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EXPERIMENTAL ARTICLES

The Bioutilization of Thiodiglycol (a Detoxication Product of Mustard Gas): Isolation of Degrading Strains and Investigation of Degradation Conditions

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Abstract—The *Alcaligenes xylosoxydans* subsp. *denitrificans* strain TD1 capable of degrading thiodiglycol (TDG), a product of mustard gas hydrolysis, was isolated from soil contaminated with breakdown products of this chemical warfare agent. The selected stable variant of TD1 (strain TD2) can grow on TDG with a lag phase of 4–8 h and a specific growth rate of 0.04–0.045 h^{-1}. Optimal conditions for the biodegradation of TDG (pH, the concentration of TDG in the medium, and specific substrate loading) were determined. TDG was found to be degraded with the formation of diglycolsulfoxide and thiodiglycolic acid as intermediate products. The data obtained can be used to develop approaches to the bioremediation of mustard gas–contaminated soils.

Key words: mustard gas, thiodiglycol, biodegradation, *Alcaligenes xylosoxydans.*

In accordance with the Chemical Weapons Convention, a conception of two-stage destruction of chemical warfare agents has been developed in the Russian Federation, with their chemical detoxication at the first stage and the destruction of detoxication products at the second stage. The incineration of the detoxication products is very expensive, since it requires much energy and necessitates technical facilities to trap aerosols containing toxic incineration products. For this reason, the microbiological utilization of organic compounds present in the breakdown products of mustard gas may be an alternative to their incineration.

Mustard gas, or bis(2-chloroethyl) sulfide, is a vesicant slightly soluble in water and hydrolyzable under alkaline conditions with the formation of the unchlorinated water-soluble compound thiodiglycol, or bis-(2-hydroxyethyl) sulfide [1]. Thiodiglycol (TDG) is a relatively persistent product of chemical warfare agent destruction, which should be removed from mustard gas–contaminated sites [2].

A search among more than 150 bacterial, yeast, micromycetous, and actinomycetous strains belonging to 35 genera failed to detect even one TDG-degrading strain. Nor was such strain found among bacteria of the genera *Pseudomonas, Corynebacterium*, and *Rhodococcus*, which are efficient degraders of diethylene glycol, a chemical analogue of TDG. Medvedeva *et al.* isolated the TDG-degrading *Pseudomonas* sp. strain 8-2 from contaminated soil samples incubated with TDG for 5 months [3]. Harvey *et al.* obtained several bacterial strains capable of utilizing TDG as the sole source of carbon and energy from mustard gas–contaminated soil samples incubated for 9 month in an enrichment chemostat culture in the presence of TDG [4]. One of those strains, which was identified as *Alcaligenes xylosoxydans* subsp. *xylosoxydans*, was employed to develop a mustard gas destruction technology combining the alkaline hydrolysis of mustard gas and the bioutilization of the resultant TDG [5, 6]. Boronin *et al.* devised the ecologically safe technology of destruction of mustard gas–lewisite mixtures, which includes the stages of chemical detoxication, the electrochemical treatment of detoxication products, and the biodegradation of electrolysis products by a selected microbial association [7].

One of the important concerned problems is the decontamination of soils polluted as the result of the storage, transportation, and destruction of chemical warfare agents. The decontamination of such soils by biological methods (bioremediation) is an attractive alternative to the costly and energy-consuming mechanical, physical, and chemical methods. Under moisture conditions, mustard gas present in contaminated soils undergoes hydrolysis to form TDG, which, due to its persistence to microbial attack, can accumulate in the soils in large amounts. The introduction of

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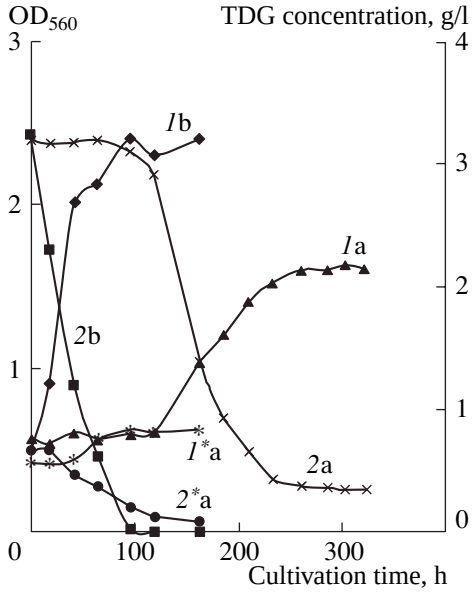

Fig. 1. Growth of two *A. xylosoxydans* strains, (a) TD1 and (b) TD2, in media with initial TDG concentrations of (*1*) 3.2 and (*1**) 0.6 g/l. Curves *2* and *2** show the TDG decline in the media with 3.2 and 0.6 g/l TDG, respectively.

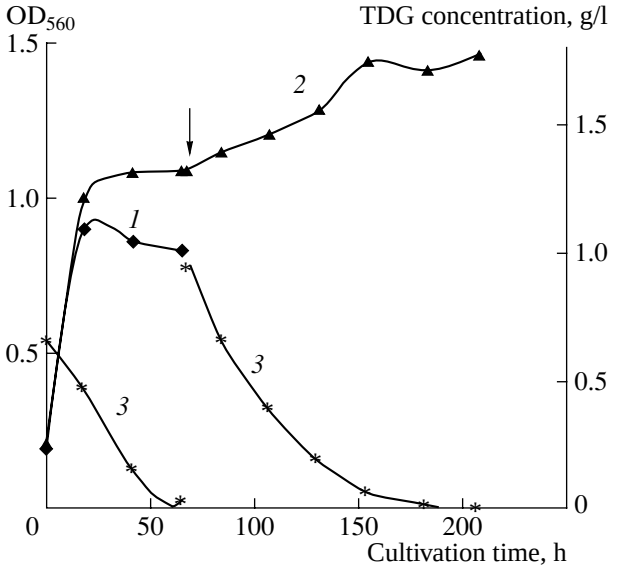

Fig. 2. Growth of *A. xylosoxydans* strain TD1 in a medium with acetate in (*2*) the presence and (*1*) absence of TDG. Curve *3* shows the TDG decline. The arrow shows the moment of TDG addition.

TDG-degrading microorganisms into the contaminated soils in combination with the addition of organic and mineral substrates and other agricultural methods may form the basis for the bioremediation of soils polluted with mustard gas and its degradation products.

The aim of the present work was to obtain microbial strains capable of utilizing TDG as the carbon source and to find conditions optimal for TDG utilization.

MATERIALS AND METHODS

The TDG-degrading strain *Alcaligenes xylosoxydans* subsp. *denitrificans* TD1 was isolated in the course of the present work from soil contaminated with breakdown products of mustard gas. Strain TD2 was selected from strain TD1.

Bacteria were cultivated at 30°C in flasks shaken at 220 rpm. The aeration rate was 0.5 g O_2/(l h). The mineral components of the medium were as follows (g/l): NH_4Cl, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 10.0; and KH_2PO_4, 1.0 (pH 7.0–7.5). The medium was supplemented with microelements at the following concentrations (mg/l): $FeSO_4 \cdot 7H_2O$, 2.5; $CaCl_2 \cdot 6H_2O$, 10.0; $CuSO_4 \cdot 5H_2O$, 2.0; H_3BO_3, 0.06; $ZnSO_4 \cdot 7H_2O$, 20.0; $MnSO_4 \cdot H_2O$, 1.0; $Na_2MoO_4 \cdot 2H_2O$, 0.3; and $NiCl_2 \cdot 6H_2O$, 0.05. Cells for inoculation were grown for 4 days on a solid medium containing, in addition to the above mineral components, 15 g/l agar, 2 g/l TDG, and 2 g/l acetate.

Bacterial growth in liquid media was monitored by measuring the culture turbidity at 560 nm in a Specol spectrophotometer or by plating culture dilutions on nutrient agar. In the latter case, the results were expressed in colony-forming units (CFU). The culture turbidity (or optical density, OD) was converted to the weight of dry biomass (DB) using an experimentally derived coefficient of 0.5 g DB/OD unit.

TDG thiodiglycolic acid (TDGA), thioglycolic acid (TGA), and diglycolsulfoxide (DGSO) were used as carbon sources; yeast extract (0.5 g/l), acetate, succinate, and glutamate (2 g/l) were used as cosubstrates. TDG was purchased from Meck (Germany) and TGA from Reakhim (Russia). TDGA and DGSO we synthesized ourselves.

TDGA was synthesized as follows: A solution containing 15.2 g $Na_2S \cdot 9H_2O$ in 40 ml water was added dropwise to a solution of sodium monochloroacetate (this was obtained by mixing 9.8 g monochloroacetic acid with a solution containing 6.4 g sodium carbonate in 14 ml water) warmed to 40°C. The mixture was stirred for 3 h, acidified with hydrochloric acid to pH 1–2, partially evaporated under a vacuum, and extracted thrice with 20 ml ethylacetate. The extracts were pooled, dehydrated with sodium sulfate, and evaporated under a vacuum. The residue was purified by crystallization from a solution in an ethylacetate–chloroform (2 : 1) mixture. The TDGA yield was 4.2 g (47% of the theoretical yield). The melting point of the product was 128–129°C. The NMR spectrum recorded at 80 MHz at pH 3.0 in D_2O had one singlet peak at 3.74 ppm.

DGSO, or bis-(2-hydroxyethyl) sulfoxide, was synthesized as described below. Eleven milliliters of 30% H_2O_2 was slowly (for about 1 h) added to a mixture of

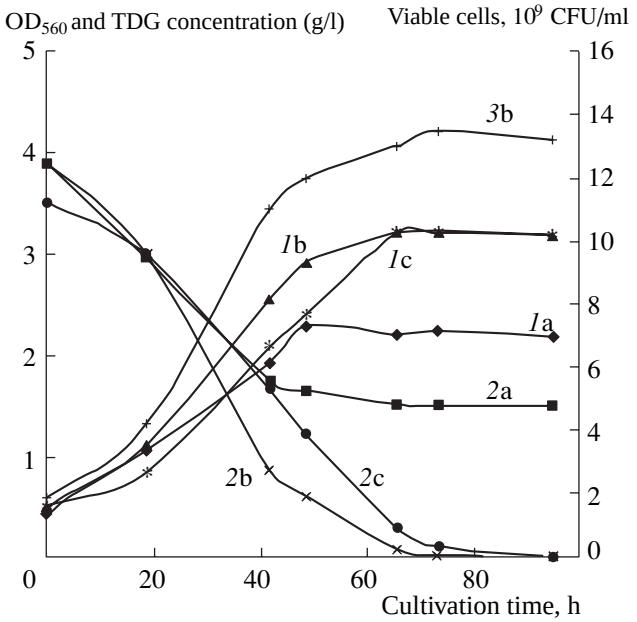

Fig. 3. (*1*) Growth of *A. xylosoxydans* strain TD2 in a medium with TDG (a) without pH control, (b) with pH controlled at a level of 7.0–7.5 by adding NaOH, and (c) with pH stabilized at the level 7.0–7.5 by supplementing the medium with $CaCO_3$. Curve *2* shows the TDG decline, and curve *3* shows the number of viable cells in the culture.

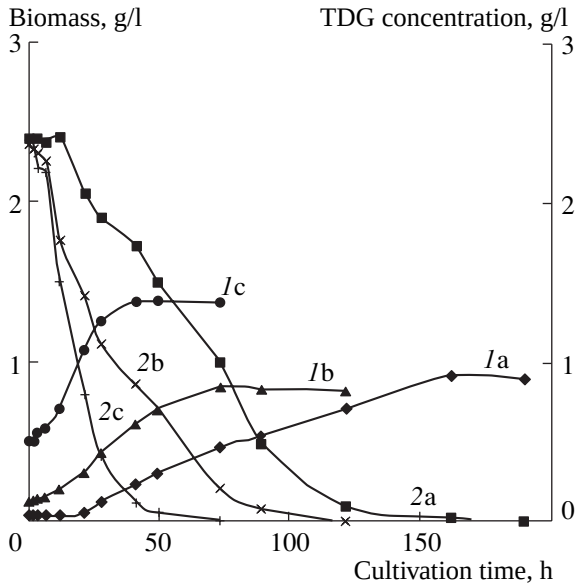

Fig. 4. (*1*) Growth of *A. xylosoxydans* strain TD2 in media with different SSL (gTDG/g biomass): (a) 125, (b) 18, and (c) 5. Curves (*2*) show the TDG decline.

10 ml thiodiglycol and 50 ml acetone cooled to 0°C. The temperature of the mixture in the course of the hydrogen peroxide addition was controlled at a level of 2–3°C. The mixture was stirred for the next 2 h at 0°C and then kept overnight in an ice bath. On the following day, the liquid phase was decanted, and crystals were purified by precipitation with acetone from a warmed aqueous solution. The DGSO yield was 3.3 g (24% of the theoretical yield). The melting point of DGSO was found to be 111°C.

TDG, TDGA, DGSO, and TGA were quantitatively analyzed by means of HPLC using an LKB-2150 chromatograph equipped with a Separon SGX C18 (5 μm) column (3.3 × 150 mm). The column was eluted at a rate of 1 ml/min with deionized water containing 3 mM H_3PO_4. The temperature of the column was maintained at 65°C. Elution peaks were recorded at 214 nm using an LKB-2151 UV detector, a NELSON ANALYTICAL 900 series interface, and an Olivetty M-24 PC. Data were processed with the aid of the Nelson Analyt-

The effect of the initial TDG concentration on the growth parameters of *A. xylosoxydans* TD2 and the accumulation of TDG degradation products in the medium

TDG concentration, g/l	Biomass, g/l	μ_{max}, h^{-1}	Biomass yield, g cells/g TDG	Concentration, g/l		
				SO_4^{2-}	DGSO	TDGA
0.8	0.27	0.022	0.33	–	0.1	0.005
1.74	0.47	0.03	0.27	–	0.21	0.006
2.6	0.72	0.036	0.28	1.6	0.3	0.014
3.24	0.9	0.042	0.27	–	–	–
7.46	1.95	0.042	0.26	5.16	0.32	0.21
9.3	2.2	0.045	0.24	–	–	–
13.9	2.7	0.029	0.19	–	–	–
15.2	3.4	0.024	0.22	–	0.53	0.52
22.7	3.2	0.018	0.14	–	–	–
26.4	3.2	0.015	0.12	–	–	–
39.3	2.4	0.004	0.06	–	0.69	8.8

Note: The symbol "–" stands for "not determined."

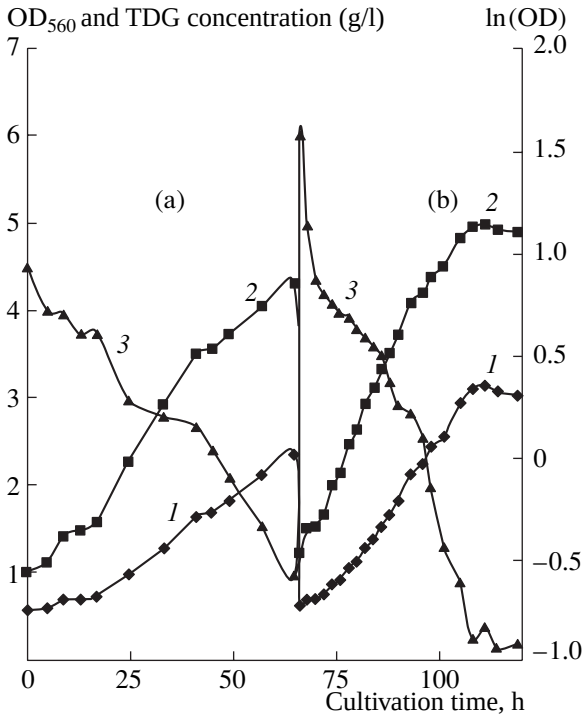

Fig. 5. (*1*) Growth of *A. xylosoxydans* strain TD2 in a fermentor at pH 7.5 and pO_2 = 35% oxygen saturation (a) before and (b) after replacement of part of the culture with fresh growth medium. Curves (*2*) show the biomass expressed in ln (OD_{560}), and curves (*3*) show the TDG decline.

ical software package. The elution times of TDG, TDGA, DGSO, and TGA under the described conditions were 2.24, 1.54, 1.26, and 1.59 min, respectively. The relationship between the concentration and peak absorbance of TDG was linear within 0.04–10.0 mM.

The concentration of SO_4^{2-} ions was determined gravimetrically [8].

RESULTS

TDG-degrading strains were isolated from soil contaminated with breakdown products of mustard gas. Samples of this soil were obtained from the State Technological Institute of Organic Synthesis, the town of Shikhany, Saratov oblast. The soil was wetted with the mineral medium to a moisture content of 40% and incubated in the presence of TDG (3 g/100 g soil) at 30°C for 3 months, after which the soil was transferred to flasks with the liquid mineral medium containing TDG and incubated for 7 days on a shaker. Four types of bacteria differing in colonial features and cell size, shape, and motility were isolated on nutrient agar. However, only one type of isolates grew on mineral agar medium with TDG. Bacteria of one of these morphotypes were able to grow on the mineral agar medium with TDG as the carbon source. Using the identification criteria of Bergey's Manual [8], a strain of these TDG-degrading bacteria, designated as TD1, was identified as *Alcaligenes xylosoxydans* subsp. *denitrificans.*

Strain TD1 began to grow in TDG-containing media after a lag phase, whose duration changed from 48 to 100–120 h when the concentration of TDG increased from 0.5 to 3 g/l (Fig. 1). In this case, the specific growth rate of the strain rose from 0.003–0.005 to 0.01 h^{-1}. Bacteria of other types isolated from the same mixed culture from which strain TG1 was obtained failed to grow not only on TDG itself but also on possible intermediates of its oxidation, TDGA, DGSO, and TGA. Their growth in TDG-containing media as components of the mixed culture can be explained by their ability to grow on the lysis products of strain TD1.

The prolonged lag phase and slow growth of strain TG1 on TDG suggest a metabolic blocking of the TDG assimilation pathway of this strain. Investigations showed that the growth dynamics of strain TD1 in media with TDG changes in the presence of easily metabolizable cosubstrates, such as acetate, succinate, glutamate, and yeast extract. Figure 2 shows the growth of strain TD1 on TDG and acetate; its growth in the presence of other cosubstrates was similar. Irrespective of the presence of TDG, strain TD1 began to grow on acetate virtually without a lag phase. The consumption of TDG took place simultaneously with that of acetate. The addition of TDG to the stationary phase culture also induced its growth without lag phase; however, the specific growth rate was again low (0.003 h^{-1}).

The selection of fast-growing variants of strain TD1 by its repeated subculturing for 6 months in media containing TDG at increasing (from 1 to 3 g/l) concentrations gave rise to bacterial variants whose lag phase was as short as 4–8 h and whose specific growth rate was 0.04–0.045 h^{-1} (Fig. 1). One of these variants with stable characteristics, designated as TD2, was chosen for further studies.

In spite of the high content of phosphates (55 mM) in the growth medium, the pH of the medium decreased to 5.0–5.5 after 48 h of cultivation, which resulted in the inhibition of growth and substrate consumption. The concentration of sulfate ions in the medium increased by 16.6 mM when strain TD2 consumed 19.6 mM TDG. When the pH of the medium was maintained at a level of 7.0–7.5 by the regular addition of the necessary amount of a sterile 20% solution of NaOH or by supplementing the medium with $CaCO_3$, strain TD2 continued to grow until TDG was depleted (Fig. 3). The strain could grow on glutamate at pH 5.2, albeit at a lower specific growth rate that at pH 7.5.

Experiments in which the initial concentration of the cells was varied from 0.02 to 0.5 g/l at the same concentration of TDG (2.5 g/l) showed that there is a correlation between specific substrate loading (SSL, g TDG/g biomass) at the early growth stage and the growth characteristics of strain TD2, such as the lag phase duration, the time of the complete consumption of TDG, specific growth rate, and the biomass yield with respect to the

TDG consumed (Fig. 4). When SSL was 125 g/g, the lag phase lasted 20 h, and the culture growth ceased after 160 h as the result of the depletion of TDG. At a SSL of 18, the lag phase lasted 7 h, and the culture entered the stationary phase after 75 h of growth. At a SSL of 5, these parameters were 3 and 42 h, respectively. In all experimental variants, the maximum specific growth rate was equal to 0.037–0.04 h^{-1} and the overall biomass yield was 0.28–0.3 g biomass per g TDG consumed. Similar regularities were revealed when the initial concentration of TDG in the medium was 20 g/l.

Experiments in which the initial concentration of TDG in the medium was varied from 0.8 to 39.3 g/l at SSL below 20 showed (table) that the specific growth rate in the exponential phase reached a value of 0.042–0.045 h^{-1} within the range of TDG concentrations from 3.5 to 10.0 g/l. At TDG concentrations of 20–25 g/l, the specific growth rate and the biomass yield decreased, although the lag phase remained short (about 4 h). In the medium with the initial concentration of TDG equal to 39.3 g/l, strain TD2 began growing after a 144-h lag phase, when the concentration of TDG decreased to 25 g/l. In this case, the specific growth rate in the exponential phase was as low as 0.004 h^{-1}.

In both sets of experiments, strain TD2 slowed down its growth before the transition to the stationary phase when relatively high concentrations of TDG remained in the medium (for instance, 4.5 g/l when its initial concentration was 7.46 g/l). Therefore, the transition of strain TD2 to the phase of growth retardation was not induced by a deficiency of the carbon source in the medium. It should be noted that the duration of the retardation growth phase increased with the initial content of TDG in the medium.

Analysis of the culture liquid showed a considerable increase in the content of sulfate ions and the presence of DGSO and TDGA, whose amount rose with the initial concentration of TDG in the growth medium (see table).

The growth dynamics and substrate consumption by strain TD2 were also studied under the conditions of batch cultivation in a fermentor at controlled values of pH and oxygen supply (pO_2). Cells for inoculation were grown on the agar and then in the liquid growth medium. The medium in the fermentor was inoculated at a dose corresponding to a substrate load of 14 g TDG/g biomass. The values of oxygen concentration and pH during cultivation were maintained at levels of 35% oxygen saturation and pH 7.5. The data presented in Fig. 5a show that, after a lag phase, strain TD2 began growing at a specific growth rate of 0.035 h^{-1}. After the culture in the fermentor had reached the retardation growth phase, a fraction of the culture was replaced with an equal volume of fresh growth medium containing TDG, and the cultivation was continued. The fraction was such that the culture OD after the culture replacement was the same as at the onset of the experiment. As can be seen from Fig. 5b, the culture continued growing after the replacement at a μ_{max} of 0.05 h^{-1} without any lag phase. The transition of the culture to the retardation growth phase was observed when the concentration of TDG in the medium was 3 g/l. The supplementary addition of mineral components to the medium at this growth stage did not increase the culture growth rate, indicating that their content in the medium before the addition was not limiting.

DISCUSSION

The data presented in this paper and those obtained by Harvey *et al.* [4] indicate that the microflora of soils contaminated with mustard gas and/or its degradation products may contain 3–4 bacterial morphotypes, of which only the species *Alcaligenes xylosoxydans* is able to utilize TDG as the sole source of carbon. Evidently, the population of this bacterium in polluted soils is heterogeneous and includes clones with different TDG-degrading abilities. Long-term selection by the repeated transfer of the exponential-phase culture of strain TD1 to fresh nutrient media allowed us to obtain a strain of *A. xylosoxydans*, TD2, which could grow at high TDG concentrations with a μ_{max} of 0.04–0.045 h^{-1} after a short lag phase. The strain retained this property even after long-term storage.

The efficiency of TDG bioutilization is affected by pH of the medium, which drops due to the formation of sulfate ions and acidic products of TDG degradation, TDGA in particular. The microbial degradation of TGA occurs at pH values exceeding those necessary for growth on easily metabolizable substrates. For instance, *A. xylosoxydans* strain TD2 can grow on glutamate at pH 5.2 but cannot utilize TDG at pH below 5.5. For comparison, the immobilized *Alcaligenes xylosoxydans* subsp. *xylosoxydans* cells cannot utilize TDG at pH below 6.0 [10].

There are literature data about the importance of SSL in the initial period of cultivation for growth dynamics of microbial cultures [11]. With *A. xylosoxidans* TD2, the reduction of SLL in medium with TDG promotes shortening of both the lag phase and the duration of the biodegradation process as a whole. The data obtained makes it possible to calculate the optimal amount of the inoculum as dependent on the initial substrate concentration. This parameter can be used for the regulation of the efficiency of TDG biodegradation process.

Generally, this strain is able to grow at sufficiently high concentrations of TDG in the medium. However, the maximum values of specific growth rate and biomass yield were observed at TDG concentrations no higher than 10 g/l. TDG at the initial concentration of 39.3 g/l was found to inhibit the reproduction of cells, leaving them metabolically active. As a result, the cells gradually reduce the concentration of TDG in the medium by oxidizing it to TDGA. When the concentration of TDG declined to 25 g/l, strain TD2 began grow-

ing, albeit at a slow specific growth rate (0.004 h^{-1}). At the same time, when the initial concentration of TDG in the medium was 26.4 g/l, the strain grew at a specific growth rate of 0.015 h^{-1}, i.e., almost fourfold faster.

The presence of distinct retardation growth phases on the growth curves of strain TD2 at TDG concentrations varied from 0.8 to 39 g/l can in fact be explained either by the inhibitory action of TDG and/or its degradation products [12] or by the limitation of cell growth by insufficient concentrations of oxygen and certain mineral components in the cultivation medium. However, the experimental results described above indicate that neither the residual TDG in the medium nor oxygen and mineral components of the medium are responsible for the limitation of the culture growth. The detection of TDGA and DGSO in the growth medium of strain TD2 suggests that its growth on TDG may be inhibited by these toxic products of the TDG degradation.

Thus, the selected *A. xylosoxydans* subsp. *denitrificans* strain TD2 efficiently degrades TDG (a breakdown product of mustard gas) and can be used for the bioremediation of soils contaminated by this chemical warfare agent as well as in the technologies for biological cleaning of the solutions formed during degassing of containers by alkaline hydrolysis of residual mustard gas.

ACKNOWLEDGMENTS

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