

Enrichment, Isolation and Susceptibility Profile of the Growth Substrate of Bacterial Strains Able to Degrade Vinyl Acetate

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Abstract

Nowadays biodegradations of harmful xenobiotics seems to be the best and cheapest method of purification of the polluted environment. VOCs are represented by vinyl acetate, which is thought to be carcinogenic. The aim of these studies was to isolate and determine the susceptibility profile for vinyl acetate of bacterial strains. The source of microorganisms was soil sampled in the area of Synthos S.A. in Oświęcim, Poland. From among 41 isolates, 4 Gram-negative strains were chosen for further analyses. As the control, one laboratory strain of *Pseudomonas fluorescens* PCM 2123 from The Polish Collection of Microorganisms (Wrocław) was used. Simultaneously, a susceptibility profile to vinyl acetate was performed on *Stenotrophomonas maltophilia* KB2 strain, aromatic compounds' degrader.

Vinyl acetate used in concentration of 3,000 ppm inhibited growth of gram-positive bacteria, and 4,000 ppm was the lethal dose for microorganisms from mixed populations. A toxicity test showed susceptibility to vinyl acetate at concentrations of 2,000 ppm. Three weeks of pre-incubation with 400 ppm of vinyl acetate magnified the level of sensitivity to 3,000 ppm of vinyl acetate for almost all strains. Although decomposition of vinyl acetate was observed even in the presence of 4,000, 5,000 and 6,000 ppm of vinyl acetate, growth was not observed. It was due to enlarged concentration of acetaldehyde, a product of hydrolysis ester bond of vinyl acetate.

Keywords: vinyl acetate, acetaldehyde, toxicity, *Pseudomonas*, *Stenotrophomonas*

Introduction

Vinyl acetate represents a large group of compounds known as volatile organic compounds (VOCs). They are emitted by a wide array of sources such as car exhaust systems, the petrochemical industry and various products used in the household, including cleaning supplies, furni-

ture polish, softening agents, hair lacquers, nail lacquers, aerosols, and paints [1]. Looking from the chemical point of view, vinyl acetate is an ester of acetic acid and vinyl alcohol, which easily polymerizes and yields polyvinyl acetate (PVAc). It's a colourless, transparent liquid with a characteristic sweet smell. The chemical character of vinyl acetate suggests that it is quite easily broken down to CO₂ and water. So far there is very little information about the biological, and especially microbiological, breakdown of vinyl

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acetate in aerobic conditions. In 1990 Nieder and co-workers [2] proposed a degradation pathway of vinyl acetate in the cells of Gram-negative strain designed as V2. V2 strain was able to grow in the presence of vinyl acetate as the only source of carbon and energy, and its metabolism went through acetic acid and ethanal, and ethanol was a temporary intermediate product [2]. Hatanaka and co-workers [3] described the isolation of *Pseudomonas* sp. Z2 strain, which was able to assimilate vinyl acetate. Little research has focused on vinyl acetate as the growth co-substrate [4]. On the other hand, there are numerous publications concerning biochemical and genetic features of esterase [EC 3.1.1.1], the enzyme responsible for cleaving ester bonds [3, 5-8].

However, vinyl acetate is very often used in experiments with higher organisms, especially rodents, and the results have shown that vinyl acetate acts as a carcinogen. The more toxic character exhibits products of hydrolytic cleavage of vinyl acetate – ethanal and acetic acid [9-11]. Acetaldehyde directly reacts with DNA, causing formation of the crosswise bonds between DNA and proteins, and chromosomal aberrations in eukaryotic cells. Toxicity of acetaldehyde, as well as the other compounds containing the aldehydic group, can be so large that reduction or even inhibition of bacterial cells growth from the genus *Pseudomonas* was observed [12].

The aim of these studies was the isolation of the environmental gram-negative strains able to grow in the presence of different concentrations of vinyl acetate. The susceptibility profile to vinyl acetate was performed on four environmental strains in order to select the best degrader. As the control, one laboratory strain of *Pseudomonas fluorescens* PCM 2123 from The Polish Collection of Microorganisms (Wrocław) was used. Simultaneously, a susceptibility profile for vinyl acetate was performed on *Stenotrophomonas maltophilia* KB2 strain, aromatic compounds' degrader. The selected strain will be immobilized on a biofilter to purify the streams of outlet gases in a bioreactor. Further characteristics of the isolated strains and kinetics of vinyl acetate biodegradation are under preparation.

Material and Methods

Vinyl acetate-degrading strains were isolated from soil samples collected in the area of Synthos S.A. in Oświęcim (Poland) (formerly Chemical Company Dwory S.A.) in August 2006. Three independent locations were chosen for sample collection. All of them were the outlet of gases arising during polymerization of polystyrene (EC_1), rubber (EC_2) and polyvinyl acetate (EC_3).

In order to isolate bacterial strains able to degrade vinyl acetate, the classical enrichment techniques with the mineral salts medium containing glucose or vinyl acetate as the carbon source was used. The composition of the mineral medium was as follows (g per litre of distilled water): EDTANa₂ × 2H₂O 0.2; MgSO₄ × 7H₂O 0.58; CaCl₂ 0.05;

FeSO₄ × 7H₂O 0.002; (NH₄)₂SO₄ 1; KH₂PO₄ 3.4; Na₂HPO₄ × 12H₂O 4.5; (pH = 7; with NaOH), with the addition of TMS solution (10 ml TMS per litre of mineral salts medium). The composition of TMS was: FeSO₄ × 7H₂O 3.82 g; CoSO₄ × 7H₂O 295 mg; MnSO₄ × H₂O 82 mg; ZnSO₄ × 7H₂O 141 mg; H₃BO₃ 6 mg; Na₂MoO₄ × 2H₂O 40 mg; NiSO₄ × 7H₂O 82 mg; CuSO₄ × 5H₂O 2.9 mg; Al₂(SO₄)₃ × 18H₂O 148 mg; Na₂WO₄ × 2H₂O 6 mg dissolved in 10 ml 32% HCl and filled up with distilled water to volume of 1 litre.

Five grams of each soil sample was suspended in 100 ml of sterile mineral medium with TMS and glucose (1 g/L), left on for three days on the rotary shaker (130 rpm) at 30°C for incubation. Such prepared suspensions were used as an inoculum for the enrichment cultures. Cultures in 250-ml flask containing 100 ml of sterile mineral salts medium with TMS and supplemented with 50 ppm of vinyl acetate were inoculated with 5 ml of soil suspension, and incubated shaking at 130 rpm at 30°C for 24 hours. If the growth of the culture was observed (measured spectrophotometrically at 550 nm), the successive dose of vinyl acetate was introduced and the cultures were left for incubation for the next 24 hours. Then, if the farther growth of the mixed population of soil microorganisms was confirmed, 5 ml of the soil suspension was transferred to the fresh medium and supplemented with 50 ppm higher dose of vinyl acetate in comparison to the previous one. That procedure was repeated until 500 ppm of vinyl acetate was confirmed to be utilized as the carbon and energy source. Because each supplement of vinyl acetate (for each soil sample) was correlated with intensive growth of the cultures, in the next step of isolation procedure the doses of vinyl acetate, introduced to the cultures, were increased by 500 ppm, and the time of incubation was extended to 7 days. Beginning from the cultures supplemented with 1,500 ppm of vinyl acetate, cultures were diluted (1 ml culture in 9 ml of 0.85% NaCl and further transfer to new 9 ml of 0.85% NaCl, adequately to the density of the cultures) and plated on nutrient agar plates. The agar plates were incubated at 30°C for 24-48h and counted.

In addition, after incubation the single, morphologically different colonies were isolated and subjected to further purification by streaking on nutrient agar plates. Each isolate was given a symbolic description for strain origin (EC1 or EC2 or EC3) and the concentration (in ppm) of vinyl acetate supporting the strain growth (e.g. 2,000 means vinyl acetate in concentration of 2,000 ppm). Gram staining, colony shape, size and colour on nutrient agar of each isolate were described. Strain was kept on agar slopes at 4°C.

Gram-negative isolates proliferated in LB medium (g/L: tryptone 10; NaCl 10; yeast extract 5) shaking (130 rpm) at 30°C. After 18h, the cells were harvested by centrifuge (5,000 × g at 4°C for 15 min) and washed with fresh mineral medium. The pellet was resuspended in 100 ml of fresh mineral medium to final optical density, about 0.1 in absorbance scale at λ = 550 nm, and 400 ppm of vinyl acetate was added as the sole source of carbon and energy. The flasks were incubated by shaking at 130 rpm at 30°C for 24 hours and the growth of bacterial strains was monitored.

Introduction of the successive dose of vinyl acetate was repeated three times every 24 hours and four bacterial strains denoted as EC3_2001, EC1_2004, EC3_3502 and EC2_3502 were found to grow in the presence of the repeated doses of vinyl acetate. These strains were chosen for further analyses. As the control, the laboratory strain *Pseudomonas fluorescens* PCM 2123 [13] (Polish Collection of Microorganisms, Institute of Immunology and Experimental Therapy in Wrocław) and *Stenotrophomonas maltophilia* KB2 [14] (Department of Biochemistry, Faculty of Biology and Environment Protection, Silesian University) were used.

Experiments concerning the degradation of various concentrations of vinyl acetate by pure cultures were conducted using polypropylene tubes (with screwed caps) of different volumes, but the liquid:space ratio (1:4) was always kept. Cells of the analyzed strains always proliferated in LB medium for 18-24 hours, later harvested by centrifuge ($5,000 \times g$ at 4°C for 15 min) and washed with fresh sterile medium. Such prepared cells were used for the experiments without adaptation. On the contrary, adaptation of the bacterial strains was conducted in mineral salt medium with TMS and 400 ppm of vinyl acetate introduced directly to the medium every 24 hours. The flasks were incubated shaking (130 rpm) at 30°C . After three weeks the cells were used as an inoculum in the experiments with bacterial strains after adaptation.

The initial amount of pure bacteria started at a cell concentration yielding about 0.1 in absorbance scale ($\lambda = 550$ nm). The tubes with bacterial suspension and different initial vinyl acetate concentrations were shaken (130 rpm) at 30°C and sampled after 24h and 72h to analyze concentrations of vinyl acetate and its metabolites (ethanal, ethanol and acetic acid) with gas chromatography. Due to volatility of vinyl acetate and the possibility of interaction between this compound and the polypropylene [15], the cultures without bacteria with different initial concentration of vinyl acetate were also conducted under the same conditions. Results of these experiments were taken into consideration

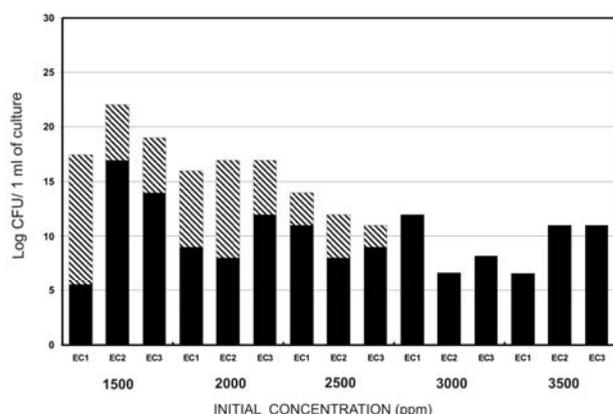


Fig. 1. Changes of bacterial cell numbers expressed as CFU/ml cultures during adaptation to increasing concentrations of vinyl acetate, where: ■ - Gram-negative bacteria, ▨ - Gram-positive bacteria.

during recalculation findings of the cultures carried out in the presence of bacteria.

Samples were taken from the cultures under sterile conditions and directly analyzed by injection of $0.15 \mu\text{l}$ samples on a Varian 3800 gas chromatograph, equipped with a 30 m length, CP-wax 52 CB column and a flame ionization detector FID. Helium was used as the carrier gas. Separation was achieved with a temperature programme ($30^{\circ}\text{C}/\text{min}$ from 70°C to 150°C). The temperature of the injector and the FID was 250°C .

Growth of the cultures was determined by the measure of absorbance at 550 nm.

Results and Discussion

The first part of the present work focused on the isolation of bacterial strains capable of growing in the presence of different concentrations of vinyl acetate. The standard enrichment culture technique relies upon selection microorganisms while the appropriate growth conditions are applied. We used increased concentrations of vinyl acetate as the discriminatory factor. A mixed population of soil microorganisms was changing while the higher concentrations of vinyl acetate were introduced. We were especially interested in monitoring the changes between Gram-negative and Gram-positive bacteria within each of the mixed populations. Due to differences in the bacterial cell structure, Gram-negative bacteria are thought to be less sensitive to many environmental factors [16]. While increased concentrations of vinyl acetate were applied, the slight gradual decrease of population number was observed. Ratio of Gram-negative to Gram-positive cells was also changing (Fig. 1). Beginning from 1,500 ppm of vinyl acetate applied to the cultures, the number of gram-positive bacteria was decreased, and after the addition of 3,000 ppm of vinyl acetate enrichment cultures consisted only of Gram-negative bacteria. The introduction of 4,000 ppm of vinyl acetate completely inhibited growth of the mixed microbial populations.

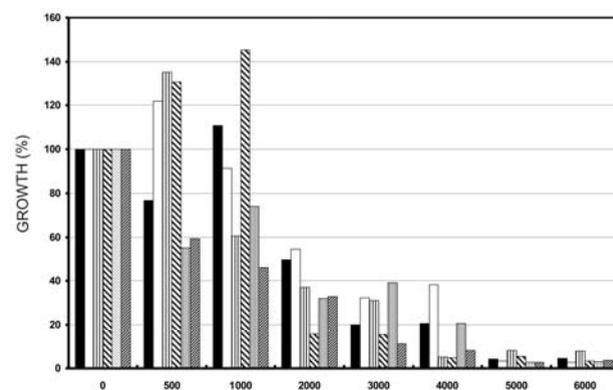


Fig. 2. Toxicity of vinyl acetate to the bacterial strains during the growth in LB medium, after 24 hours of incubation, where: ■ - EC3_2001, □ - EC1_2004, ▨ - EC3_3502, ▩ - EC2_3502, ▤ - *Pseudomonas fluorescens* PCM 2123, ▥ - *Stenotrophomonas maltophilia* KB2.

Table 1. The presence of intermediates during degradation of different concentrations of vinyl acetate after 24 and 72h of incubation, where: + - presence of compound, — lack of metabolite, ↑ - increase of concentration, ↓ - decrease of concentration.

Strain	Metabolite	Vinyl acetate concentration (ppm)													
		500		1000		2000		3000		4000		5000		6000	
		24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h	24h	72h
EC3_2001	Acetaldehyde	—	—	+	—	+	↓	+	↑	+	↑	+	↑	+	↑
	Ethanol	+	—	+	—	+	↑	+	↑	+	↑	+	↑	+	↑
	Acetic acid	+	—	+	—	+	↑	+	↑	+	↑	+	↑	+	↑
EC1_2004	Acetaldehyde	—	—	—	—	+	↓	+	↑	+	↑	+	↑	+	↑
	Ethanol	+	—	+	—	+	↑	+	+	+	+	+	+	+	+
	Acetic acid	+	—	+	—	+	↑	+	↑	+	↑	+	↑	+	↑
EC3_3502	Acetaldehyde	+	—	+	—	+	↓	+	↑	+	↑	+	↑	+	↑
	Ethanol	+	—	+	—	+	↑	+	↑	+	+	+	+	+	+
	Acetic acid	+	—	+	—	+	↓	+	↑	+	↑	+	↑	+	↑
EC2_3502	Acetaldehyde	—	—	—	—	+	↓	+	↑	+	↑	+	↑	+	↑
	Ethanol	—	—	—	—	+	↑	+	↑	+	+	+	+	+	+
	Acetic acid	—	—	+	—	+	↑	+	↑	+	↑	+	↑	+	↑
PCM 2123	Acetaldehyde	—	—	—	—	+	↓	+	↑	+	↑	+	↑	+	↑
	Ethanol	—	—	+	—	+	↑	+	↓	+	+	+	+	+	+
	Acetic acid	—	—	+	—	+	↓	+	↑	+	↑	+	↑	+	↑
KB2	Acetaldehyde	—	—	—	—	+	—	+	↑	+	↑	+	↑	+	↑
	Ethanol	—	—	+	—	+	↑	+	↑	+	↑	+	↑	+	↑
	Acetic acid	—	—	+	—	+	↑	+	↑	+	↑	+	↑	+	↑

Generally 41 morphologically distinct colonies growing on the nutrient agar were described, and 14 bacterial strains belonged to Gram-positive ones. The ability of 27 Gram-negative strains to grow in the presence of vinyl acetate was tested. The test results indicated that four strains, denoted as EC3_2001, EC1_2004, EC3_3502 and EC2_3502, were able to grow continuously, while vinyl acetate in concentrations of 400 ppm was applied every 24 hours for four days. No flocculation of cells or temporary lack of growth were observed, and that's why these four strains were chosen for further experiments.

In order to determine the toxic impact of different initial vinyl acetate concentrations on survivability of the environmental strains and the control ones, very nutritive LB medium was used (Fig. 2). The most sensitive strain turned out to be EC2_3502, whose growth in the presence of 2,000 ppm of vinyl acetate achieved less than 20% in contrast to the control (culture without vinyl acetate). The presence of 500 and 1,000 ppm of vinyl acetate stimulated the environmental strains to better growth, while the control strains *Pseudomonas fluorescens* PCM 2123 and *Stenotrophomonas maltophilia* KB2 reached about 60% growth in comparison to the control cultures. All tested strains reduced their growth to less than 50% in the presence of 2000 ppm and more of vinyl acetate. From among

the tested strains, EC1_2004 and *Pseudomonas fluorescens* PCM 2123 were less sensitive to higher concentrations (2,000–4,000 ppm) of vinyl acetate.

Adaptation of microorganisms to the increased concentrations of the given xenobiotics or toxic compounds constitutes the important stage during their biodegradation. Mechanisms of adaptation can be of different types. Enzymatic mechanism relies upon the induction of the appropriate enzymes, genetic ones count upon the gene transfers and modifications of DNA (point mutation, recombination, gene duplication) [17, 18]. In our work, three weeks of incubation in the presence of 400 ppm of vinyl acetate resulted in the better growth of pre-incubated cells in the presence of 500 1,000 and 2,000 ppm of xenobiotics (Fig. 3). The only strain whose growth in the presence of 3,000 ppm vinyl acetate exceeded growth in the control culture (without growth substrate; calculated as 100% growth) after 72 hours of incubation was *Stenotrophomonas maltophilia* KB2, regardless of previous pre-incubation or its shortage. This strain was isolated from the activated sludge of the sewage treatment plant in Bytom Miechowice in Poland using classical enrichment technique with phenol as a selection factor [14]. It degrades phenol and some of its derivatives mainly through so-called *meta*-cleavage pathway. The first intermediate of catechol degradation is then

2-hydroxyomuconic semialdehyde, containing aldehydic group. Probably the ability to strain KB2 to metabolize this compound makes it less sensitive to the presence of other compounds containing the same chemical group.

The introduction of 2,000 ppm of vinyl acetate did not inhibit the growth of strains EC1_2004 and KB2 after 72 hours of incubation. Their growths exceeded five times the

growth in the control culture while pre-incubated cells were used. Lack of earlier adaptation to vinyl acetate diminished growth of EC1_2004 strain in the presence of 2,000 ppm of substrate. When lower (500 and 1,000 ppm) concentrations of vinyl acetate were used as the only source of carbon and energy for the tested strains, the elongation of incubation time favoured the larger increase of biomass (Fig. 3). It also

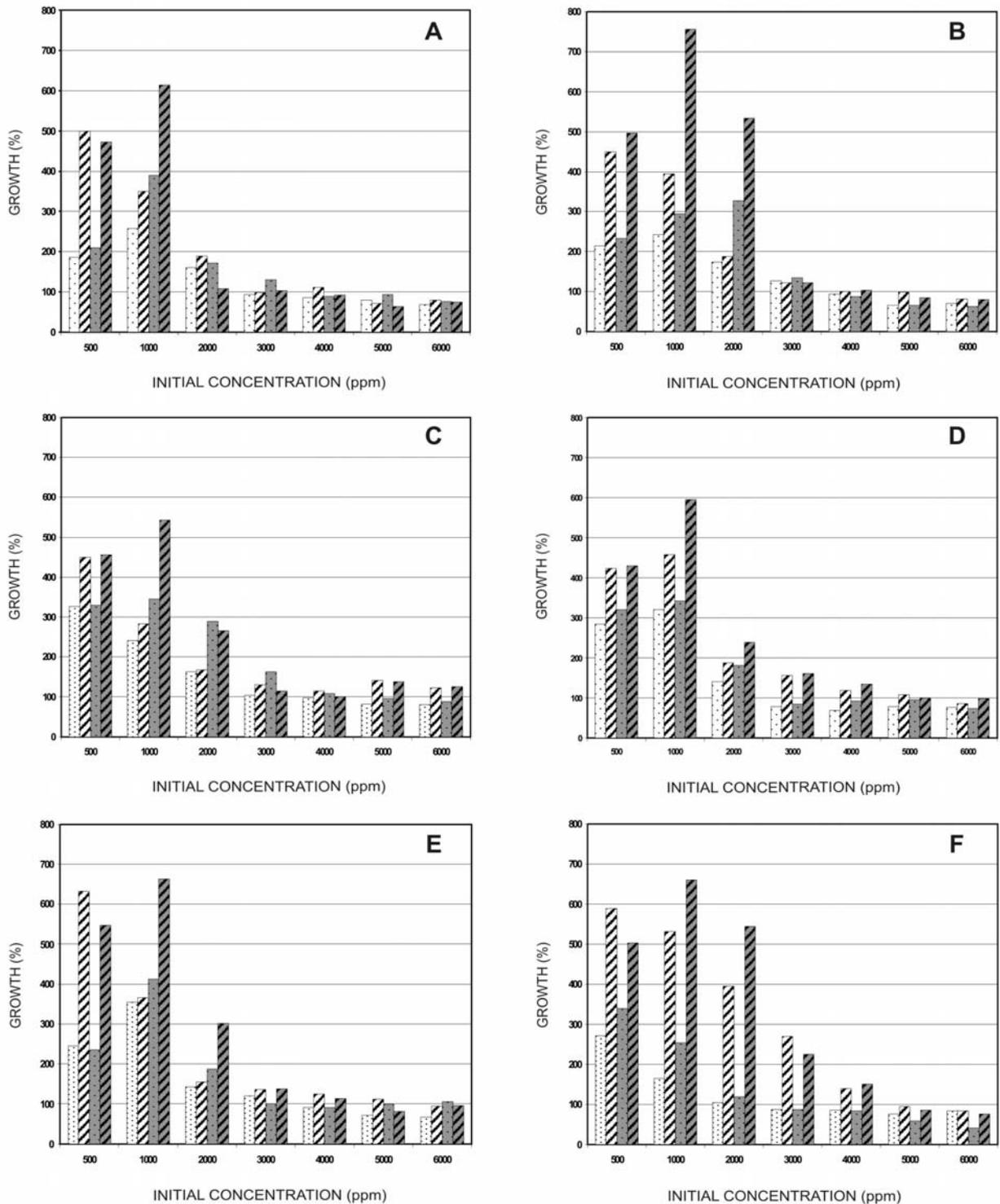


Fig. 3. Comparison of bacterial cells' growth without adaptation (dotted columns: □ and ▤), and after adaptation to vinyl acetate (striped columns: ▨ and ▩) in the presence of various initial concentrations of vinyl acetate, after 24h (white background of columns: □ and ▨) and 72h (grey background of columns: ▤ and ▩) of incubation, where: A – EC3_2001, B – EC1_2004, C – EC3_3502, D – EC2_3502, E – *Pseudomonas fluorescens* PCM 2123, F – *Stenotrophomonas maltophilia* KB2.

facilitated percentage of vinyl acetate biodegradation, independently of the applied initial concentration of vinyl acetate (Fig. 4). Vinyl acetate disappeared from the bacterial cultures, conducted even in the presence of really high concentrations of vinyl acetate (4,000-6,000 ppm), where no growth was observed (Fig. 3). Lack of growth is typical for

so-called co-metabolic cultures, where the easily assimilated source of carbon and energy is introduced next to xenobiotics. Growth substrate is metabolized, but energy obtained in this process is used to transform xenobiotics [19]. The stable amount of biomass was also observed during biodegradation of chlorinated aliphatic hydrocarbons [20].

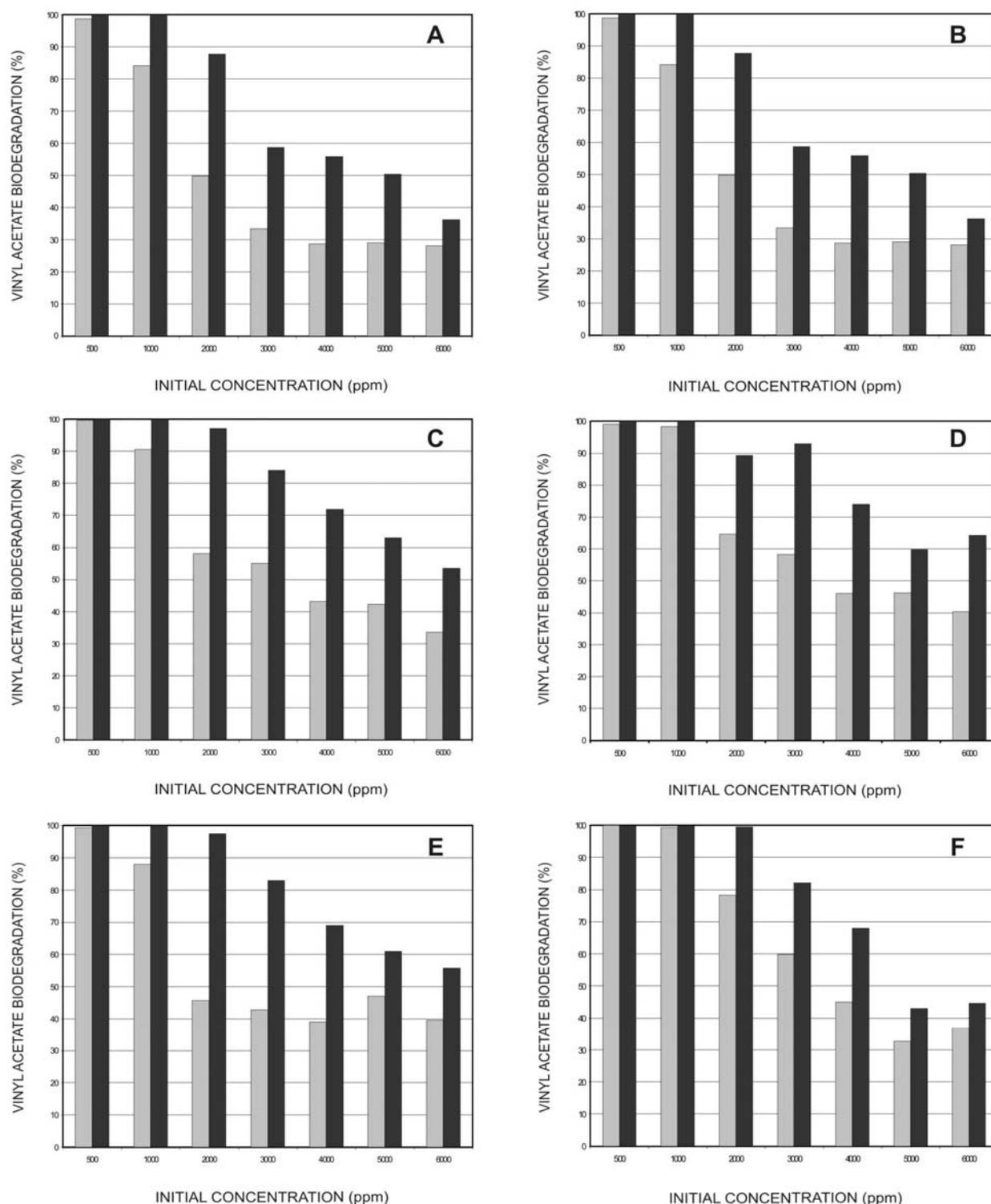


Fig. 4. Percentage of vinyl acetate biodegradation in the cultures with different initial concentration of vinyl acetate as the growth substrate, after 24h (□) and 72h (■) of incubation, where: A – EC3_2001, B- EC1_2004, C – EC3_3502, D – EC2_3502, E – *Pseudomonas fluorescens* PCM 2123, F – *Stenotrophomonas maltophilia* KB2; all pre-incubated with 400 ppm of vinyl acetate.

Biodegradation of vinyl acetate takes place through the activity of dehydrogenases, and reduction of acetaldehyde to ethanol needs accessibility of NADH [2]. Complete decomposition of 500 and 1,000 ppm of vinyl acetate within 24 hours of incubation was observed only for strains EC2_3502 and *Stenotrophomonas maltophilia* KB2. These two strains, as well as EC3_3502 and *Pseudomonas fluorescens* PCM 2123, also hydrolyzed the ester bond of vinyl acetate supplied at higher concentrations after 72 hours of incubation. Two remaining strains EC3_2001 and EC1_2004 degraded only about 50% of introduced vinyl acetate in concentration of 3,000 ppm and higher. The ability to biodegrade the different concentrations of vinyl acetate seems to be the individual characteristic of the isolated strains, although strain EC1_2004 can be thought to be the best one. In the toxicity test, when LB medium was used, EC1_2004 achieved almost 50% growth in the presence of 2,000 ppm of vinyl acetate, in contrast to the control culture (Fig. 2). Two control strains, *Pseudomonas fluorescens* PCM 2123 and *Stenotrophomonas maltophilia* KB2, are also good degraders of vinyl acetate, and their susceptibility profile to vinyl acetate, especially of strain PCM 2123, permit using them for vinyl acetate decomposition.

The ability to survive in the presence of different concentrations of vinyl acetate stems also from accumulation of intermediates of vinyl acetate degradation. Analyses of the cultures' liquids showed interesting relations among the presence of particular intermediates, the ability to grow, and the applied initial concentrations of vinyl acetate. Generally vinyl acetate in concentration of 3,000 ppm and higher reduces growth of the tested strains (Fig. 3), and it's correlated with the appearance of acetaldehyde and its accumulation in the cultures (Table 1). When the lower doses of vinyl acetate are applied (500-2,000 ppm), only the temporary presence of acetaldehyde or its lack is observed. Accumulation of ethanal can be problematic in the long-term cultures, that's why resistance to acetaldehyde is a desired characteristic. Some type of defence against the toxic effect of aldehyde is its transitory reduction to ethanol by the action of alcohol dehydrogenase [EC 1.1.1.1]. Nieder et al. [2] verified the ability of V2 strain to grow in the presence of acetaldehyde. They found out that 60% of introduced dose of acetaldehyde was metabolized and used for growth, and the remaining 40% was reduced to ethanol. We also observed the presence of ethanol in the culture liquid after 24 hours of incubation (Table 1). But elongation of incubation time allows for the complete oxidation of ethanol, when 500 and 1,000 ppm of vinyl acetate were introduced as the growth substrate. Beginning from the cultures with 2,000 ppm of vinyl acetate, ethanol was present in the culture liquid also after 72 hours of incubation, and its concentration increased (EC3_2001 and KB2 strain) or remained at the same level (the rest of tested strains). Accumulation of acetic acid was also observed in the cultures with 3,000 ppm of vinyl acetate as the initial dose of growth substrate. The shortage of its assimilation can be due to the presence of ethanal and ethanol in the cultures as the toxic agents. Prolongation of incubation time to more

than 72 hours does not seem to overcome the toxic impact of acetaldehyde taking into consideration its destructive effect for DNA and proteins in the cells [9-12].

Conclusions

The present studies have shown that Gram-positive bacteria are thought to be more sensitive to vinyl acetate. Vinyl acetate in the concentration of 3,000 ppm inhibited growth of Gram-positive strains, and 4,000 ppm impeded completely growth of all microorganisms.

The susceptibility profile for vinyl acetate determined in nutrient medium, displayed that 2,000 pm of vinyl acetate limited growth of bacteria by a half or more independently of their origin.

The susceptibility profile for vinyl acetate determined in mineral medium depended on the earlier adaptation to vinyl acetate or its shortage and time of incubation. Prolongation of incubation promotes biodegradation of vinyl acetate in lower concentrations (500 and 1,000 ppm). Adaptation in the presence of 400 ppm of vinyl acetate enables the better growth of all strains apart from EC3_2001. Distinct influence of preincubation has been shown for the *Stenotrophomonas maltophilia* KB2 strain which is able to grow even in the presence of 3,000 ppm of vinyl acetate.

Growth in the presence of vinyl acetate is not only limited by the initial concentration of growth substrate, but also by the type and amount of intermediates. Especially increased concentrations of acetaldehyde inhibit the microbial growth. Temporary transformation of acetaldehyde to ethanol is probably a way for bacteria to protect themselves from the toxic influence of ethanal.

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