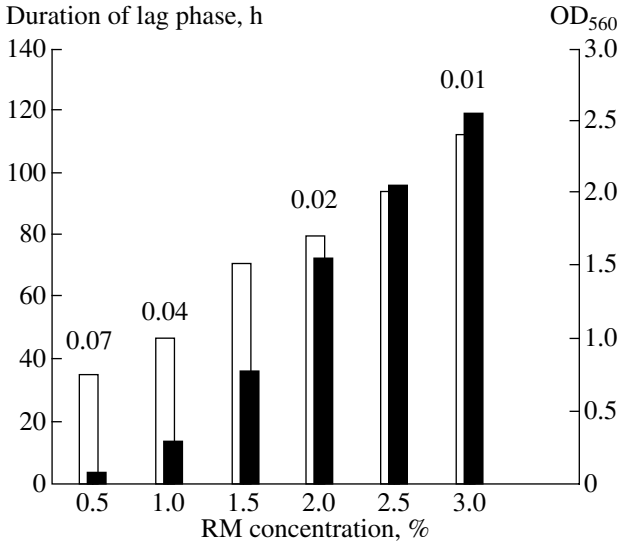

Fig. 2. Effect of the mustard gas RM concentration on biomass yield (light columns), duration of lag phase (black columns), and maximal specific growth rate (figures above columns) of strain *P. putida* SH1.

The cessation of the growth of strain SH1 in the medium with mustard gas RM was shown to proceed at a high residual concentration of total carbon in the culture liquid, which could be due both to the exhaustion of bioutilizable components and to the accumulation of growth-inhibiting metabolites in the culture liquid or in the cells. To test this assumption, the stationary-phase culture was centrifuged; the supernatant was supplemented with mustard gas RM or glutamate and inoculated with logarithmic- or stationary-phase cells of strain SH1 grown in the RM-containing medium.

As seen from the table, biomass content was almost the same in the mineral medium and in the supernatant supplemented with RM or glutamate, regardless of the inoculum age (logarithmic- or stationary-phase cells). No cell growth in supernatant without addition of a carbon source was observed. These results indicate that toxic products of mustard gas degradation were not accumulated either in the cells or in the culture liquid; therefore, the cessation of cell growth in the stationary phase occurred because of the depletion of bioutilizable substrate(s).

To reveal RM components that were utilized by bacteria as carbon sources, the growth of strain SH1 on MEA and EG, whose content in RM was rather high, was studied. The time course of strain growth on the mixture of MEA and EG was similar to that on the medium with 1% RM; the maximum biomass level in both variants was 1.2–1.4 OD units. When the mixture of MEA and EG was added to the stationary-phase culture grown in RM-containing medium, cell growth resumed (Fig. 1, curves *3* and *4*). This indicates that they were the RM components that were primarily utilized by bacteria as carbon sources.

Two strains of *A. xylosoxydans* and eight strains of *P. putida* (polycyclic hydrocarbon degraders) were also studied for their ability to grow on RM, MEA, and EG. Except for strain *P. putida* SH1, none of the strains studied grew in media with the above carbon sources. The results

Growth of *P. putida* SH1 in supernatant-based medium and MS medium with various carbon sources

Medium	Carbon source	Growth phase of inoculum	Biomass, OD units
MS medium	RM	Logarithmic	1.4
Supernatant	RM	Logarithmic	1.6
Supernatant	Glutamate	Logarithmic	1.1
Supernatant	Absent	Logarithmic	0.1
Supernatant	RM	Stationary	1.4
MS medium	RM	Stationary	1.5
MS medium	Glutamate	Stationary	1.2
MS medium	Absent	Stationary	0.1

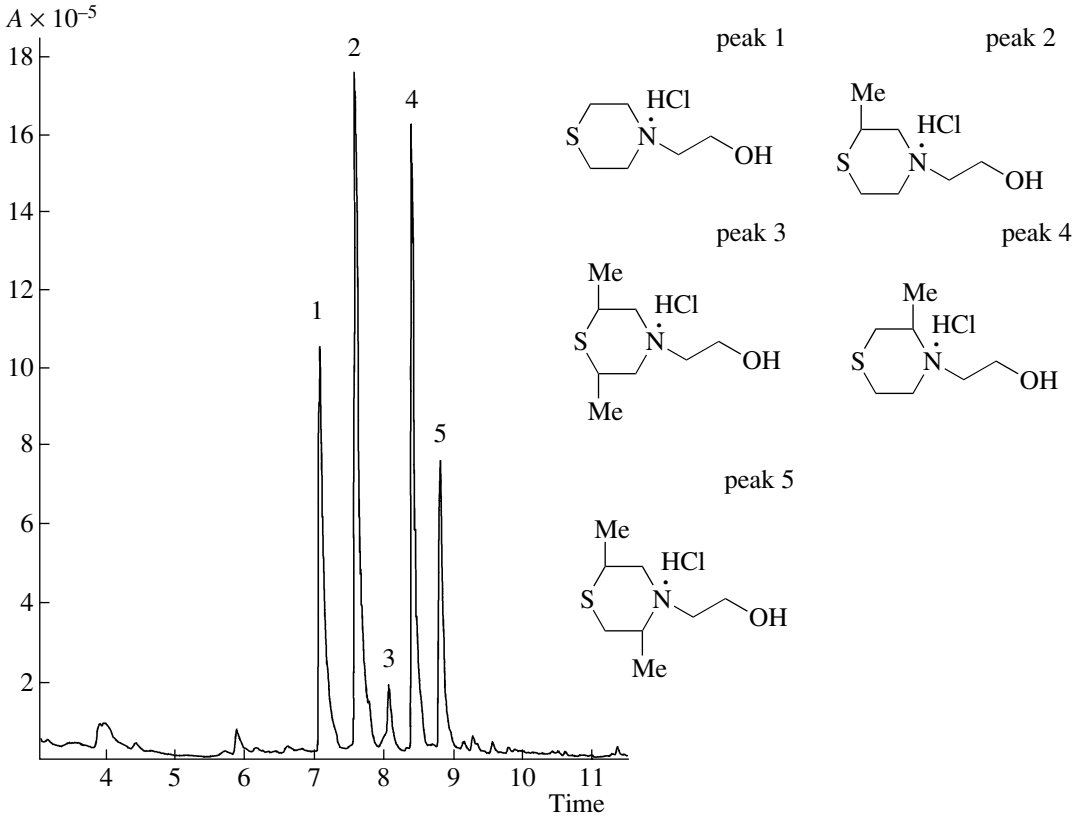

Fig. 3. Chromatogram of the culture liquid extract at the onset of *P. putida* SH1 cultivation in medium with mustard gas RM.

obtained are indicative of the adaptation of strain *P. putida* SH1 to these substrates in contaminated soil.

To study the degradative capacities of strain SH1, the culture liquid of bacteria grown in the medium with mustard gas RM was extracted with chloroform, benzene, heptane, or diethyl ether and analyzed by gas-liquid chromatography and mass spectrometry. The most complete extraction of RM components was attained by the treatment of samples with chloroform.

The main organic components revealed in extracts of mustard gas RM were 1,4-perhydrothiazines (PHT) (thiomorpholines). In the course of strain SH1 growth in the medium with mustard gas RM, the areas of chromatographic peaks corresponding to different PHT derivatives decreased. Identification of these peaks was performed by mass spectrometry with the use of a mass-spectrum database.

As seen from Fig. 3, the identified sulfur-containing PHT derivatives included *N*-(2-hydroxyethyl)-1,4-perhydrothiazine hydrochloride (peak 1), *N*-(2-hydroxyethyl)-2-methyl-1,4-perhydrothiazinehydrochloride (peak 2), *N*-(2-hydroxyethyl)-2,6-dimethyl-1,4-perhydrothiazinehydrochloride (peak 3), *N*-(2-hydroxyethyl)-3-methyl-1,4-perhydrothiazine hydrochloride (peak 4), and *N*-(2-hydroxyethyl)-2,5-dimethyl-1,4-perhydrothiazinehydrochloride (peak 5).

The amounts of these components were determined by the method of absolute calibration with the use of solutions of synthesized *N*-(2-hydroxyethyl)-1,4-perhydrothiazine. The relative percentage of every component was calculated from the areas of chromatographic peaks registered with an integrator by taking the sum of all peak areas related to PHT as 100%.

Since PHT represent the basic part of the organic phase in mustard gas RM [7], emphasis was placed on the biodegradation of these compounds. Variations in the content of each of the PHT derivatives were determined in the course of strain SH1 growth in the medium with 1% mustard gas RM. A control variant contained medium with inactivated microbial cells or without cells; it was incubated under the same conditions.

As seen from Fig. 4a, in the course of strain SH1 cultivation, peak areas of each of the PHT derivatives decreased. The total amount of these components reduced by 50%; the ratio of individual derivatives remaining constant. In control variants, no changes in the PHT concentrations were observed (Fig. 4b). From the results obtained, we inferred that only microorganisms can be responsible for the decrease in PHT content of RM.

The addition of the mixture of MEA and EG to the stationary-phase culture resulted in the resumption of

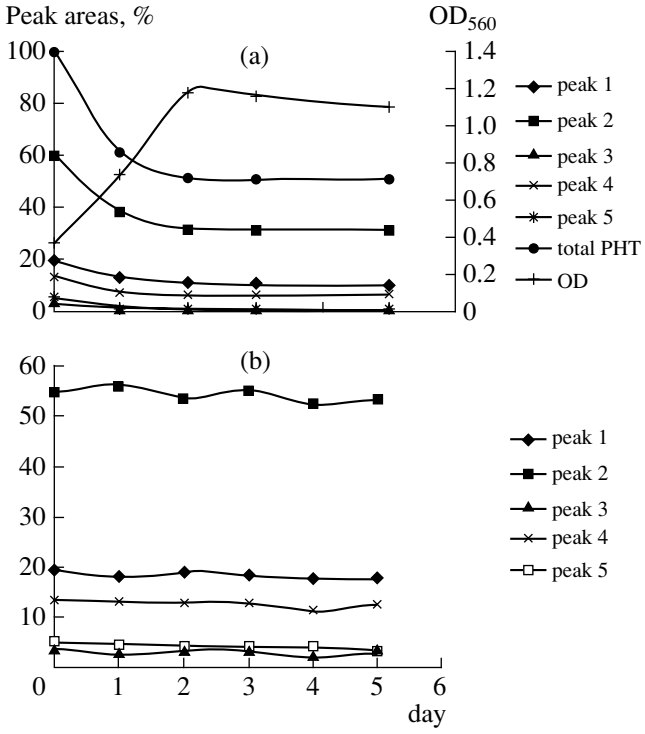

Fig. 4. Time courses of PHT concentrations in medium with 1% RM (a) inoculated with *P. putida* SH1 and (b) without bacteria or in the presence of inactivated cells. The total amount of all PHT derivatives in the initial medium is taken as 100%.

cell growth (Fig. 1, curve *3*) and the subsequent decrease in the total amounts of PHT to 18% of their initial concentrations. When mustard gas RM (1%) was added to the culture at the stationary phase, biomass also increased but the degradation level comprised only 60% of the total amount of PHT added to the medium.

The transition of the culture to the stationary phase under a sufficiently high residual concentration of PHT in the absence of toxic metabolites in the culture liquid testifies that PHT were not used as carbon sources for cell growth but were transformed by growing cells.

ACKNOWLEDGMENTS

This work was supported by a grant from the project "Environmental Biotechnology" within the Russian Federal Scientific and Technical Program "Research and Development in Priority Directions of Science and Engineering."

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