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# DECOLOURIZATION OF TRIPHENYLMETHANE DYES AND ECOTOXICITY OF THEIR END PRODUCTS

Common usage of dyes is always connected with pollution of surface water deposits. The goal of the research was to investigate the capability of bacteria and fungi as dyes decolourizing agents. The ecotoxicity of decolourization products was tested with *Daphnia magna* and *Lemna minor*. According to ACE 89/BE 2/D3 Final Report Commission EC, dye control samples were classified as toxic. High decolourization effectiveness was not always connected with detoxification. Crystal violet was the most difficult dye to remove (27–76% decolourization) and brilliant green, the easiest (62.5–88.6%). Thymol blue and cresol red were easily removed by fungi (>80%) and poorly by bacteria (<30%). Decolourization of these dyes was mostly connected with an increase of toxicity.

## 1. INTRODUCTION

Triphenylmethane (TPH) dyes are synthetic aromatic water-soluble organic colorants used by a wide number of industries [1]–[3]. The coloured effluents lead to pollution of surface water deposits. Dyes may affect photosynthetic activity in water because they reduce light penetration, cause deficiency of oxygen and deterioration of life conditions [4]. The majority of dyes are highly toxic and mutagenic. Synthetic origin and complex aromatic structure make them more resistant to biodegradation [1], [3], [5]–[7]. Bacteria and fungi are well known organisms with high potential for degradation of different pollutants, using different metabolic pathways. It is well documented that the effectiveness of degradation depends on the dye structure, concentration, adaptation of microorganisms, their activity and biomass concentration [7], [8]. Decolourization process of TPH is mainly based on sorption and/or biotransformation. The well known bacterial strains highly capable of colour removing are as follows: *Pseudomonas* sp., *Bacillus* sp., *Kurthia* sp., *Shewanella* sp., *Aeromonas hydrophila*, and *Desulfovibrio* sp. [1], [3], [7]. Various fungal strains, e.g. *Pseudozyma*

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*rugulosa*, *Candida krusi, Rhodotorulla* sp., *Phanerochaete chrysosporium, Funalia trogi, Coriolus versicolor,* and *Cyathus species*, can decolourize effluents as well [1], [3], [6], [9].

Biological processes are not always connected with detoxification of pollutants. Especially cleavage of aromatic rings can lead to products more toxic than initial compounds. Evaluation of the toxicity of TPH biotransformation products seems to be very important step before the application of any biotechnology to ensure environmental safety.

The goal of the research was to determine bacterial and fungal TPH-degradation and detoxification capabilities. Toxicity tests were done with *Daphnia magna* and *Lemna minor*.

## 2. MATERIALS AND METHODS

The triphenylomethane dyes used in this study can be itemized as follows: brilliant green (BG), crystal violet (CV), thymol blue (TB) and cresol red (CR). Bacterial strains used in the experiment were isolated from municipal sewage on the Bushnell–Hass medium with naphthalene as a carbon source (0.25% w/v). Four of the most active bacterial strains were used in experiment and identified according to API 20NE and API 20E as: *Chryseomonas luteola* (A3), *Pseudomonas aeruginosa* (B2), *Burkholderia cepacia* (B3). Strain A1 was not identified. Fungal strains were isolated by means of tissue culture method: *Polyporus picipes* (RWP17) and *Trametes versicolor* (L4), *Clitocybe dealbata* (RWP1) and *Morchella* sp. (SM).

Colour removal test was performed in tubes filled with 10 $cm^3$ of the Kimura medium [10]. The control samples and samples inoculated with 1 $cm^3$ of bacterial or fungal suspension were done in triplicate. Inoculum was prepared in physiological salt from 48 h bacterial slants and 72 h fungal cultures. Biomass samples were cultivated at 26 °C (bacterial biomass for 48 h and fungal biomass for 5 days). Two modifications were used: alive biomass for estimation of biodegradation effectiveness and autoclaved biomass (121 °C, 1.5 atm. for 20 minutes) for estimation of sorption effectiveness. Dyes were sterilized by filtration (millipore cellulose filters ∅ 0.20 μm) and then added to samples. Dye concentration in each sample was 0.05 $g/dm^3$.

After 7 days of experiment the samples were centrifuged (10000 rpm for 15 min) and the absorbance of the supernatant obtained was measured. The wavelength was experimentally determined with UV VIS Spectrophotometer (HP Diode Array Spectrophotometer 8452A) as the wave with maximal absorbance.

Both sorption ($S$) and dye removal by living biomass ($R$) were calculated according to the following formulae: $S[\%] = ((C - Sa)/C)\cdot 100\%$ and $R[\%] = ((C - Sna)/C)\cdot 100\%$; where $C$ – the concentration of dye in control sample (mg), $Sa$ – the residue

concentration of dye in autoclaved samples (mg), *Sna* – the residue concentration of dye in samples with living biomass (mg).

The zootoxicity test was done according to OECD 202 and that of phytotoxicity– according to OECD *Lemna* sp. growth inhibition test No. 221. $TU_a$ (acute toxic unit) was determined for both tests. Samples were classified according to ACE 89/BE 2/D3 Final Report Commission EC. The toxicity of natural bacterial and fungal products on the Kimura medium was also evaluated.

## 3. RESULTS AND DISCUSSION

Triphenylmethane dyes are resistant to biodegradation because of their aromatic structure and many functional groups. Metabolic pathways of dyes transformations may be characteristics of each group of microorganisms. Sometimes biological processes are connected with formation of toxic intermediates. Environmental safety demands both pollutant removal and their detoxication. The valuation of decolourization effect should be related to ecotoxicity assessment. By now most of the decolourization projects were concentrated mainly on colour removal.

Decolourization effectiveness as well as toxicity of the dyes studied and the final products of their biotransformation depended on the kind of the strain used and on the structure of the dye tested (figures 1–4 and the table). Such corelations are well documented in literature [1], [11]–[16].

Table

Effectiveness of bacterial and fungal decolourization

| Strain | *R* (%) | *S* (%) | *R* (%) | *S* (%) | *R* (%) | *S* (%) | *R* (%) | *S* (%) |
|---|---|---|---|---|---|---|---|---|
| | Crystal violet | | Brilliant green | | Thymol blue | | Cresol red | |
| A1 | 26.8±0.4 | 18.5±1.2 | 79.3±2.1 | 14.4±0.5 | 4.6±0.3 | 0 | 15.2±3.3 | 10.2±0.1 |
| A3 | 42.0±1.1 | 7.2±0.9 | 88.6±1.0 | 50.2±4.2 | 29.1±0.1 | 0 | 18.4±2.2 | 12.9±0,4 |
| B2 | 40.5±0.9 | 28.2±0.4 | 85.1±2.6 | 13.4±2.2 | 17.7±1.8 | 0 | 26.4±0.2 | 22.9±0.6 |
| B3 | 30.3±3.1 | 7.6±0.2 | 79.9±0.4 | 0.7±0.5 | 2.7±0.1 | 0 | 0.2±0.0 | 0.2±0.0 |
| RWP1 | 72.4±0.4 | 70.4±5.1 | 72.1±0.9 | 48.1±1.4 | 94.0±0.2 | 90.6±2.1 | 74.8±6.1 | 70.6±0.2 |
| RWP17 | 75.9±2.2 | 59.7±7.4 | 62.5±1.1 | 53.1±0.2 | 95.1±0.8 | 89.7±2.5 | 89.2±0.7 | 76.8±4.1 |
| SM | 73.2±8.1 | 68.3±3.2 | 76.6±0.7 | 71.1±7.4 | 96.2±0.2 | 93.0±0.1 | 74.5±0.2 | 64.4±2.2 |
| L4 | 64.0±2.2 | 61.4±1.2 | 75.8±0.3 | 42.0±1.1 | 31.4±7.1 | 31.4±3.4 | 97.9±2.2 | 95.5±0.7 |

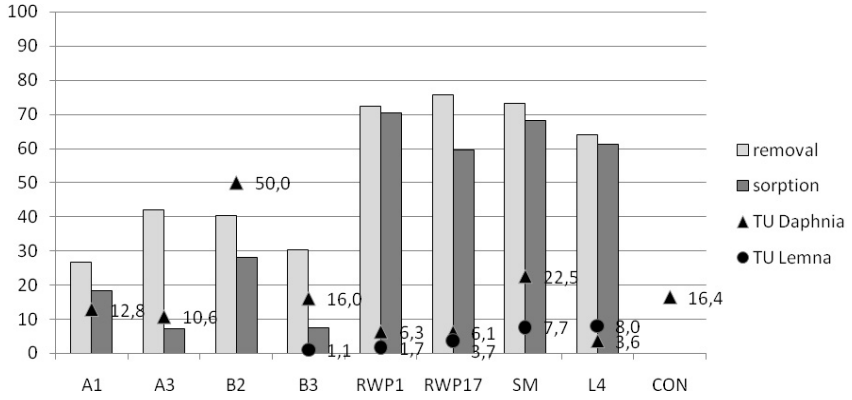


Fig. 1. Removal of crystal violet by bacterial and fungal strains and TU value for *Daphnia magna* and *Lemna minor*

Dye decolourization by microorganisms may take place in two ways: either by adsorption or degradation. Sorption is frequently the first step of biodegradation process. Visual changes of the biomass colour can suggest what is the way of decolourization [12], [14], [15], [17]. Decolourization effectiveness and toxicity of crystal violet samples with bacteria and fungi are shown in figure 1. Higher elimination of crystal violet was observed in the samples with fungal strains. This removal ranged from 64% for strain L4 up to 75.9% for strain RWP17. Sorption was also high: from 59.7 (RWP17) up to 70.4% (RWP1). Strain RWP17 biodegraded CV with the highest efficiency (75.9%). Removal of CV by bacterial strains was lower (26.8–42%) but their contribution to biological processes was higher than that observed in fungal samples. Crystal violet is cytotoxin and mitotoxin commonly used in medicine [3], but there is little description of the properties of the metabolic products of this dye. The most effective were strains: A3 – *Chryseomonas luteola* (42% removal and only 7.2% sorption) and B2 – *Pseudomonas aeruginosa* (40.5% removal and 28.2% sorption). Also *Burkholderia cepacia* (B3) fairly effectively removed CV, and sorption constituted only 7.2% of its removal. TU values after the process differed, depending on the strain tested. Bacterial and fungal strains did not produce toxins on a pure Kimura medium. Bacterial products of decolourization proved to be zootoxic. The class of toxicity of the control sample either with dye or with bacterial intermediates was the same (IV class). The intermediates of fungal strains were less toxic than dye (except SM strain) which manifested itself as the change of toxicity class from IV to III. Decolourization products of bacterial strains (except B3 intermediates) as well as the control sample with dye were not toxic to *Lemna minor*. The phytotoxicity of fungal samples increased, hence they could be found in the III class of toxicity, which was probably connected with the mouldling of *Lemna minor*. In this case, it is impossible to properly estimate the toxicity of intermediates.

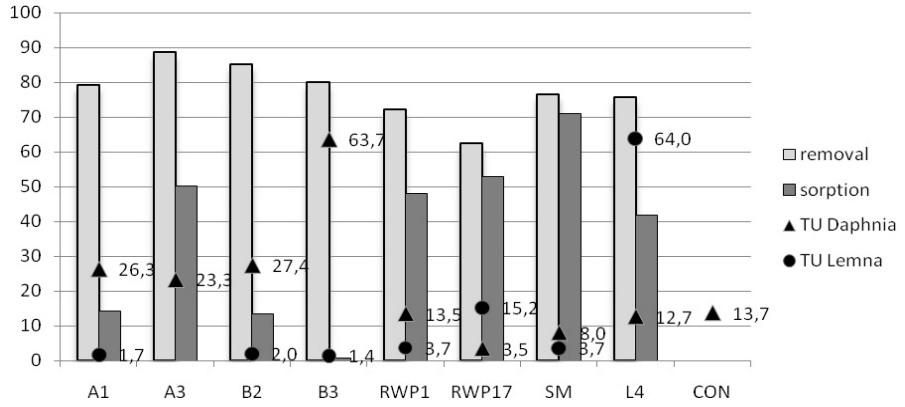


Fig. 2. Removal of brilliant green by bacterial and fungal strains and TU value for *Daphnia magna* and *Lemna minor*

Brilliant green removal and changes in toxicity of samples are shown in figure 2. Decolourization effectiveness was better for bacterial strains, but high BG removal was connected with an increase in sample zootoxicity (TU=13.7 in control and 23.3 for A3 and 63.7 for B3). The control sample and the bacterial samples were in the same class of toxicity (IV). The samples with bacteria were phytotoxic (III class of toxicity), apart from the sample with A3 strain whose toxicity was the same as that of the control with dye. *Chryseomonas luteola* and *Pseudomonas aeruginosa* were again the most effective in BG removal which ranged from 79.3% (A1) up to 88.6% (A3). Sorption was not practically observed for B3 strain (0.7%) and was the highest for A3 (50.2%). The B2 and B3 strains made the greatest contribution to biological BG decolourization (72.3 and 79.2%, respectively). Impressive results were also obtained by other authors for *Bacillus subtilis*, *Aeromonas hydrofilla*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas cepacia* [11]. KUMAR SANI [1] reported that the more complex the chemical structure of dye, the lower the rate of TPH removal by *Kurthia* sp. In samples with the dyes concentration of 10 μM, after 30-min incubation a colour removal ranged from 8% for structurally complicated ethyl violet to 100% for structurally simple brilliant green.

In fungal samples, as in the case of crystal violet, highly effective sorption of BG and a decrease in sample zootoxicity were observed. Two strains were active in sample detoxification (TU for RWP17 was 3.5 and for SM was 8.0 – III class of toxicity). The phytotoxicity of fungal samples was high (III class) and reached even IV class for strain L4. The most active in colour removal were strains SM and L4 (76.65 and 75.8%, respectively) and the least effective was strain RWP17 (62.5%). A significant difference in a colour removal due to sorption and biological removal was observed only for strain L4 (33.8%). Numerous white rot fungi were highly effective in the removal of dyes. A special attention is paid to the processes of the white rot of wood

carried out by *Pchanerochaete chrysosporium* and *Trametes* sp. [5], [6], [14], [15], [17]–[20]. *P. chrysosporium* tested by RADHA [17] removed 95% of methyl violet during 120 h. Six of seven fungal strains tested decolourized 99–100% of brilliant green during one week. TYCHANOWICZ [21] proved that *Pleurotus pulmonaris* is able to remove 93% of methyl violet for 6 days. The process conditions affect the activity of ligninolytic enzymes, hence they determine its efficiency [5], [17], [20], [21].

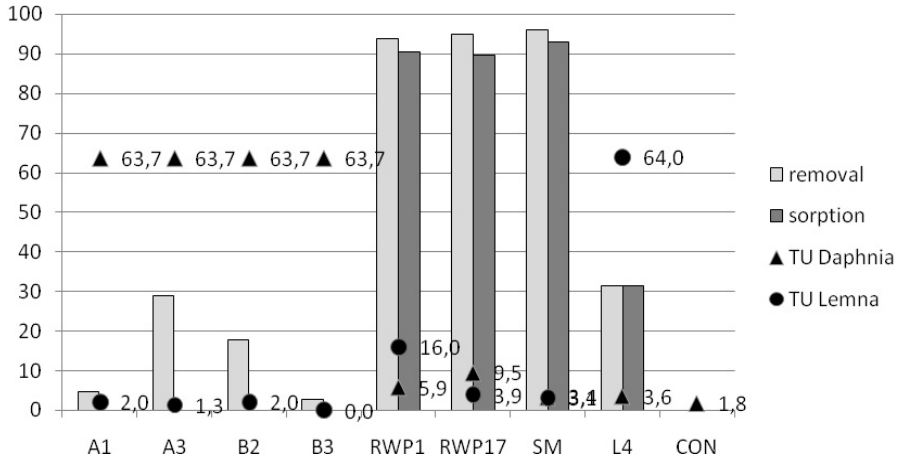


Fig. 3. Removal of thymol blue by bacterial and fungal strains and TU value for *Daphnia magna* and *Lemna minor*

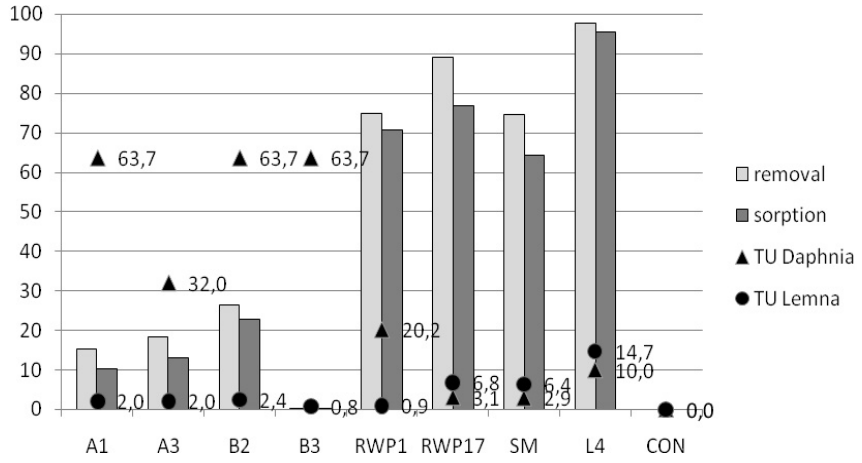


Fig. 4. Removal of cresol red by bacterial and fungal strains and TU value for *Daphnia magna* and *Lemna minor*

Thymol blue (figure 3) and cresol red (figure 4) were removed more effectively by fungal strains than by bacterial strains. Fungal strains had a great ability to sorpb dyes. The highest delcolourization of thymol blue was in the samples with strains RWP1, RWP17 and SM (about 95%), while sorption reached 89.7–93%. Only the strain L4 was not so active (31.4% removal). The zootoxicity of process products was not higher than that noticed in the control with dye. Finally the class of toxicity was the same (III). High phytotoxicity was observed for strain RWP1 and again for L4 (IV class in contrast to non-toxic control). The effectiveness of bacterial decolourization was very low. No dye removal by strains A1 and B3 was observed (4.6 and 2.7%, respectively). Strains A3 and B2 eliminated 29.1% and 17.7% of colour, respectively. The dye tested was removed only by biodegradation (sorption was not observed). Biological processes increased the sample toxicity to *Daphnia magna* (from III class in control to IV class of toxicity in samples with bacteria) as well as to *Lemna minor* (from I to III class of toxicity).

Cresol red was removed mainly by sorption. Decolourization was higher in the samples with fungal strains than in those with bacterial ones. The most effective was L4 strain (97.9%) and the lowest removal was observed in the samples with strains RWP17 and SM (about 75%). The removal by sorption was similar to the removal by living biomass. After the end of experiment the toxicity of the samples with fungi was higher than that of the control with dye. An increase of toxicity was observed also for bacterial strains. In the test with *Daphnia magna* and in the test with *Lemna minor*, IV and III/II classes of toxicity were obtained, respectively. Strain B3 did not remove CR. The highest decolourization was observed in the samples with strain B2 (26.4% removal and 22.9% sorption). The differences between the effectiveness of sorption and biological removal were not signifficant, but changes in sample toxicity can suggest that a real contribution of biodegradation to CFR removal is bigger than can be calculated from these values.

The effectiveness of colour removal for all dyes tested was connected with dye structure and the presence of functional groups. In the case of cresol red, sulphonic group could be responsible for its lower biodegradability. The influence of dye structure on decolourization effectiveness was proved by AN [12]. The bacterial strain tested, i.e. *Citrobacter* sp., removed faster and more effectively structurally simpler crystal violet and methyl red than more complicated genthian violet, malachit green and brilliant green. Similar dependence was also reported by TYCHANOWICZ [21], SHARMA [16], JANG [13] and KUMAR SANI [1].

## 4. SUMMARY

High effectiveness of dye decolourization was not always connected with a reduction of dye toxicity. Crystal violet was the most resistant dye (its decolourization was

on the level of 27–76%). A lower removal of CV was associated with a decrease in sample toxicity. High decolourization of brilliant green by bacteria (79.3–88.6%) could be related to toxicity increase. Lower effectiveness of fungal process (62.5–76.6%) was connected with toxicity decrease. Thymol blue and cresol red turned out to be resistant to degradation by bacteria (<30% removal). The transformtion of these dyes led to an increase in sample toxicity. Fungal strains decolourized both dyes with high effectiveness (>80%) connected with the rise of toxicity.

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## DEKOLORYZACJA BARWNIKÓW TRÓJFENYLOMETANOWYCH I OKREŚLENIE EKOTOKSYCZNOŚCI POWSTAŁYCH PRODUKTÓW

Powszechne stosowanie barwników prowadzi do zanieczyszczenia wód powierzchniowych. Celem badań było określenie możliwości dekoloryzacji barwników przez bakterie i grzyby. Ekotoksyczność produktów dekoloryzacji badano na *Daphnia magna* i *Lemna minor*. W oparciu o ACE 89/BE 2/D3 Final Report Commission EC próbki kontrolne z barwnikiem zaklasyfikowano jako toksyczne. Wysoka efektywność usunięcia barwnika nie zawsze wiązała się z detoksykacją próbek. Barwnikiem najtrudniej usuwanym był fiolet krystaliczny (usunięcie barwy 27–76%), a najłatwiej – zieleń brylantowa (62.5–88.6%). Błękit tymolowy i czerwień krezolowa były łatwo usuwane przez szczepy grzybowe (>80%), słabo natomiast przez bakterie (<30%). Dekoloryzacja tych barwników wiązała się ze wzrostem toksyczności.