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The Effect of Glutaraldehyde on the Kinetics of Selected Microbiological Processes

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Abstract

The effect of glutaraldehyde (GA) in different concentrations on microbiological activities of *Bacillus licheniformis*, *Desulfotomaculum ruminis* and *Thiobacillus ferrooxidans* bacteria used in the processes of denitrification, desulfurication and iron (II) oxidation, respectively, has been tested. For the sake of comparison, the effect of formaldehyde (FA) on the activities of the same organisms in the same conditions has been studied. The tolerated and toxic concentrations of the aldehydes were determined.

Keywords: bacteria, *Bacillus licheniformis, Desulfotomaculum ruminis, Thiobacillus ferrooxidans*, toxicity, denitrification, desulfurication, glutaraldehyde (GA), formaldehyde (FA), indigocarmine

Introduction

Glutaraldehyde has been widely used in analytical chemistry [1, 2], organic chemistry [3, 4], chemistry of polymers [5] and medicine [6]. Its molecule comprises two aldehyde groups undergoing fast reactions with proteins already in normal conditions (water solution, low temperature). In an alkaline environment the rate of the reaction significantly increases [7]. GA is used for immobilisation of labile molecules of biologically active proteins and enzymes [8]. It is active towards all kinds of bacteria and spores, therefore it is widely applied for cold sterilisation of clinical and surgical instruments [9].

The activity of GA extends over fungi and viruses. The main advantage of the reaction between GA and proteins is the irreversibility and stability of its products in a wide range of pH, ionic strength and temperatures. An additional advantage offered by GA used in solutions is that it does not cause metal corrosion and does not damage rubber products. The high reactivity of GA, still ambiguous structure of its water solutions and the mechanism of the reaction with proteins, have been the subject of many studies [1-10].

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Increasing use of GA in all kinds of applications inevitably means that its increasing amounts penetrate the natural environment, consequently the question arises on its possible impact. Protection of the natural environment demands recognition of the mechanism and effects of GA interactions and finding methods to eliminate the possible harmful influence of this chemical substance. In particular the toxic concentrations of GA and its susceptibility to biological decomposition should be established. With regard to the above, it seems interesting to determine the character of GA influence on the processes of denitrification and desulfurication, closely related to the cycles of biogenic conversion of nitrogen and sulphur compounds.

Moreover, the influence of GA on the activity of the *Thiobacillus* bacteria, commonly found in the water accompanying coal beds, whose source of life sustaining energy are the processes of iron (II) oxidation and reduction of inorganic sulphur compounds.

This paper reports the results of this study. Determination of the limits of admissible levels of GA concentrations will help develop appropriate procedures of optimum detoxication from ecological and ecotoxicological points of view.

Materials and Methods

1. Denitrification. The denitrification bacteria from the genus *Bacillus* were isolated and identified in the way described earlier [11].

Kinetic study was performed at 37°C and pH = 7.5 in closed glass reactors of 20 cm^3 in capacity, containing 10 cm^3 of lactate medium composed of [g/dm^3]: $N_{NO_3^-}$ = 1.40; $Fe(NO_3)_3 \cdot 9H_2O$ = 0.44; NH_4Cl = 0.25; $MgSO_4 \cdot 7H_2O$ = 0.50; $CaCl_2$ = 1.00; $Na_2HPO_4 \cdot 12H_2O$ = 2.50; $C_{org.}$ = 3.27 and microelements [12]. The medium was inoculated with 4% vol. of the inoculum collected after 24 hours of the bacteria growth (the phase of logarithmic proliferation). Then, a 50% water solution of GA in the portions of 2, 5, 10, 20, 30, 50, 100 and 200 ppm was added. The rate of denitrification was controlled by measuring the concentrations of nitrates and nitrites at certain time intervals.

2. Desulfurication. The bacteria involved in desulfurication were isolated and identified as *Desulfotomaculum ruminis* by the method described in [13].

Kinetic study was performed at 37°C in anaerobic conditions (helium) at pH = 6.8 – 7.2, in sealed glass reactors containing 50 cm^3 of sterile modified Starkey medium containing [g/dm^3]: $MgSO_4 \cdot 7H_2O$ = 2.00; Na_2SO_4 = 2.42; NH_4Cl = 1.00; K_2HPO_4 = 5.00; $CaCl_2$ = 0.13; Mohr salt = 0.5; sodium lactate = 5.00 and microelements [13]. The GA tested wad added to the reactors at concentrations of: 3, 5, 7, 10, 12, 15 ppm and after deoxidation the medium was inoculated with 4% vol of the inoculum collected from the phase of logarithmic proliferation (after 24h). The rate of the reaction was determined by measuring the concentration of sulphates and sulphides at certain time intervals.

3. Iron (II) oxidation. The *Thiobacillus ferrooxidans* bacteria were isolated from the water from the Siersza colliery and they were grown in the Silverman 9K medium [14].

Kinetic study was performed at 35°C and pH = 2.2, in glass reactors of 50 cm^3 in capacity, containing 20 cm^3 of the medium 9K and 10% vol of the inoculum collected from the phase of logarithmic proliferation (after 22 hours of incubation). At the next step a proper amount (as specified in the results) of GA in 50% water solution was added to the reactors. The reaction was conducted in a thermostated shaker (Elpan 357) rotating at 150 c.p.m with an amplitude 9. The rate of the process of oxidation was controlled by measuring the concentration of iron (II) ions.

The instruments and media used were sterilised for 20 min. at 120°C. Analogous experiments were performed in the same conditions on the reference samples, i.e. without GA. For analysis we have taken values obtained as averages of three measurements. In this way we measured the effects of the substance tested on microbiological processes eliminating the influence of chemical processes.

4. Analytical methods. The concentration of nitrates was measured potentiometrically by an ion-selective electrode "Detektor."

The concentration of nitrites was measured spectrophotometrically (Beckman spectrophotometer DU-640) at λ= 520 nm [15].

The concentration of sulphides was measured by the iodometic method in CdS precipitate [16].

The concentration of sulphates was determined by the complexometric method [16].

The concentration of iron (II) was measured by the spectrophotometric phenantroline method (Beckman DU 640) [17].

5. For the sake of comparison the effect of formaldehyde, the most commonly applied active conservation agent was tested in the same conditions as GA.

6. The indicator of chemical activity producing changes in the environment was the time of oxidation of indigo-carmine manifested as a loss of colour under the effect of GA.

Results and Discussion

Thanks to the high activity of GA towards the vegetative forms of micro-organisms and bacteria and virus resting spores and fungi sclerotium, its solutions belong to the most commonly used sterilising substances [7]. Since the mechanism of sterilisation depends on the chemical nature of the agents, the results obtained for GA were compared with the influence of widely applied formaldehyde.

As follows from the results of chemical activity measured as the time of discoloration of indigo-carmine, in an experiment carried out for 4 hours 20 cm^3 of GA is needed for the discoloration of this compound. In the same conditions formaldehyde did not cause the discoloration, which means that the chemical activity of these compounds is different. The dependence of the time needed for discoloration of indigo-carmine on the concentration of GA is shown in Fig. 1.

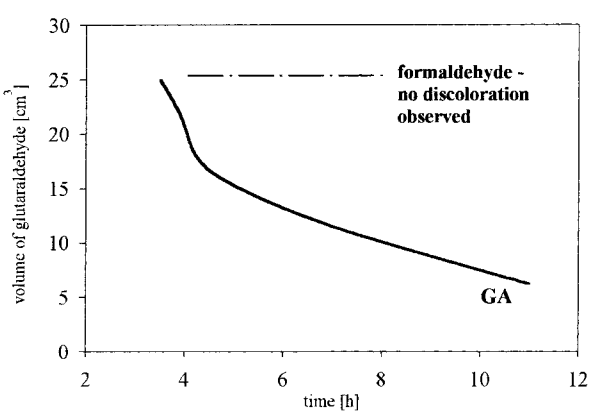

Fig. 1. The effect of glutaraldehyde (GA) concentration on the time of discoloration of indigo carmine (0.03 g indigo carmine/100 cm^3, temp. 25°C).

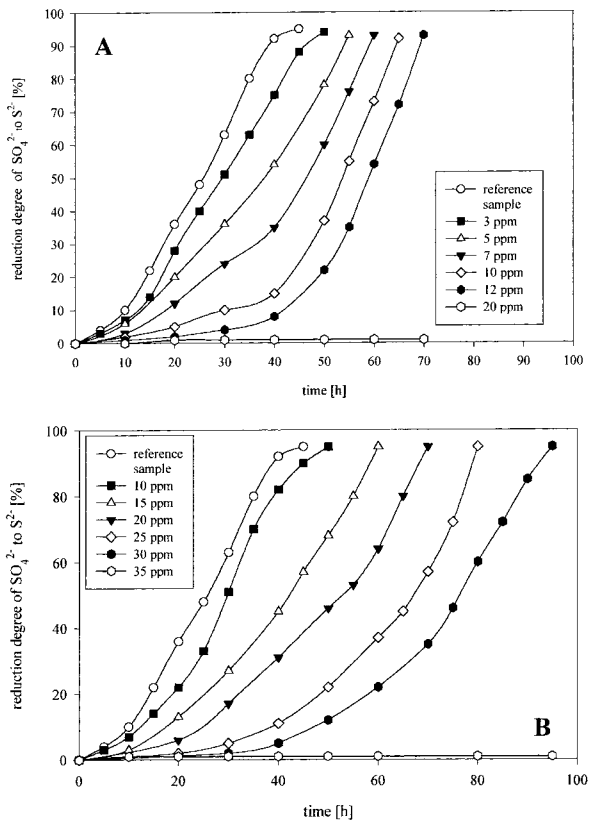

Fig. 2. The effect of glutaraldehyde (A) and formaldehyde (B) concentration in the media on kinetics of dissimilatory sulphate reduction (*Desulfotomaculum ruminis* bacteria, pH = 6.8 – 7.2, temp. 37°C).

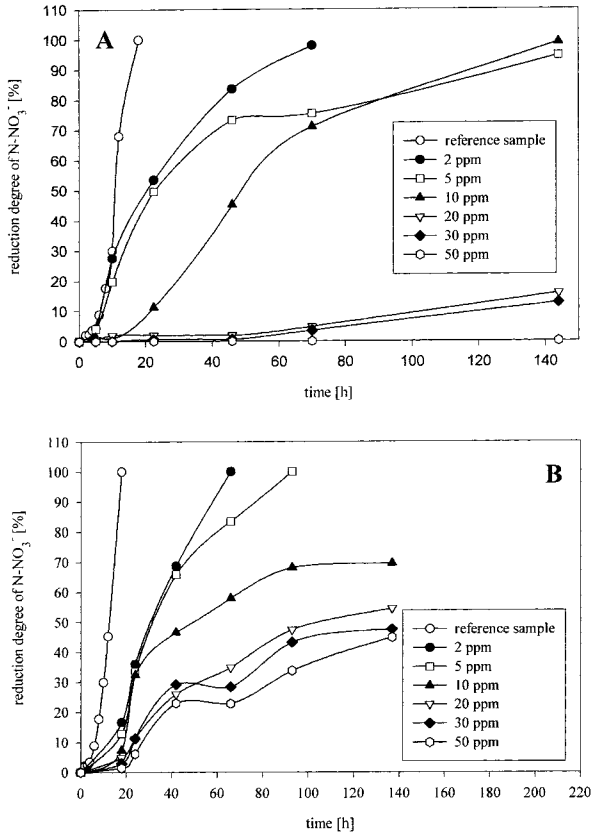

Fig. 3. The effect of glutaraldehyde (A) and formaldehyde (B) concentration in the media on kinetics of denitrification (*Bacillus lichenformis* bacteria, pH = 7.5, temp. 37°C).

Biological activity of GA and formaldehyde (FA) was tested in the reactions of desulfurication and denitrification taking place with the involvement of the bacteria *Desulfotomaculum ruminis* and *Bacillus licheniformis.* The influence of GA and FA on the kinetics of iron (II) oxidation with the bacteria *Thiobacillus ferrooxidans* was also studied.

Fig. 2 presents the kinetic curves illustrating the effect of GA and FA on the activity of anaerobic bacteria *Desulfotomaculum ruminis*, whose source of life-sustaining energy were simple organic compounds providing carbon and sulphates providing electrons. As follows from the curves, with increasing concentration of the two aldehydes in the media, the induction period related to the process of proliferation and adaptation of the micro-organisms increases and the reaction rate decreases. This means that the bacteria activity decreases and GA shows about twice stronger inhibition effect than FA.

For example, at the presence of GA at a concentration of 12 ppm GA the degree of sulphates reduction is the same as at the presence of 20 ppm FA. Results of the activity tests indicate that the bacteriostatic activity of glutaraldehyde introduced to the medium begins from the concentration of 20 ppm, whereas that of FA begins from 35 ppm. At these concentrations the process of desulfurication is irreversibly ceased, which means that the life-sustaining processes of the bacteria have stopped.

The influence of GA and FA on the kinetics of the process of denitrification taking place with the involvement of *Bacillus licheniformis* bacteria, is illustrated in Fig. 3. The microorganisms can proliferate in aerobic and anaerobic conditions using as a final electron acceptor not only oxygen but also nitrates and simple organic compounds as a source of carbon.

According to the results of the kinetic studies, the bacteria are more susceptible for the inhibiting effect of the aldehydes, as indicated for example by short induction periods of denitrification. Only at GA in concentrations higher than 20 ppm is a significant inhibition of the induction period to about 5 hours observed. At a concentration of 50 ppm GA in the medium the process of denitrification is totally inhibited. The flattening of the kinetic curves of denitrification run in the medium containing 5 ppm GA appearing after 40 hours of the process, visible in Fig. 3, is related to the presence of transient nitrite intermediates. Fig. 4 presents the kinetic curves of decomposition of nitrites formed at the first stage of denitrification [12]. The nitrites appearing in the process of denitrification in an unstable transient state do not undergo decomposition to gas nitrogen in the conditions of the reaction and remain undecomposed at a level of 80 mg/dm^3.

The inhibitory effect of formaldehyde on denitrification is similar (Fig. 3B). With increasing concentration

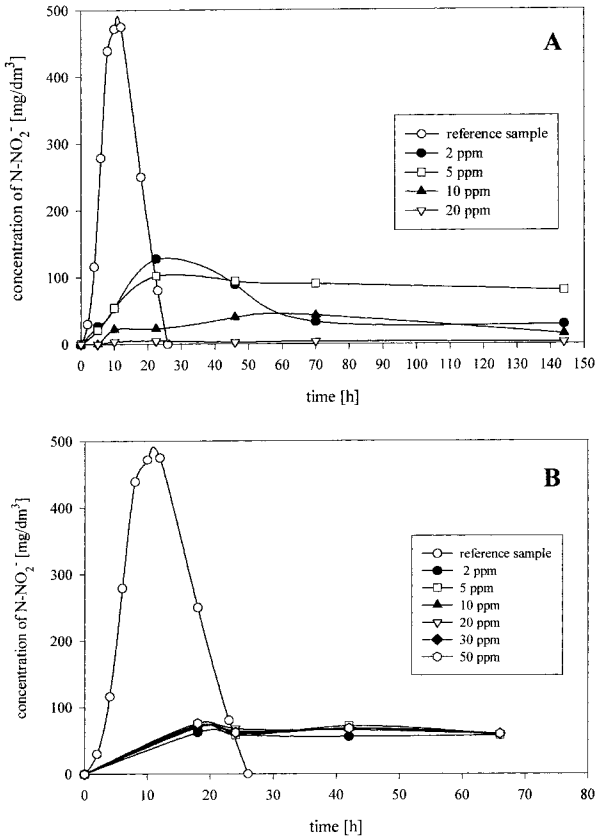

Fig. 4. The effect of glutaraldehyde (A) and formaldehyde (B) concentration in the media on kinetics of NO_2^- reduction (*Bacillus lichenformis* bacteria, pH = 7.5, temp. 37°C).

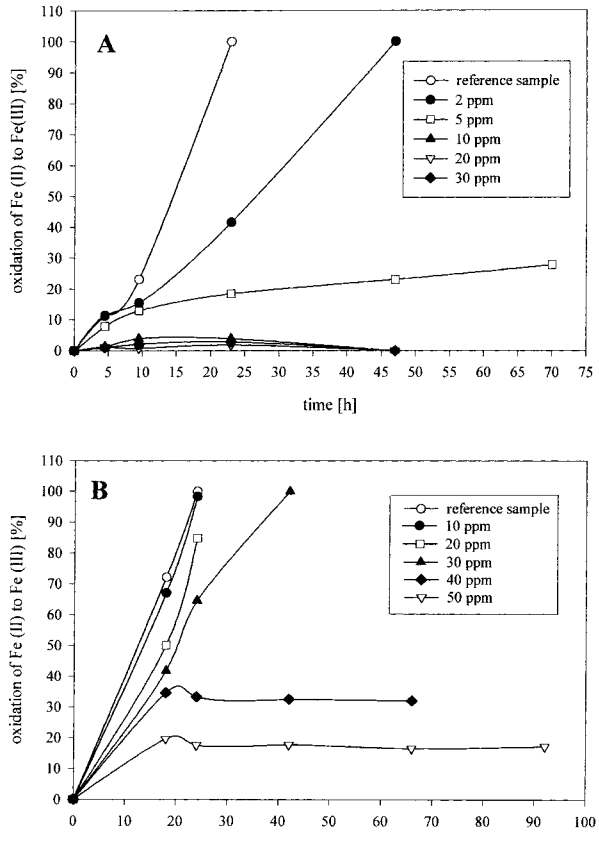

Fig. 5. The effect of glutaraldehyde (A) and formaldehyde (B) concentration in the media on kinetics of Fe(II) oxidation (*Thiobacillus ferrooxidans* bacteria, pH = 2.2, temp. 35°C).

of FA to 50 ppm, the degree of denitrification decreases to about 40%. In FA concentrations higher than 50 ppm, a significant inhibition of proliferation of the bacteria *Bacillus licheniformis* is observed. After 30 hours of the reaction the process of denitrification is temporarily stopped, which is related to the inhibition of the reduction of nitrites at a level of 50 mg/dm^3 (Fig. 4), which has not changed in the course of the process.

The influence of the concentration of GA and FA on the activity of the microorganisms in the process of iron (II) oxidation in a medium containing the bacteria *Thiobacillus ferrooxidans* is illustrated by the kinetic curves shown in Fig. 5. The source of life-sustaining energy of the bacteria *Thiobacillus* are the reactions of oxidation of sulphur and sulphides to sulphates or iron (II) to iron (III). The source of carbon needed for cell metabolism is carbon dioxide.

The shape of these curves differs significantly from the shape of those describing the processes of reduction of sulphates or nitrates. The oxidation of Fe(II) in the presence of 2 ppm GA starts without any induction period. After about 5 hours the process stops and only after adaptation of the bacteria to the medium does it start again. In the medium containing already 5 ppm of GA, the process of iron (II) oxidation is significantly inhibited for the duration of the experiment. Increasing concentration of GA result in its further inhibition and at a concentration of 30 ppm GA the process is stopped.

The inhibitory effect of FA is manifested starting from its presence at a concentration of 10 ppm (Fig. 5B). Interestingly, at concentrations of 40 and 50 ppm FA inhibits the process of iron (II) oxidation at the levels of 35 and 20% of conversion, respectively. A further increase of FA concentration to about 100 ppm practically does not affect the degree of Fe(II) oxidation which remains at a level of 20%. This observation means that a certain state of dynamical equilibrium is reached between the process of microbiological oxidation of Fe(II) to Fe(III) and the process of reduction of Fe(III) caused by the presence of FA.

Table 1 presents the values of biological activity determined for GA and FA in the processes studied. The Table also gives the ranges of concentrations of GA and FA tolerated, inhibiting and toxic towards the bacteria used in the experiment.

The bacteriostatic effect of the aldehydes studied is different and depends on their chemical activity. The most sensitive to GA are the bacteria *Thiobacillus ferrooxidans* as the toxic concentration of GA is 10 ppm, while for the bacteria *Bacillus licheniformis* the toxic concentration of GA is 50 ppm. The sensitivity of the bacteria studied to FA increases in the sequence:

Desulfotomaculum ruminis > *Thiobacillus ferrooxidans* ≥ *Bacillus licheniformis*

Table 1. Microbiological activity of glutaraldehyde and formaldehyde.

Bacteria	Compound	Concentration [ppm]		
		Tolerated	Inhibiting	Toxic
Desulfotomaculum ruminis	GA	< 3	5 – 12	> 20
	FA	< 10	15 – 30	> 35
Bacillus licheniformis	GA	< 2	5 – 10	> 50
	FA	< 2	5 – 50	> 80
Thiobacillus ferrooxidans	GA	< 2	2 – 5	> 10
	FA	< 10	20 – 40	> 80

The mechanism of the inhibitory and toxic effect of the aldehydes studied on the bacteria used in the processes of desulfurication, denitrification and iron (II) oxidation is undoubtedly related to the reactions of the aldehydes with free amine and amide groups of proteins forming bonds among peptide chains. As a consequence the cell membranes shrink and intracellular pressure increases leading to irreversible changes and death of cells. Moreover, the aldehydes can react with nucleic acids and show mutagenic and cancerogenic effects [7]. The mechanism of GA activity towards microorganisms involves deactivation of sulfhydryl, carboxylic and amide groups of cellular peptides. Its high bacteriostatic effectiveness determines its wide application in chemistry and for sterilisation wherever needed. Its additional advantage is that it has no corrosive effect on metals and thus can be safely used for sterilisation of surgical instruments and endoscopes.

The concentration ranges of GA and FA in which they are tolerated and toxic to the bacteria *Desulfotomaculum ruminis, Bacillus licheniformis* and *Thiobacillus ferrooxidans* are of significance when working out effective and environmentally friendly methods of waste or pollutant deactivation.

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