

Isolation of Microorganisms Capable of Styrene Degradation

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Received: May 5, 2005

Accepted: May 5, 2006

Abstract

The results of a study on the isolation of microorganisms decomposing styrene are presented in this paper. Bacteria showing such an ability were isolated from the bed of an experimental biofilter purifying exhaust gases from a cable factory's coil-wire varnishing division. In order to isolate the bacteria, styrene was introduced into the medium in the amounts of 0.5, 1.0, 1.5 and 2.0 ml/l. Also, the air in the desiccators, where the microorganism culture was performed, was saturated with styrene. The study also describes the ability of isolated bacteria strains to degrade styrene. These additional studies were carried out in flask cultures (periodically) and gas wash bottle cultures (semi-continuously). Isolated bacteria metabolized styrene that was contained in the air at maximum concentrations of about 1300 mg m⁻³. The most active bacterial isolates for styrene utilization were: *Streptomyces halstedii*, *Bacillus megaterium*, *Sphingobacterium spirtivorum*, *Bacillus cerus*. The efficiency of styrene biodegradation was up to 100%, and the rate was about 23 g m⁻³h⁻¹.

Keywords: microorganism isolation, styrene, styrene biodegradation.

Introduction

There is an increase in hazardous substance emissions to the natural environment that comes with industrial development. The substances emitted into the atmosphere may be a threat to human and animal health as well as cause changes in the troposphere and stratosphere compositions that are difficult to reverse. Transport, petrochemical, coking and chemical industries, including plastics production and processing, dye and varnish production, etc., are the main anthropogenic sources of that pollution type. Waste dumps are also a significant hydrocarbon source.

Substances introduced into the environment are often transformed through various complicated reactions in the air, which may result in an increase in their toxicity [1]. It

is therefore necessary to search for methods for removing the volatile organic compounds, including styrene, from gases originating during processes in different industries. One of the most important goals in these studies is the adaptation for the above-mentioned purpose of pollutant degradation processes that occur in the natural environment with the assistance of microorganisms [2]. In many cases, they are more efficient, and less costly than physicochemical methods [3-7]. Research has led to the formation of a new group of biological methods for pollutant degradation. Used in this research are spontaneously reproducing microorganisms, as well as those isolated from the environment and adapted for use on an industrial scale at high xenobiotic concentrations. In some countries (e.g. Germany or Netherlands), systems for exhausting gas purification such as bio-scrubbers and biofilters are widespread [8].

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The aim of this paper was to isolate the microorganisms that show a high metabolic activity in relation to styrene from a biofilter bed and to characterize their biodegradation efficiency. Earlier studies have dealt with this subject [9-10].

Styrene is one of the substances most damaging to the natural environment. Its production exceeded 5.3 million tons in the USA in 1993 with increases in recent years [11].

Materials and Methods

Source of Microorganisms

We used a bed of an experimental biofilter purifying exhausting gases from a wire-producing factory, "Załom", near Szczecin that was installed for the purposes of an earlier research project [12]. Besides various compounds, purified exhaust gasses also contained small amounts of styrene [13]. The biofilter was filled with a compost of a municipal and industrial waste mixture. The material for analyses were samples taken from two layers of the biofilter's bed. These were 10 and 30-cm layers from its upper surface at a total bed thickness of 40 cm.

Isolation of Microorganisms

An attempt was made to find out which of the microorganisms occupying the biofilter bed are capable of growth on a medium where styrene is the only carbon and energy source. At the same time, optimum styrene concentrations for microorganism growth were evaluated. At first, an inoculation of compost microorganisms on a complex MPA medium was made (MPA typical formula in g dm^{-3} : tryptone 4.0, casein acid hydrolysate 5.40, meat extract 0.40, yeast extract 1.70, sodium chloride 3.50, agar 10.00). Then, cultures were conducted on Kojima's medium without yeast extract [14], to which various styrene amounts were added (0.5, 1.0, 1.5 and 2.0 ml/l of medium). These quantities exceeded styrene solubility in pure water, thus it is impossible to find out to what extent styrene is dissolved and to what extent it was only dispersed and partially immobilized in a solid medium containing agar. Using a velvet punch, the colonies grown on a complex medium ("mother") were transferred onto a mineral medium with the tested compound added [15]. Also, efforts were made to evaluate the influence an additional styrene source in gaseous form on microorganism growth at its optimized concentration in a liquid (1.5 ml/l). Therefore, open vials with styrene (10 cm^3) were put into the desiccator (25-litre capacity), where a culture with an optimum styrene amount in a solid medium was performed. Incubation was conducted at 30°C for 2 weeks. Then, in order to obtain pure cultures, single colonies were isolated from mediums where grown microorganisms were selected by means of repli-

cation. Isolated strains grown on MPA medium, formed as slants in test tubes. For the next analyses, strains were preserved in a refrigerator at 4°C.

The statistical significance of received microorganisms counts were determined with help of ANOVA calculations. For the determination of LSD the Duncan test was used.

Studies on Degradation Activity – Flask Experiment

Suspensions of microorganisms were prepared by washing out the cultures from the slants using 3 cm^3 of 0.85% NaCl solution. Then, 1 cm^3 of suspension was taken and introduced into the flasks containing 40 cm^3 of sterile mineral medium- Kojima's medium without yeast extract. Aliquots of 10 cm^3 of such prepared samples were put into sterile orange 'Supelco' brand bottles of 125 cm^3 capacity. The bottles were hermetically sealed with a plastic cap with a hole under which was a silicone septum covered with teflon. Liquid styrene (1 μl) was injected into the bottles using a Hamilton syringe. That amount was insufficient to saturate the liquid medium with styrene. Thus, the styrene concentration in the gaseous phase at the beginning of measurement was the effect of its partition from the liquid to air, amounting to about 5 g m^{-3} . According to Henry's rule, its concentration in the medium, being an aqueous solution, was about 40 mg dm^{-3} . Also, control samples not inoculated with microorganisms containing a sterile medium were prepared. During the measurements, the cultures were shaken using to-and-fro shakers. Samples for chromatographic analysis by means of a "head space" technique were taken directly after the injection of styrene and later in convenient time intervals. Samples from the gaseous phase were taken using sterile insulin syringe, puncturing the septum with the needle. Each experiment was repeated 3-4 times.

Metabolic activity was estimated by comparing the

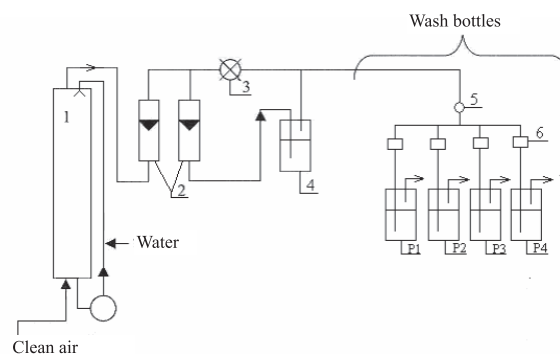


Fig. 1. Diagram of experimental apparatus; 1 – humidifying column; 2 – rotameters; 3 – valve; 4 – gas wash bottle with styrene; 5 – port for sample intake at inlet; 6 – bacterial filters; 7 – outlet of gas wash bottle (sample intake); P1 to P4 – gas wash bottles with active strain suspension.

rate of styrene consumption vs. time for studied and control samples, as well as the time required for the decrease of styrene concentration to below its detectability limit (i.e. about 4 mg m^{-3}). On the concentration vs. time figures, the curve slope at a given point is the reaction rate—the rate of styrene consumption. Therefore, the larger slope, the faster the bio-degradation process, which points to a higher activity in the strain studied. At the same time, a shorter time of styrene disappearance in a given sample should be expected.

To improve the readability of the kinetic curves on Fig. 3 the difference of dimensionless styrene concentrations in tested and control samples $\Delta C = C_{T_i}/C_{T_0} - C_{C_i}/C_{C_0}$ vs. time is presented: where C_i – running concentrations, C_0 – starting concentration.

Studies on Degradation Activity – Gas Wash Bottle Experiment

The biodegradation of styrene vapors in mixtures with air was studied using an experimental kit consisting of reactors with a suspension of microorganisms in mineral medium (Fig. 1). Four parallel working gas wash bottles (reactors) of 250 cm^3 capacity were applied, to which 190 cm^3 of mineral medium and 10 cm^3 of active strain suspension (inoculum) was added. A mixture of styrene vapors with air at concentrations beginning from 60 up to 1290 mg m^{-3} was forced through the gas wash bottles using a membrane pump. Bacterial filters (6) were installed between pump and gas wash bottles to protect the culture in the gas wash bottle against infection with microorganisms

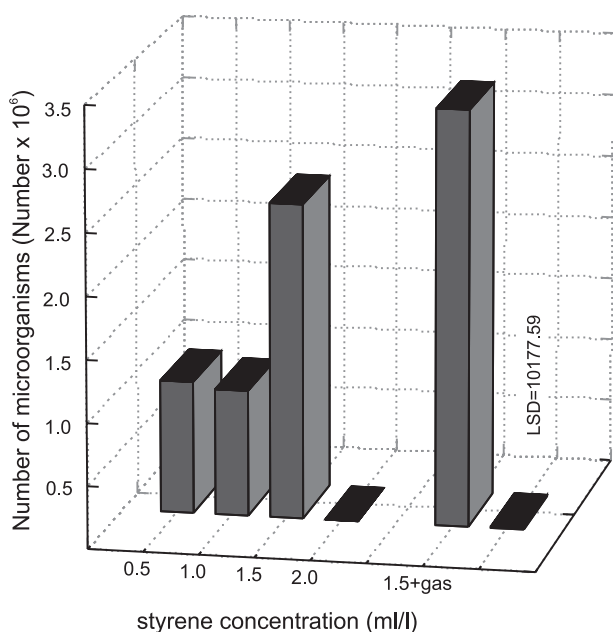


Fig. 2. Number of microorganisms (per 1 g of bed's dry matter) on mineral medium at various styrene concentrations as well as the growth on medium with optimum styrene concentration at the presence of its gaseous form (right marginal column).

present in the air. The medium was removed and replaced with a new one every several days, when a gradual loss of styrene degradation ability was observed. Then, 20 cm^3 of old microorganism culture (suspension from reactor) was added into 180 cm^3 of fresh medium. At the end of the suspension culture experiment, inoculations from the gas wash bottles onto solid substrates were made to ensure that culture infection did not occur. A uniform morphological picture of the colony grown on a solid substrate confirmed the lack of infection.

Analytical Control of Processes

Chromatographic analyses were performed using a gas chromatograph CHROM 4 (Laboratori Pristoj, Praha). The parameters of chromatograph work are listed below.

Detector: flame-ionizing (FID); column: steel packed of 1.5 m length and 3 mm diameter; column filling: SE – 30, 5% on Chromosorb W – HP, 60/80 mesh; injector temperature: 150°C ; column temperature: 110°C ; detector temperature: 150°C ; nitrogen flow: $40 \text{ cm}^3 \text{ min}^{-1}$; air flow: $100 \text{ cm}^3 \text{ min}^{-1}$; hydrogen flow: $30 \text{ cm}^3 \text{ min}^{-1}$.

Aliquots of $250 \mu\text{l}$ of gaseous sample were injected onto the chromatographic column using a single 0.5 ml insulin syringe. This ensured the sample was sterile but it did not significantly worsen sample intake accuracy, which was checked to compare the accuracy of injections made in this way with those using a gastight Hamilton syringe. A mixture of styrene with air of $C_{wz} = 798 \text{ mg m}^{-3}$ concentration prepared every time in the laboratory played the role of the standard. It was prepared by injecting $1 \mu\text{l}$ of liquid styrene into a bottle of 1130 cm^3 capacity and thermostating for an hour. Then, chromatograph calibration was carried out. The detectability threshold for styrene was about 4 mg m^{-3} . Analyses were performed two times. If the difference was greater than 10% the analysis was repeated. When the opposite were true, the average from two measurements was taken as a result of the analysis.

Bacteria Identification

The tentative identification of the four most active cultures was done by gas chromatography fatty acid methyl ester analysis performed by Microbial ID (Newark, DE, USA).

Results and Discussion

The results achieved in present paper point to the existence of microorganisms capable of utilizing styrene as the only carbon and energy source in the beds of biofilters used in industry (Fig. 2).

Styrene concentration was one of the more important

parameters affecting the number of microorganisms that were able to grow. In performed experiments, isolated microorganisms grew on mediums containing styrene at concentrations of 0.5–1.5 ml/l (0.05–0.15% v/v) at the beginning. No microorganism growth was observed on mediums with styrene at a concentration of 2.0 ml/l (0.2% v/v). Due to the physicochemical properties of styrene, particularly

its solubility in water and volatility, it should be assumed that its concentration in the medium was slightly lower than the initial one.

An initial value of 1.5 ml/l was the optimum for microorganism development. With this amount of styrene in the medium, the number of microorganisms was higher by about 40% than when using styrene at concentrations

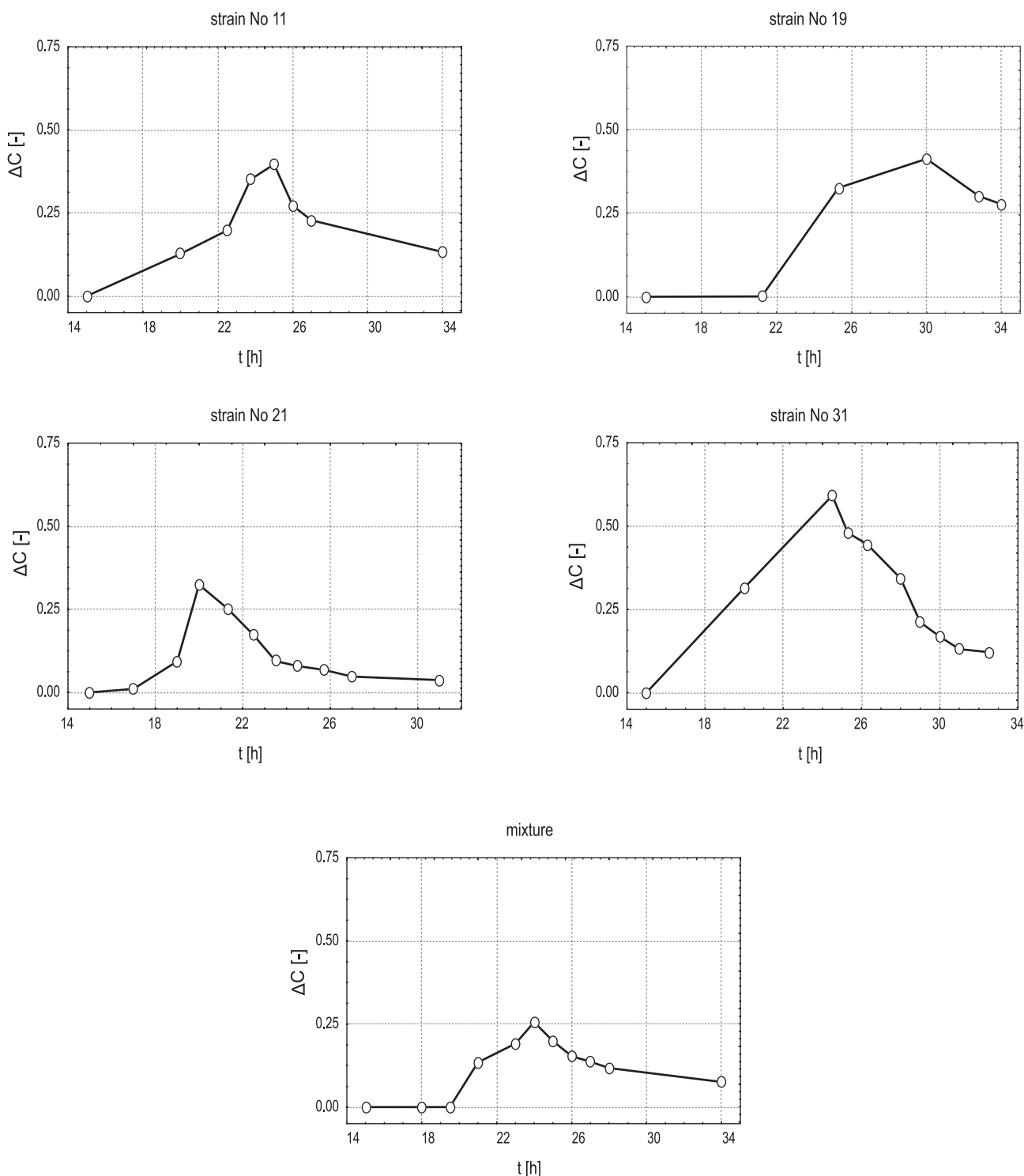


Fig. 3. Kinetic of styrene degradation by *Streptomyces halstedii* (No 11), *Bacillus megaterium* (No- 19), *Sphingobacterium spiritivorum* (No- 21), *Bacillus cerus* (No- 31): where difference of styrene dimensionless concentrations in tested and control samples $\Delta C = C_{T_i}/C_{T_0} - C_{C_i}/C_{C_0}$, C_i – running concentrations, C_0 – starting concentration.

of 0.5 and 1.0 ml/l.

Microorganisms with a higher tolerance towards high styrene levels were isolated by Arnold et al. as well as Sielicki et al. [9, 16]. Bacterial strains isolated by them showed the ability to degrade styrene at concentrations of 0.02-0.2% and 0.2–0.5%, respectively.

The results achieved in the present study also point out that the incubation of media with an additional source of styrene in gaseous phase positively affected the increase of population by 32%. Similar methods of microorganism culturing and isolation with styrene in gaseous form were applied by Hartmans et al. [17]. Microorganism cultures may be also conducted on liquid mediums in hermetically closed flasks, which minimizes the styrene losses into the atmosphere [9-10].

The increase in the number of microorganisms able to grow in the presence of styrene in a gaseous form may be explained by the fact that the presence of gaseous styrene inhibited its volatilization from the medium and made it possible to complete the losses in the medium due to microorganism activity. Thus, a continuous supply of styrene was ensured in the medium at which the concentration was not too toxic for microorganisms.

In total, 28 strains with varying morphology were isolated in the present experiments. Then, the most active strains, which degraded styrene at the highest rates, were selected using a shake flask experiment. While shake flask experiments were poorly reproducible, certain strains were selected for further study based on their generally high rates of styrene degradation. The highest metabolic activity towards styrene was observed in 4 strains, namely: No 11- *Streptomyces halstedii*, No- 19 *Bacillus megaterium*, No- 21 *Sphingobacterium spiritivorum* (*Flavobacterium*), and No- 31 *Bacillus cereus* (Fig. 3). The lowest activity was observed for strains Nos. 12, 24, 25, 28, and 29. All the tests were performed for no longer than 45 hours. A decrease in styrene concentration below its detectability threshold was observed in the most active strains within the range of 20-30 hours. The decrease in styrene concentration after a period of 27 hours was approximately 98% for strains 11 and 19. A 92% and 96% decrease in styrene concentration was recorded for strains 21 and 31, respectively, over the same time period.

A decrease in styrene concentration was also recorded in the control sample not containing the microorganisms, which suggested that some of the styrene underwent an abiotic process and some styrene may also have passed through the septum. If this effect was taken into account, the time required to lose styrene in particular samples would be longer.

A mixture of the active strains (No 11, 19, 21, 31) usually showed slightly higher activity than single strains under comparable conditions. Statistically, however, at usually used significance level 0.05 these differences were not important. Similar conclusions were drawn by Buitron and Gonzales [18] after studies on phenol compound degradation by activated acclimated sludge and isolated

from this sludge, and acclimated strains *Aeromonas* sp., *Pseudomonas* sp., *Flavimonas* sp., and *Chryseomonas* sp. The activated acclimated sludge was from one to two orders of magnitude faster than the pure strains, when biodegradation rate was calculated in terms of viable biomass.

In studies carried out by Braun-Lulleman [19] the metabolic activity of *Bjerkandera adusta*, *Trametes versicolor*, *Pleurotus ostreatus* (F6), *Pleurotus ostreatus* (G241), and *Phanerochaete chrysosporium* strains in relation to styrene was determined. The time needed for almost complete styrene decomposition was similar to that achieved in experiments described in the present paper and was within the range of 20-50 hours. Those authors observed that all strains, after 48 hours, almost completely degraded the styrene at 88 and 176 μM concentrations. A level of 352 μM was toxic for all strains except from *Pleurotus ostreatus*. In the case of the *Pleurotus ostreatus* (F6) and *Pleurotus ostreatus* (G241) strains, a complete styrene degradation at the two lowest concentrations occurred after 24 hours of incubation. At a 352 μM concentration, only the *Pleurotus ostreatus* (F6) strain was able to complete styrene elimination after 48 hours, while 10% of the styrene remained during the culture of the *Pleurotus ostreatus* (G241) strain. It is worth mentioning that 88 μM is only slightly higher than that applied in present flask experiment.

The activeness of the bacteria selected in such a way was unequivocally confirmed and quantitatively characterized in a gas wash bottle experiment. On the basis of chromatographic analysis results and data referring to the passing of styrene vapors and air through gas wash bottles as well as the volume of the suspension, the ability to eliminate the pollution (specific biodegradation rate), the gas wash bottle mass styrene load and the total biodegradation efficiency were calculated.

$$EC = \frac{G \cdot (C_1 - C_2) \cdot 10^{-3}}{V} \quad (1)$$

$$M = \frac{G \cdot C_1 \cdot 10^{-3}}{V} \quad (2)$$

$$S_u = \frac{(C_1 - C_2)}{C_1} \cdot 100 \quad (3)$$

where:

C_1 – inlet styrene concentration [mg m^{-3}];

C_2 – outlet styrene concentration [mg m^{-3}];

G – gas flow rate [$\text{m}^3 \text{s}^{-1}$];

V – volume of suspension [m^3];

M – gas wash bottle mass styrene load [$\text{g m}^{-3} \text{s}^{-1}$];

S_u – efficiency of biofiltration [%];

EC – elimination capacity, specific biodegradation rate [$\text{g m}^{-3} \text{s}^{-1}$] and [$\text{g m}^{-3} \text{h}^{-1}$].

Examples of data for two measurement days, selected

Table 1. Examples of results for styrene biodegradation in gas wash bottles on May 30th, 2003 and June 2nd.

Number of gas wash bottle	C_1 [mg m ⁻³]	C_2 [mg m ⁻³]	$G \cdot 10^6$ [m ³ s ⁻¹]	$M \cdot 10^4$ [g m ⁻³ s ⁻¹]	Su [%]	$EC \cdot 10^4$ [g m ⁻³ s ⁻¹]	EC [g m ⁻³ h ⁻¹]
	Date						
P1 (strain 11)	651.0	386.4	1.80	58.6	40.6	23.8	8.6
P2 (strain 19)		357.0	1.74	56.6	45.2	25.6	9.2
P3 (strain 21)		483.0	1.74	56.6	25.8	14.6	5.3
P4 (strain 31)		470.4	1.80	58.6	27.7	16.3	5.9
P1 (strain 11)	556.0	108.5	1.87	52.0	80.5	41.8	15.1
P2 (strain 19)		49.0	1.94	53.9	91.2	49.2	17.7
P3 (strain 21)		122.5	1.87	52.0	78.0	40.5	14.6
P4 (strain 31)		134.25	1.94	53.9	75.9	40.9	14.7

from the whole set of the results, are presented in Table 1.

After the initialization of measurements, a low efficiency of biodegradation processes was observed at the beginning; however, after 7 days it increased up to 100% for strains Nos. 19 and 21, and it was slightly lower for the two remaining ones. Good efficiency also was observed in the subsequent part of measurements, despite an increase in styrene concentration and gas flow. The replacement of the medium with a new one caused a significant increase in process efficiency, which proves the exhaustion of some components in the medium or an inhibiting effect of accumulating metabolites. This is clear from the data in Table 1 – the medium was replaced on May 30th, 2003. A maximum biodegradation rate in the suspension ($EC = 22.91 \text{ g m}^{-3} \text{ h}^{-1}$) was achieved on June 5th, 2003 at an inlet concentration of $C_1 = 722 \text{ mg m}^{-3}$ and a load of $70.03 \times 10^{-4} \text{ g m}^{-3} \text{ s}^{-1}$ for strain No 31. These values correspond to those achieved in other laboratories. For instance Neal et al.[20], using suspension reactors similar to gas wash bottles, achieved a biodegradation rate in the range of 4.3 to 29.2 $\text{mg dm}^{-3} \text{ h}^{-1}$ for mass loads from 4.4 to 30.2 $\text{mg dm}^{-3} \text{ h}^{-1}$.

Conclusions

1. The optimal styrene concentration for microorganism growth on mineral mediums was 1.5 ml/l. The introduction of an additional amount of styrene in a gaseous form caused an increase in microorganism count by over 30%.
2. Tests performed using the two methods revealed that among the 28 isolated strains, four of them were characterized by a high rate of styrene biodegradation: *Streptomyces halstedii*, *Bacillus megaterium*, *Sphingobacterium spiritivorum*, and *Bacillus cerus*. The highest rate was shown by *Bacillus cerus* ($EC = 22.91 \text{ g m}^{-3} \text{ h}^{-1}$ at $C_1 = 722 \text{ mg m}^{-3}$ concentration). The activity of a mixture of the above-mentioned strains did not significantly differ from that showed by individual strains.

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