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DIVERSITY OF MIXED MICROORGANISM POPULATION AFTER SCREENING IN THE PRESENCE OF SELECTED VOCs

ZRÓŻNICOWANIE MIESZANYCH POPULACJI MIKROORGANIZMÓW PO SKRININGU W OBECNOŚCI WYBRANYCH LOTNYCH ZWIĄZKÓW ORGANICZNYCH

Abstract: Biological methods of productive gases treatment from Volatile Organic Compounds are based on the catalytic activities of degradative enzymes from environmental microorganisms. That is why screening for microorganisms able to degrade xenobiotics is performed. In order to isolate microorganisms able do degrade selected VOCs (*vinyl acetate and styrene*), soil sampling was performed in the area of Synthos S.A. in Oswiecim (Poland) (formerly Chemical Company "Dwory" S.A.) in August 2006. Two independent localizations were chosen for the collection of samples, and they were the outlet of gases arising during polymerisation of polyvinyl acetate and polystyrene. Different selection media were applied. They consisted of mineral salts solution, buffer components, and selective factor. As the selective factor increasing concentrations (50÷4000 mg/dm^3) of vinyl acetate or constant concentration of styrene (100 mg/dm^3) were applied. There was no increase of styrene concentration due to the significant drop in the amount of mixed population of microorganisms after application of that selective factor. Isolation, determination of microorganisms' amount on the grounds of colony morphology and results of the Gram staining of cells, were carried out after introduction of vinyl acetate in the concentrations of 1500, 2000, 2500, 3000 and 3500 mg/dm^3, and at the end of 6 weeks adaptation to styrene. Presence of selected VOCs caused significant changes in the amount and composition of mixed population of microorganisms. Both, vinyl acetate and styrene, resulted in the decrease of the initial number of populations. The ratio of Gram-negative to Gram-positive cells was changing in the presence of selected VOCs. In the beginning Gram-negative bacteria predominated. Increasing concentrations of vinyl acetate brought about gradual decrease in the number of Gram-positive bacteria, and finally after application of 3000 mg/dm^3 of vinyl acetate mixed populations consisted of only Gram-negative bacteria. Different chemical structure of styrene probably caused almost complete decay of Gram-negative bacteria in the presence of that selective factor. Differences in the structure of the bacterial cell envelopes are most likely the reason of increased survivability of Gram-positive bacteria, mainly filiform cells of *Actinomycetes*.

Keywords: isolation of microorganisms, Volatile Organic Compounds, biological waste gases treatment

Volatile Organic Compounds are group of compounds of different structure and toxicity, eg vinyl acetate and styrene, used in these studies. Biological methods of productive gases treatment from Volatile Organic Compounds are based on the catalytic activities of degradative enzymes from environmental microorganisms. That is why screening for microorganisms able to degrade xenobiotics is performed. It is usually based on the enrichment culture technique while the selection of the appropriate phenotypes and genotypes is performed. Introduction of the selective factors can occur on the same level (the same concentration) or the increased concentration of chemical can be introduced gradually. There are two main mechanisms of adaptation of microorganisms to new xenobiotics: enzymatic and genetic [1, 2]. The first one is connected with induction of the

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appropriate enzymes, and the second mechanism is connected with horizontal gene transfer and mutations. Time of adaptation can last from hours to months and years.

Diversity of mixed populations of microorganisms as the result of introduction of selective factors of different structure, $P_{o/w}$, and concentration may demonstrate how unpredictable are results of biological treatment of waste gases of mixed composition. The logarithm of the partitioning coefficient of a solvent in a defined octanol-water mixture (log $P_{o/w}$) is commonly used as a measure of the lipophilicity of a solvent. Aromatic solvents with a log $P_{o/w}$ below 4.0, such as eg styrene, accumulate in the cytoplasmatic membrane of bacteria, causing disorganization of the cell membrane structure and impairment of the vital membrane functions [3]. On the contrary vinyl acetate is thought not to bioaccumulate [4].

The aim of this work was to compare composition of mixed soil populations of microorganisms exposed to VOCs of different structure.

Methods

Soil samples were collected in the area of Synthos S.A. in Oswiecim (Poland) (formerly Chemical Company Dwory S.A.) in August 2006. Two independent localizations were chosen for the collection of samples, and they were the outlet of gases arising during polymerisation of polyvinyl acetate (PA) and polystyrene (PS).

Different selection media were applied. Composition of mineral medium used for isolation of microorganisms able to degrade vinyl acetate was described previously [5]. In order to isolate microorganisms able to styrene degradation the second mineral medium was used. The composition of that mineral medium was as follows (g per dm^3 of distilled water): K_2HPO_4 0.2; KH_2PO_4 0.1; $MgSO_4 \cdot 7H_2O$ 0.08; $CaCl_2$ 0.06; yeast extract 0.01; iron(III) citrate 0.016 (dissolved in small amount of hot water). Regardless of mineral medium type used for screening of microorganisms TMS (*Trace Mineral Solution*) in amount of 1 cm^3 per dm^3 was added. The composition of TMS was as described elsewhere [5]. As the selective factor increasing concentrations (50÷4000 mg/dm^3) of vinyl acetate or constant concentration of styrene (100 mg/dm^3) were applied.

Cultures for isolation of microorganisms able to vinyl acetate degradation were grown in 100 cm^3 of the mineral medium in 250 cm^3 Erlenmayer flasks closed with cellulose stopper and aluminium foil. And cultures for the isolation of bacteria able to survive in the presence of styrene were conducted in 250 cm^3 glass bottles fitted with rubber stoppers in 50 cm^3 of the mineral medium of the second type.

All incubations were carried out at 30°C on a rotary shaker (130 rpm) in the dark. Cell growth was monitored spectrophotometrically at λ = 550 nm.

Gram staining of bacterial cells was performed by the standard procedure [6]. Photos of bacterial cells after staining were done under the light microscope (× 1250 magnification).

Results and discussion

Procedure of isolation of microorganisms able to live in the presence of vinyl acetate and styrene is shown in Figure 1. Microorganisms present in the original soil samples were promoted to grow in the presence of glucose. Before introduction of the first dose of VOCs the initial number of bacteria was determined by counting colonies after growth on the

nutrient agar plates. 20 cm^3 of the culture liquid was transferred to small chamber and left in 4°C in order to keep the original population of microorganisms.

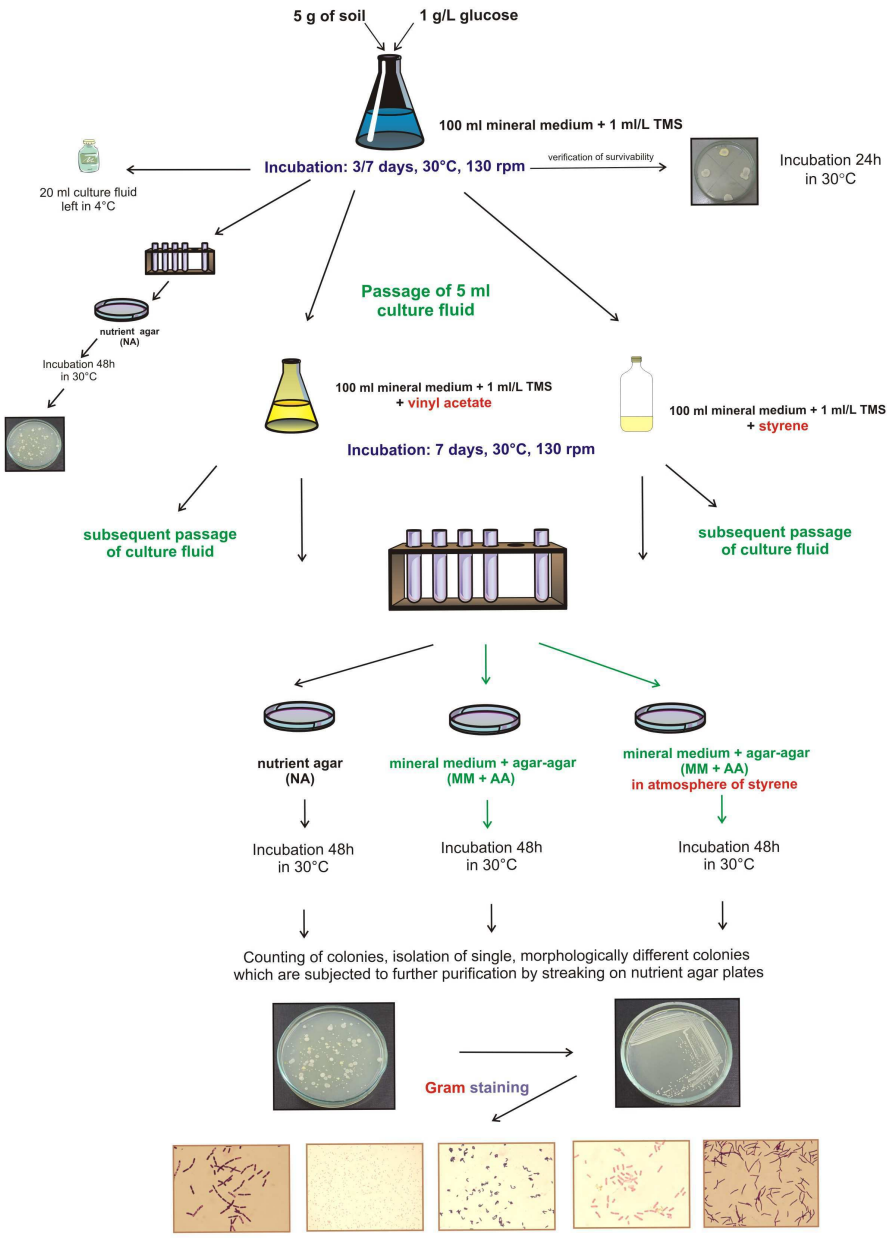

Fig. 1. General scheme of microorganisms' screening from soil samples in the presence of selected VOCs

After passage of 5 cm^3 of culture liquid to the fresh mineral medium and introduction of the appropriate dose of VOCs, incubation in 30°C for 7 days took place. After time of incubation estimation of number and composition of mixed population were performed. The initial dose of vinyl acetate was 50 ppm. Because the growth of microorganisms' population was observed from the beginning of adaptation, the increased concentrations of vinyl acetate were applied to the cultures. Until 200 ppm of vinyl acetate was introduced to the batch cultures, the gradual growth of bacteria was observed (Fig. 2). When higher concentrations of selective factor were used, the number of Gram-positive bacteria was decreased and finally only Gram-negative bacteria were present in population. 4000 ppm of vinyl acetate completely inhibited microbial growth. Biochemical and physiological analyses showed that isolated strains belonged mainly to genus *Pseudomonas* and *Alcaligenes.* There is little information in literature about microorganisms able to vinyl acetate [5-7], but hydrolytic activity of esterase responsible for cleavage of ester bond seems to be widespread.

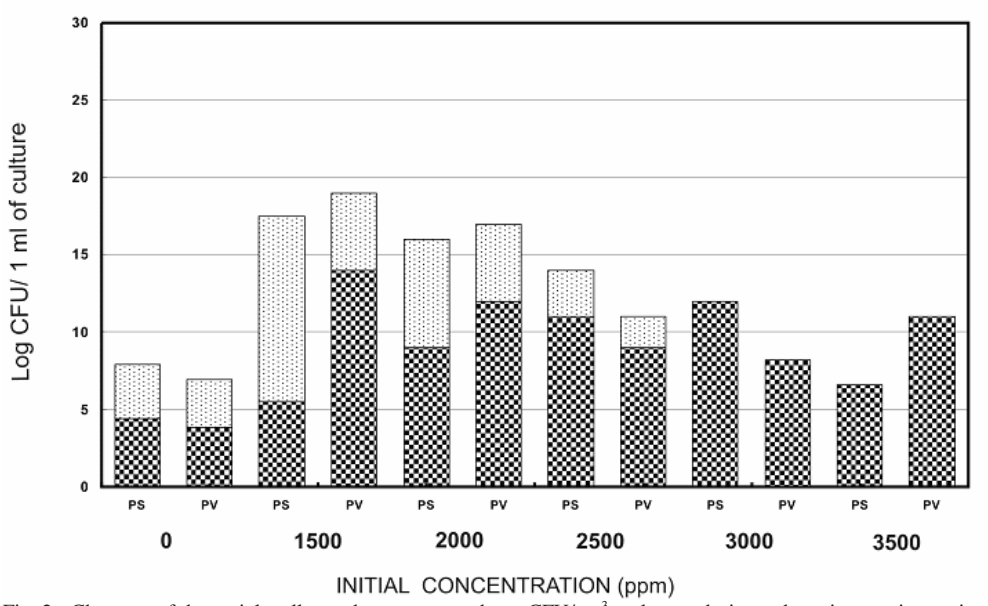

Fig. 2. Changes of bacterial cell numbers expressed as CFU/cm^3 cultures during adaptation to increasing concentrations of vinyl acetate, where: checked - Gram-negative bacteria, dotted - Gram-positive bacteria

Styrene as selective factor completely changed composition and number of mixed population of microorganisms. There was no growth in the batch cultures, where styrene was applied as the only source of carbon and energy, but bacterial cells remained alive. They grew both on nutrient agar as well as on the mineral medium in the atmosphere of styrene. Gram-positive bacteria predominated in mixed population exposed to styrene (Table 1), mainly filiform cells of *Actinomycetes*. Similar results were obtained by Przybulewska and Arnold et al [8, 9]. They carried out isolation of microorganisms from the bed of an experimental biofilter purifying exhaust gases from a cable factory's coil-wire

varnishing division. Microorganisms belonging to genera: *Bacillus, Streptomyces* and *Sphingobacterium.* On the contrary many Gram-negative strains, such as e.g. *Pseudomonas fluorescens* ST, *Pseudomonas putida* CA-3 or *Xanthobacter* sp. are described in literature as the best styrene decomposer [10]. Apart from bacteria fungi are also described as volatile organic compounds degrader [11].

Table 1

Number [log CFU/1 cm^3 of culture fluid] of Gram-negative G(-) and Gram-positive G(+) bacteria of mixed populations in the beginning and after 6 weeks of exposure to 200 mg/dm^3 of styrene, grown on nutrient agar (NA) and mineral medium (MM) in the presence of styrene (ST)

Medium / Soil sample	Time of screening procedure [week]							
	0				6^{th}			
	NA		MM + ST		NA		MM + ST	
	G (-)	G (+)	G (-)	G (+)	G (-)	G (+)	G (-)	G (+)
PS	4.6	3.9	4.5	3.5	0.1	7.4	0.2	6.7
PV	4.4	2.8	4.2	2.6	0.2	6.9	0.1	6.9

Different chemical structure of styrene probably caused almost complete decay of Gram-negative bacteria in the presence of that selective factor. But according to the literature Gram-negative bacteria are thought to be more resistant to different xenobiotics [3, 12, 13]. Presence of outer membrane seems to be the better protection than a more extensively linked peptidoglycan of Gram-positive strains. There are some known mechanisms of organic solvent tolerance among Gram-negative bacteria. The most popular and well described are modifications in cell envelope to increase cell membrane rigidity and decrease permeability, special solvent-inactivating enzymes and active efflux of solvents by means of solvent efflux pumps [3, 12, 13]. It is believed that organic solvent emulsifying/deactivating/ solubilising enzymes/substances could play a very important role in diminishing solvent toxicity in Gram-positive bacteria. Tolerance to some organic solvent does not mean ability to its degradation [13]. The results of these studies showed that from among microorganisms coming from the same soil sample, the Gram-positive strains are more tolerant to styrene ($P_{o/w}$ 3.6), and Gram-negative strains - to vinyl acetate ($P_{o/w}$ 0.73). Knowledge of bacteria tolerance to different VOCs and xenobiotics seems to be very important while the composition of microorganisms' population used in bioremediation processes is established.

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References

[1] Chmiel A.: Biotechnologia. Podstawy mikrobiologiczne i biochemiczne. WN PWN, Warszawa 1998.
[2] Van der Meer J.R., Vos W.M., Harayama S. and Zehdner A.J.B.: *Molecular mechanisms of genetic adaptation to xenobiotic compounds*. Microbiol. Rev., 1992, **56**, 677-694.
[3] Segura A., Duque E., Mosqueda G., Ramos J.L. and Junker F.: *Multiple response of Gram-negative bacteria to organic solvents*. Environ. Microbiol., 1999, **1**(3), 191-198.

[4] MSDS for vinyl acetate monomer - http://www.jubl.com/msds/msds-VAM-(Vinyl-acetate-monomer)%20.pdf
[5] Greń I., Gąszczak A., Szczyrba E. and Łabużek S.: *Enrichment, isolation and susceptibility profile to the growth substrate of bacterial strains able to degrade vinyl acetate*. Polish. J. Environ. Stud., 2009, **18**(3), 383-390.
[6] Seeley H.W., Vandemark P.J. and Lee J.J.: *Microbes in action. A laboratory manual of microbiology*. 4th ed. W.H. Freeman and Company, New York 1991, 78-79.
[7] Nieder M., Sunarko B. and Meyer O.: *Degradation of vinyl acetate by soil, sewage, sludge, and the newly isolated aerobic bacterium V2*. Appl. Environ. Microbiol., 1990, **56**, 3023-3028.
[8] Przybulewska K., Wieczorek A. and Nowak A.: *Isolation of microorganisms capable of styrene degradation*. Polish. J. Environ. Stud., 2006, **15**(5), 777-783.
[9] Arnold M., Reittu A., von Wright A., Martikainen P.J. and Suihko M.-L.: *Bacterial degradation of styrene in waste gases using a peat filter*. Appl. Microbiol. Biotechnol., 1997, **48**, 738-744.
[10] Mooney A., Ward P.G. and O'Connor K.E.: *Microbial degradation of styrene: biochemistry, molecular genetics, and perspectives for biotechnological applications*. Appl. Microbiol. Biotechnol., 2006, **72**, 1-10.
[11] Qi B., Moe W.M. and Kinney K.A.: *Biodegradation of volatile organic compounds by five fungal species.* Appl. Microbiol. Biotechnol., 2002, **58**, 684-689.
[12] Fernandes P., Ferreira B.S. and Sampaio Cabral J.M.: *Solvent tolerance in bacteria: role of efflux pumps and cross-resistance with antibiotics.* Int. J. Antimicrob. Agents, 2003, **22**, 211-216.
[13] Sardessai Y. and Bhosle S.: *Tolerance of bacteria to organic solvents*. Res. Microbiol., 2002, **153**, 263-268.

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Abstrakt: Biologiczne metody oczyszczania gazów poprodukcyjnych z lotnych związków organicznych wykorzystują zdolności katalityczne enzymów degradacyjnych szczepów środowiskowych. W tym celu poszukuje się w środowisku mikroorganizmów zdolnych do rozkładu różnych substancji o charakterze ksenobiotycznym. W celu pozyskania szczepów zdolnych do rozkładu wybranych lotnych związków organicznych (VOC), to jest octanu winylu i/lub styrenu, pobrano próbki glebowe w sierpniu 2006 roku na terenie firmy Synthos S.A. w Oświęcimiu (dawniej Dwory S.A.) z dwóch niezależnych stanowisk, będących miejscami wylotu różnych rodzajów zanieczyszczeń poprodukcyjnych w procesie syntezy polioctanu winylu oraz polistyrenu. Zastosowano różne podłoża selekcyjne, w których obok roztworu soli mineralnych i składników buforujących jako czynnik selekcyjny wykorzystano albo wzrastające stężenia octanu winylu (50÷4000 mg/dm^3), albo stałe stężenie styrenu (100 mg/dm^3). Nie zwiększano wprowadzonej dawki styrenu, gdyż od pierwszego tygodnia adaptacji zaobserwowano drastyczne zmniejszanie liczebności mieszanej populacji po zastosowaniu tego czynnika selekcyjnego. Izolację szczepów, określanie ich liczebności na podstawie różnic morfologicznych kolonii wyrosłych na podłożu z agarem odżywczym oraz komórek barwionych metodą Grama wykonano po zastosowaniu dawek 1500, 2000, 2500, 3000 i 3500 mg/dm^3 octanu winylu oraz po 6 tygodniach prowadzenia adaptacji do obecności styrenu. Obecność wybranych lotnych związków organicznych spowodowała znaczne zmiany w liczebności i składzie mieszanych populacji mikroorganizmów. Obydwa związki przyczyniły się do spadku wyjściowej liczebności populacji. Różnice w działaniu obu związków ujawniły się w zmianie stosunku bakterii Gram-dodatnich do Gram-ujemnych badanych populacji, który w momencie rozpoczęcia hodowli adaptacyjnych był przesunięty w kierunku szczepów Gram-ujemnych. Wzrastające stężenia octanu winylu przyczyniły się do zmniejszenia liczebności szczepów Gram-dodatnich aż do całkowitego ich zaniku po zastosowaniu dawki 3000 mg/dm^3 octanu winylu. Zupełnie odmienna struktura chemiczna styrenu spowodowała praktycznie całkowity zanik szczepów Gram-ujemnych. Odmienna budowa kompleksu ścianowo-błonowego bakterii Gram-dodatnich i Gram-ujemnych jest prawdopodobnie przyczyną zwiększonej przeżywalności szczepów Gram-dodatnich, wśród których przeważały nitkowate formy mikroorganizmów należących do promieniowców.

Słowa kluczowe: izolacja mikroorganizmów, lotne związki organiczne (VOC), biologiczne metody oczyszczania gazów