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DEGRADATION OF BTEX BY MICROALGAE *Parachlorella kessleri*

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Abstract

Biological methods for wastewater treatment are becoming more accepted over the world. This study it was focused on the benzene, toluene, ethylbenzene and xylenes (BTEX) biodegradation under model conditions by *Parachlorella kessleri*. BTEX-mixture was added to the cultures as the sole carbon source, at concentration of 100 μg L^{-1}. It was observed loss of BTEX after 24, 48 and 72 h, respect-tively. Benzene and xylenes of 40 % was degraded within 48 h. The highest toluene degradation of 63 % was achieved. Only 30 % of ethylbenzene was degraded after 72 h. On the other hand the elementary analysis of algal biomass was assayed before and after the biodegradation process. After biodegradation the ratio C/N was increased 2.7 times from the value of 14.69 to 39.68. These results favor the biomass after biodegradation process and make it attractive for further use as a suitable substrate for subsequent processing into biofuels of third generation.

Keywords: algae; BTEX; biodegradation; environment; pollutants.

1. Introduction

Benzene, toluene, ethylbenzene and xylenes (BTEX) are monoaromatic hydrocarbons commonly found together in crude oil and oil products. These compounds are also produced as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics and synthetic fibers [1-2].

Nowadays they are important contaminants present in surface and ground waters, which usually originate from the leakage of underground petroleum storage tanks; spills at oil production, refineries, pipelines and distribution terminals; and industrial wastewaters [3]. Clean-up of the BTEX contaminated soil and groundwater is desirable in order to avoid public health hazards. Bioremediation, expected to be an economical, energy efficient and environmentally sound approach to other remediation processes such as chemical or physical ones has been developed as a soil and groundwater clean-up technique [4-5]. Under proper conditions, microorganisms are able to degrade all of the BTEX compounds and the use of bioreactors for water decontamination can be a feasible alternative [6-15].

The ability of BTEX degradation of certain microorganisms is known since 1908, when Störmer [16] observed the capacity of the bacteria *Bacillus hexabovorum* to grow aerobically in a medium containing toluene and xylene. The ability of natural microorganisms in the soil in BTEX degradation was first demonstrated by Gray and Thornton in 1928 [17]. Since then, several studies have been carried out in order to find out efficient microorganisms for BTEX degradation, so they could be used in environmental remediation for this mixture [18]. Lugowski *et al.* [19] developed and patented a process for the detoxification of liquid effluent streams. A mixture of thermophilic aerobic bacteria, comprising predominantly *Pseudomonas* spp., were used to degrade a wide range of aromatic hydrocarbons, such as phenols, toluene, aniline, benzothiazole, lindane (hexachlorocyclohexane), and their combination [20-22].

Under aerobic conditions, many substrate interactions have been observed during biodegradation of BTEX combinations. Abuhamed *et al.* [23] observed that the inhibition effect of toluene on benzene was higher than the inhibition effect of benzene on toluene. Moreover, the inhibitory effects of p-xylene on toluene and ethylbenzene degradation were much more pronounced than on other BTEX compounds. One possible reason may be due to the competitive metabolism in which p-xylene inhibited the utilization of toluene and ethylbenzene because of competition for the active binding site of an enzyme [24-27]. Chang and his co-workers investigated the substrate interactions of BTEX by a mixed culture isolated from a gasoline-contaminated site, and demonstrated that the simultaneous presence of benzene and toluene were degraded with a slight inhibitory effect on each other, ethylbenzene was the most potent inhibitor of BTEX degradation, the presence of p-xylene inhibited the degradation of benzene, toluene, and ethylbenzene, whereas the presence of either benzene or toluene enhanced the degradation of ethylbenzene and the xylenes [28]. Reardon *et al.* [29] found that toluene significantly inhibited the biodegradation rate of benzene by *Pseudomonas putida* F1. Not only bacterial but also algal cells are used in wastewater remediation systems. For instance, González *et al.* [30] achieved fixation rates of 3 g CO_2 L^{-1} d^{-1} in continuous pilot column photobioreactors. However, despite the merits of microalgae-based CO_2 capture technologies, process economics still require the use of low - cost photobioreactors (i.e. HRAPs- High Rate Algae Ponds) and a cheap nutrients source (i.e. wastewater). This suggests that CO_2 removal in photobioreactors should be coupled to wastewater treatment in HRAPs and that this combination could be indeed synergistic [31-32].

Microalgae enhance the removal of nutrients, organic contaminants, heavy metals, from domestic wastewater and furnish an interesting raw material for the production of high-value chemicals (algae metabolites). Photosynthetic oxygen production also reduces the need for external aeration, which is especially advantageous for the treatment of hazardous pollutants that must be biodegraded aerobically. Recent studies have therefore shown that when proper methods for algal selection and cultivation are used, it is possible to use microalgae to produce the O_2 required by acclimatized bacteria to biodegrade hazardous pollutants such as polycyclic aromatic hydrocarbons, phenolics, and organic solvents. In this work we used algal cells as an appropriate system for biodegradation of high toxic organic contaminates.

2. Experimental

2.1 Materials and methods

Biodegradation experiments

In this work the mixture of BTEX (each 100 μg L^{-1}) contained benzene purity standards (99 %), toluene (99 %), ethylbenzene (99.80 %) and xylene (99 %, mixture of isomers) (Supelco) were used for biodegradation assay. All chemicals used in this study were of analytical grade supplied by Merck (Germany), and the solutions were prepared using deionized water.

Parachlorella kessleri, strain LARG/1 (Botanical institute in Bratislava, Slovakia), was batch cultured in mineral medium (MM) containing the following stock solutions (ST): $(ST)_1$: NH_4Cl 15 mg L^1, $MgCl_2 \times 6H_2O$ 12 mg L^{-1}, $CaCl_2 \times 2H_2O$ 18 mg L^{-1}, $MgSO_4 \times 7H_2O$ 15 mg L^{-1}, KH_2PO_4 1.6 mg L^{-1}; $(ST)_2$: $FeCl_3 \times 6H_2O$ 64 mg L^{-1}, $Na_2EDTA \times 2H_2O$ 100 mg L^{-1}; $(ST)_3$: H_3BO_3 185 μg L^{-1}, $MnCl_2 \times 4H_2O$ 415 μg L^{-1}, $ZnCl_2$ 3 μg L^{-1}, $CoCl_2 \times 6H_2O$ 1.5 μg L^{-1}, $CuCl_2 \times 2H_2O$ 0.01 μg L^{-1}, $Na_2MoO_4 \times 2H_2O$ 7 μg L^{-1}; $(ST)_4$: $NaHCO_3$ 50 mg L^{-1}. Stock solutions were mixed in the ratio $(ST)_1$:$(ST)_2$:$(ST)_3$:$(ST)_4$ = 10 :1 :1 :1 and the volume was adjusted to 1000 mL, pH was modified to 7.4. Experiments were carried out in a sterile laminar box JOUAN MSC 12 (USA). The cell density of inoculum was 5×10^4 cells per mL. Cultivation was carried out in a chamber with a stabile temperature in the range of (22 ± 3)°C with four linear fluorescent bulbs (7400 lux; Testo 545, Germany).

The effect of BTEX mixture on the growth of *P. kessleri* was evaluated microscopically according the OECD test 201 [33] by direct cells counting using the Bürker grid. Erlenmeyer flasks (250 mL) with MM were inoculated with *P. kessleri* (10^4 cells per mL) and the mixture of BTEX (100 μg mL^{-1}) was added. Erlenmeyer flasks were sealed with cotton wool

and were cultivated under shaking by an orbital platform shaker GFL 3020 (Czech Republic) with the frequency of 120 min^{-1}, 72 h in order to assure aerobic conditions. The growth of *P. kessleri* was quantified until confluent growth microscopically (0, 24, 48, 72 h). All experiments were carried out in three parallels. The concentration of oxygen was measured by microoxymax system [34].

Determination of BTEX

The BTEX concentration was determined according to EN ISO 17025:2005. BTEX concentration was analyzed by gas chromatography (Purge & Trap Tekmar 3000 (U.S.A) with GC/FID detector). Samples (5 mL) under nitrogen stream ECD (Messer, Bratislava, Slovakia, flow rate of 40 mL min^{-1}) were withdrawn from the vial using purge and trap technique and were analyzed using gas chromatography (Varian 3400 CX ,U.S.A). Analytes were desorpted to column CP Select 624 CB (Varian U.S.A) with dimensions D (30 m) × ID (0.53 mm) × OD (30 mm). Chromatographic separation was performed with a temperature program: 50ºC (hold 1.00 min), 5ºC min^{-1} to 100ºC, 50ºC min^{-1} to 220ºC (hold 2 min) Injector Varian with temperature 100°C. Detector – FID (flame ionization detector) was operating at 300°C.

Because of the possibility of volatization of BTEX during the experiments, control experiments were carried out at the same operating conditions of the biodegradation experiment. Sterile controls were prepared by autoclaving cells for 30 min at 120°C to check the adsorption of BTEXs to the cells and a control of non-inoculated sterile MM was prepared to check the volatilization of BTEX during the experiment. The concentration of BTEX remained unchanged. The rate of BTEX degradation was calculated as a tangent of the degradation curve.

Elementary analysis of algae

Elementary analysis was carried out on a vario macro cube (Elementar Analysen Systeme GmbH, Germany). Working temperature of the combustion tube was 1150°C and that of the reduction tube was 850°C. Helium (99.996 %) was used as the carrier gas (120–125 kPa) at the flow rate of 600 mL min^{-1} and oxygen (99.995 %) (200.0 kPa) as oxidizing agent. Sulfanilamide was used as the standard for elementary analysis.

3. Results and discussion

First the effect of BTEX on the growth of *Parachlorella kessleri* was monitored. The growth curve with and without BTEX mixture (control) is shown on Figure 1. The growth of *P. kessleri* was minimally inhibited. The growth inhibition was observed after 48 h (13 %). It was reported that microorganisms were inhibited by higher concentration of formed metabolic intermediates like catechol or methyl catechol [23]. During the biodegradation experiments the concentration of BTEX in the mixture was monitored in three parallels. For sampling, aliquots of the culture medium were regularly taken by purge and trap technique for gas chromatographic (GC) analysis of BTEX (5 mL) during three consecutive days. Results of biodegradation are shown in Figure 2 and listed in Table 1.

Table 1 Results of biodegradation of BTEX compounds

Time (h)	Benzene		Toulene		Ethylbenzene		Σ Xylenes	
	BD (%)	R_d (μg L^{-1} h^{-1})	BD (%)	R_d (μg L^{-1} h^{-1})	BD (%)	R_d (μg L^{-1} h^{-1})	BD (%)	R_d (μg L^{-1} h^{-1})
24	40 ± 4		43 ± 6		50 ± 4		39 ± 5	
48	64 ± 5	0.63 ±0.15	63 ± 3	0.3±0.07	62 ± 7	0.49±0.17	56 ± 2	0.68±0.17

All the values are in mean (n = 3) ± S.D.,R_d – degradation rates; BD – biodegradation
Results of biodegradation are defined according to the relationship:
% BD = (C_0-C_d) / C_0 × 100; C_0 – Initial BTEX concentration (μg L^{-1}), C_d – BTEX concentrations (μg L^{-1}) after biodegradation.

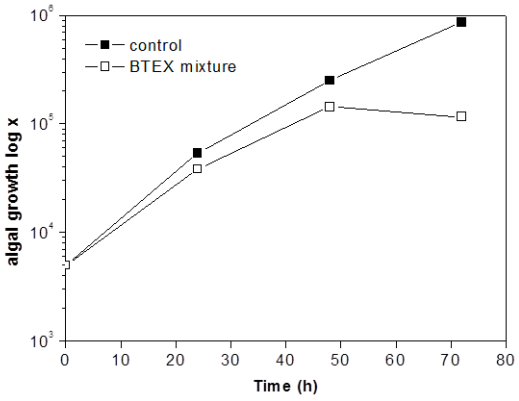

Fig. 1. The growth of *P. kessleri* in the presence of BTEX mixture

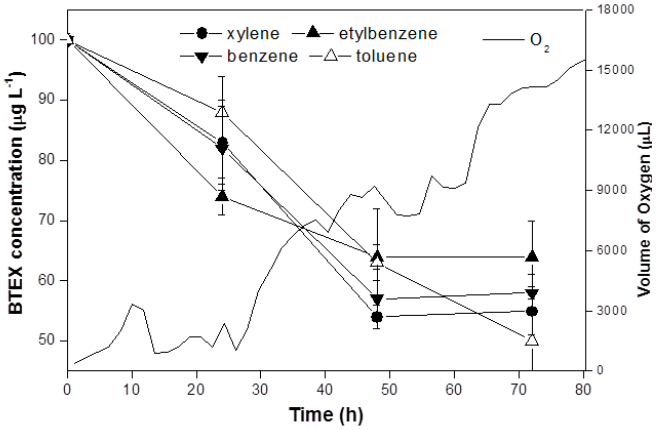

Fig. 2. The biodegradation of BTEX compounds with simultaneous oxygen detection

Since the biological test was carried out in a completely closed system, a decree of BTEX was caused exclusively by *P. kessleri* by consumption of BTEX as a sole carbon source. 40 % of benzene and xylene were degraded within 48 h with the rate of degradation 0.65 ± 0.15 µg L^{-1} h^{-1}. The fastest (0.73 ± 0.07 µg L^{-1} h^{-1}) and highest degradation of toluene (63 %) was achieved. The slowest biodegradation was observed in the case of ethylbenzene. The main part of all BTEX was degradates within 48 h. Between the 48–72 h only a minor decrease of BETX was observed (Figure 1).

It is extremely rare that a single microorganism is capable of completely degrading a pollutant or a mixture of xenobiotics. Under environmental conditions, the combined action of microalgae and other microorganisms might be a rather important process for the elimination of these undesired compounds from the environment. The degradation of pollutants under these conditions usually involves the combined actions of two or more microorganisms. In the case of algae, it is clear that although the complete degradation of aromatic pollutants is rare [35-36]. To higher degradation degree helps a two phase degradation system, but such conditions are hardly to design in the natural environment [23]. In natural conditions, organic compounds may undergo partial or complete degradation and can be volatilized. Most of the organic compounds appear to undergo some degree of transformation in cells before being sequestered in vacuoles or bound to cellular structures. Most of these compounds are metabolized, but only a few xenobiotics are mineralized. The xenobiotics can be oxidized by microsomal cytochrome P450. Other enzymes involved in xenobiotic metabolism in plants are peroxygenases, peroxidases, glutathione, S-transferases, carboxylasesterases, O-glucosyltransferases and N-malonyltransferases [37]. Two possible mechanisms for break of BTEX are known from the literature, an anaerobic pathway and the oxygenic pathway [35]. Presumably, the oxygen needed for mixtures of BTEX breakdown is likely provided by the photosynthetic activity of algae. In our work we have monitored the status of oxygen during the biodegradation process. In Figure 2 it is shown that the oxygen content increased while the biodegradation process. In biodegradation processes, aromatic compounds can be either electron donors or electron acceptors depending on the oxidation state of the pollutants.

Enzymes of both *orto* and *meta* cleavage pathways which are commonly found in bacteria were assayed spectrophotometrically using extracts of phenol-induced cells [38-39]. Axenic cultures of algae *Ochromonas danica* were found to cleave catechol in the 2-3 position resulting in the formation of 2-hydroxymuconic semialdehyde. The algal extracts also oxygenated 3-methylcatechol, 4-methylcatechol, 4-bromocatechol and 4-fuorocatechol to the corresponding ring cleavage products. Figure 3 shows the catabolic pathway substituted aromatic compounds in algal cells [40]. In aerobic degradation of aromatic compounds, well defined channels within the biodegradation pathways have evolved for most commonly encountered aromatic compounds. This evolution is not at all surprising in view of the vast turnover of aromatic compounds in the carbon cycle. Structurally diverse pollutants are first transformed into a few key intermediates through a number of peripheral pathways, which are then further channeled via a few central pathways to the central cellular meta-

bolism. Concerning that these aromatic rings are depredated with the same pathway it is clear that in the mixture they act as competitors. According our results toluene partly inhibits the degradation of benzene and xylenes and most inhibits the degradation of ethylbenzene. After the biodegradation process elementary analysis of dry algal cells was assayed. Based on the results of elemental analysis, it can be argued that the degradation of BTEX mixture increases the concentration of carbon in the dry algae from 19 to 41 % (Table 2).

Table 2 Outcomes of elementary analysis for used substrate.

Substrate	N (%)	C (%)	H (%)	S (%)
C. kessleri	1.29	18.49	10.40	0.82
C. kessleri after biodegradation	1.42	41.1	6.11	0.19

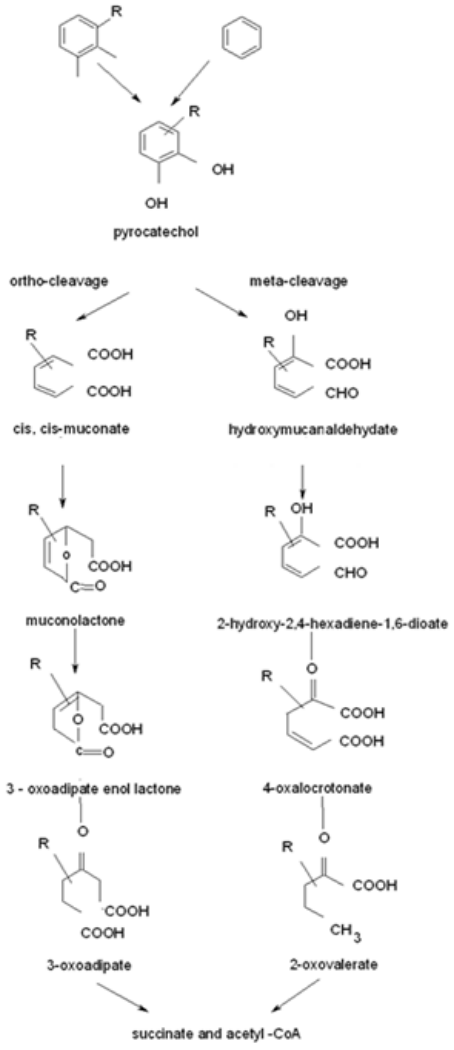

Fig. 3. The predicted scheme of BTEX biodegradation [35,40]

After the biodegradation process the ratio C/N was increased. These results favor this process and make it attractive for further use of algae as a suitable substrate for subsequent processing into biofuels of third generation.

4. Conclusions

Photosynthetic organisms transform the energy of solar photons into the free energy of chemical bonds that provide for nearly the entire energy supply of Earth's biosphere. This enormous capacity of algae to transform the solar radiation into energy-rich compounds and to remove CO_2 from the atmosphere justifies the current interest of science and

industry. In this work we have used algae as a model system for BTEX. Optimization of BTEX biodegradation in laboratory conditions provides an opportunity to obtain a high activity of the consortia able to biodegrade BTEX. The selected media should not contain simple chemical compounds (such as: lactate, ethanol and acetate) that could act as a potential carbon source for the algae, because they could greatly inhibit the biodegradation process through utilization of simple organic compounds as the source of carbon in the first place. The maximum degradation of 63 % was achieved in the case of toluene after 48 h. Utilization of the remarkable potential of algae to accumulate elements and compounds from the environment and perform novel biological transformation may be a novel way of cleaning up of the environment of toxic elemental and organic pollutants for sustainable development. Algae are considered as a potent source of biofuels of higher generation that will not compete for land with food production and that will contribute to biological capture of atmospheric CO_2 to mitigate the global climate change.

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