ARCHITECTURE CIVIL ENGINEERING ENVIRONMENT
The Silesian University of Technology
No. 2/2020

doi: 10.21307/ACEE-2020-019

ISOLATION AND SCREENING OF BACTERIA WITH ABILITY TO DECOLORIZE SELECTED SYNTHETIC DYES – PRELIMINARY RESULTS

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Received: 31.01.2020; Revised: 22.04.2020; Accepted: 22.04.2020

Abstract
Commonly used synthetic dyes cause serious problems with their efficient removal from sewage. The bioaugmentation of sewage treatment systems with highly decolorizing bacteria may be a solution. The aim of the study was the screening of bacteria with high ability to remove synthetic dyes (brilliant green (BG), crystal violet (CV), erythrosine (Er). The bacteria were isolated from municipal sewage, compost and rotten beech wood. Mineral and nutrient solid growth media supplemented with dyes (BG or EB) at a concentration 0.1 gL^{-1} were used. At second stage of screening the liquid nutrient broth supplemented with one of dye (BG, CV or Er at concentration 0.1 gL^{-1}) was used. The contents of dyes in samples (after 96 h) were measured spectrophotometrically. The largest number of decolorizers were obtained from wastewater, then from compost and the rotten wood. In the case of BG and CV even small differences in the structure of the molecules affect the results of dyes removal. The structurally simpler BG was definitely better removed than CV. The results of the removal of Er were worse than BG but better than CV. Bacteria isolated at mineral medium removed dyes with higher efficiency.

Keywords: **Bacteria; Brilliant green; Crystal violet; Decolorization; Erythrosine; Fluorone dyes; Triphenylmethane dyes; Screening; Synthetic dyes.**

1. INTRODUCTION

The low costs of production, the wide spectrum of colors and the durability of coloration caused increase of synthetic dyes production and the limitation and even elimination of usage of natural dyes in most of branches of the economy [1, 2, 3]. The triphenylmethane dyes are derivatives of methane and are one of the groups of most used synthetic dyes. They are applied mainly at textile industry (for dyeing the wool, silk, cotton, nylon), also at leather, paper, plastic, food industry and at analytics and medicine sector [1, 3, 4, 5, 6, 7, 8, 9, 10]. Erythrosine is a fluorone dye which has not toxic properties. In some countries it is permitted to use it for dyeing a food, cosmetics and sometimes pharmaceuticals [11, 12, 13]. Complicated chemical structure of this substances make them resistant for biological decomposition. Such properties cause the problems with efficient removal of them from dyed sewages. A great amount of this chemicals are toxic, mutagenic, carcinogenic, allergenic and biocidal [1, 10, 14, 15, 16, 17]. The problems with removal of dyes from sewages by conventional methods, the widespread use and annually increasing amount of their consumption promote the accumulation of these xenobiotics mainly in surface water [1, 10, 14, 15, 16, 17]. The effect is the deterioration of the physico-

chemical and biological properties of aquatic ecosystems. Direct and indirect exposure of organisms of all trophic levels and human being to these substances increases. Removal of synthetic dyes from sewages is carried out by physical and chemical methods (e.g. flocculation, coagulation, sorption, precipitation, oxidation, membrane technics etc.). Despite the high efficiency, these methods are not commonly used because of high costs and generation of large amounts of the after-process sludges, which are hazardous wastes difficult to manage [16, 18]. Most commonly used biological methods of treatment of colored wastewaters are conducted at different conditions by different organisms including microorganisms [2, 3, 5, 6, 18, 19, 20, 21, 22]. Biological technologies are environmentally friendly and cost effective. A main problem at treatment of colored wastewaters is their complex, variable composition and often toxic properties, so the conventional biological techniques as activated sludge show lower than expected efficiency. A suitable composition of microbiocenosis, adapted for such kind of sewages is very important for an efficient process. The selection and multiplication of adapted microflora for the purpose to creating biopreparation for bioaugmentation of treatment systems may be an interesting solution [4, 16, 18, 23, 24, 25, 26, 27, 28]. The most popular sources of obtaining decolorizing bacteria are industrial sewages, especially textile wastewater [8, 17, 24]. The aim of the study was to show if other source materials may be used for izolation the bacteria with high abilities of decolorization of synthetic dyes. The screening was led for decolorization triphenylmethane brilliant green (BG) and crystal violet (CK) and fluorone erythrosine (Er).

2. MATERIALS AND METHODS

2.1. Dyes used in study

For the screening four synthetic dyes belonging to three chemical groups were used:

– triphenylmethane dyes – brilliant green (BG) and crystal violet (CV);

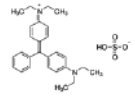
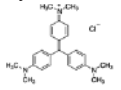
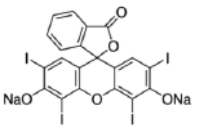
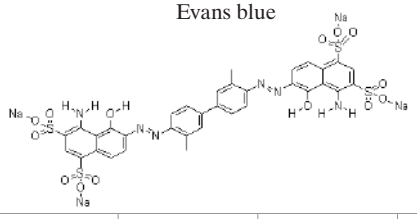
– azo dye – Evans blue (EB);

– fluorone dye – erythrosine (Er).

All dyes were produced by Sigma Aldrich.

The spectral properties of dyes, including the wavelength corresponding to the maximum absorbance, were determined experimentally using the Hitachi 1900 UV-VIS spectrophotometer. The characteristics of all used dyes are shown in Table 1.

Table 1.
Dyes used at the experiment – characterization (based on [29])

Triphenylmethane dyes			
Brilliant green 			
Molecular formula: $C_{27}H_{34}N_2O_4S$	Molecular weight: 482.63 g/mol	Absorbance – λ max.[nm]: 624	Nr C.I.: 42040
Crystal violet 			
Molecular formula: $C_{25}H_{30}N_3Cl$	Molecular weight: 407.98 g/mol	Absorbance – λ max.[nm]: 590	Nr C.I.: 42555
Fluorone dyes			
Erythrosine 			
Molecular formula: $C_{20}H_6I_4Na_2O_5$	Molecular weight: 879.84 g/mol	Absorbance – λ max.[nm]: 527	Nr C.I.: 45430
Disazo dyes			
Evans blue 			
Molecular formula: $C_{34}H_{24}N_6Na_4O_{14}S_4$	Molecular weight 960.79 g/mol	Absorbance – λ max.[nm]: 606	Nr C.I.: 23860

Aqueous solutions of dyes (BG, CV, EB, Er), prepared in sterile distilled water were mechanically sterilized (cellulose syringe filters with the pore diameter 0.2 μm) before usage. After sterilization the real concentration of dyes in aqueous solutions was estimated (spectrophotometer UV-VIS Hitachi 1900). The solutions of dyes were used for supplementation of solid and liquid bacteria growth media. The initial concentration of dyes in samples was at the level 0.1 gL^{-1}.

2.2. Bacteria growth media

The culture of bacteria was carried out on the solid and liquid growth media. Two solid growth media,

different in compositions were used for isolation, purification of strains and confirmation of their decolorization capabilities. Nutrient agar (BTL) and minimal mineral medium MM were used (composition gL^{-1}: 1.75 g K_2HPO_4; 0.75 g KH_2PO_4; 0.025 g $MgSO_4x7H_2O$; 0.25 g $(NH_4)2SO_4$; 20 g agar-agar). Before using the growth media were autoclaved (121°C, 15 min.). The sterile media were supplemented with the solutions of BG or EB (dye concentration in medium 0.1 gL^{-1}). At the second stage of the experiment the sterile (autoclaved at 121°C for 15 min.) liquid nutrient broth (BTL) was used.

Dilution of source materials and preparation of bacteria suspensions were done in the sterile physiological salt (0.85% water solution of NaCl). For storage and multiplication of bacteria the nutrient agar slants (BTL) were used.

2.3. Preparation of source materials and isolation of decolorizing bacteria

The main criterion for the choose in materials for isolation of bacteria strains was expected presence of microorganisms active at decomposition of the complex organic matter. The used source materials were:

– municipal sewages (aeration chamber of a wastewater treatment plant in Bytom in Silesia region, south Poland) (S);
– compost from household composter (K);
– rotten beech wood (collected from the local forest) (P).

The compost and the rotten wood were pretreated (after grinding they were sieved through 2 mm mesh sieves).

The solutions of raw materials (sewages, compost, rotten wood) were prepared in physiological salt (ranged: sewages and compost 10^{-1}–10^{-8}; rotten wood 10^{-1}–10^{-5}). Diluted samples were sieved by the spread plate method on solid media at two series:

– series 1 – cultivation on nutrient agar supplemented with BG or EB;
– series 2 – cultivation on MM agar supplemented with BG or EB.

Bacteria were cultured at temperature 26°C for 96 h. For further experiment bacteria strains were chosen grown as colonies with the decolorized area around them and/or having strongly dyed biomass what pointed on the dye sorption process. Chosen strains were purified and checked in terms of their decolorization abilities (on growth media supplemented with BG or EB) and then they were multiplied on agar slants.

Obtained strains were signed with codes, which among others define the source of obtaining, kind of medium and the strain number:

– S, K, P – bacteria strain obtained from sewage (S), compost (K) and rotten wood (P);
– Z, B – the dye used for supplementation of the medium from which the strain was obtained: Z – brilliant green, B – Evans blue;
– number – ordinal number signed every next isolated strain;
– m – if it is present in the code it means that bacteria strains were obtained from MM medium.

2.4. Decolorization potential of obtained bacteria strains

Abilities of decolorization of three synthetic dyes (two triphenylmethane dyes: brilliant green (BG), crystal violet (CV) and fluorone erythrosine (Er)) by isolated bacteria were estimated. Decolorization experiment was conducted on liquid medium (10 ml of medium in each tube). Each strain was tested in three series (BG, CV, and Er) in four replications. For each series the control series without biomass were performed. Samples were inoculated with the bacteria suspensions prepared in physiological salt from 48 hours aged biomass. The optical density of all biomass suspensions was estimated by the McFarland scale and was set at the level of $15x10^8$ cfu/ml. Tubes with the sterile liquid media were inoculated with 0.1 ml of given bacteria suspension. Samples were incubated at temperature 26°C for 48 h in the aim of multiplication of biomass. The next step was the introduction into tubes the sterile water solutions of dyes (BG, CV or Er)) in volumes which allowed to obtain the initial dyes concentration in samples on level 0.1 g/l. The samples with dyes were incubated at temperature 26°C for 96 h. After every 24 h the visual estimation of changes were done and in decolorized samples the percentage removal of dyes were spectrophotometrically estimated. The rest of the samples were incubated maximum up to 96 h and then measured.

In order to determine the dyes removal, the samples were centrifuged (5000 rpm/10 min). The dyes concentrations were estimated in obtained supernatants spectrophotometrically (spectrophotometer UV-Vis Hitachi U-1900) at a proper wavelength (BG – 624 nm; CV – 590 nm; Er – 527 nm). The dyes con-

centrations in samples were calculated based on performed calibration curves. The percentage removal of dyes was calculated by the formula (1):

$$R\% = (K\text{-}B/K) \times 100\% \quad (1)$$

where:

R% – percentage removal of dye [%];

K – concentration of dye in control sample (sample without biomass) [mg/l];

B – concentration of dye in sample with bacteria biomass [mg/l].

3. RESULTS AND DISCUSSION

A complex and variable composition of colored sewages (for example from textile industry) make them difficult for treatment. The efficiency of treatment of such wastewater at biological systems depends on many factors. Besides the conditions of process the most important factor is the presence of microorganisms with high potential of adaptation to relative changeable habitat conditions [1, 14, 16, 17].

Particularly important is the presence of microflora showing the ability to decompose a wide spectrum of structurally complex organic compounds. Such properties usually are associated with the ability to produce enzymes with low substrate specificity. The changeable composition of colored wastewater causes that the microorganisms selection taking place in the treatment system may be too slow. Supporting such systems with properly developed biopreparations containing appropriately selected microorganisms is still a promising method of improving their effectiveness [16, 18, 23, 24, 26, 27].

The aim of conducted screening was to obtain the bacteria strains with abilities of efficient removal of different synthetic dyes (triphenylmethane brilliant green and crystal violet and fluorone erythrosine). The decolorizers of synthetic dyes are most often obtained from industrial sewages, especially from textile wastewater [8, 17, 24, 30].

3.1. Decolorization potential of bacteria isolated from different materials

The screening studies showed that compost, rotten wood as well as municipal sewages may be a proper sources for obtaining the bacteria strains able to decolorize the synthetic dyes. All used materials had the common feature – the complex composition and the ongoing biological decomposition processes.

The highest efficiency of removal of the used synthetic dyes was reached in the case of triphenylmethane brilliant green (BG) (Fig. 1–3). The most numerous group of bacteria with abilities to decolorize BG was obtained from sewage - 18 strains (Fig. 1), then from compost – 9 strains (Fig. 2), and from rotten wood – 6 strains (Fig. 3). The BG removal efficiency higher than 50% after 96 h of incubation was observed in the case of 83% of strains from sewage, 78% of strains from compost and 100% of strains from rotten wood. The removal of BG higher than 80% was obtained in the case of 50%, 56% and 66% of strains respectively and respectively 39%, 11% and 33% of them reach this level already after 48 h of culture. The average removal of BG by the bacteria strains isolated from the sewage was on the level of 76.9%, from the compost 71.6%, from the rotten wood 83.2%.

It is noteworthy that the highest efficiency of decolorization of BG was observed in the case of the strains isolated from rotten wood. It may be connected with the complexity of this substrate (polymers, mainly cellulose and lignin) what requires the production of special groups of enzymes necessary for utilization of such complex substrates, what probably allowed to decompose also the complex aromatic structure of BG.

The second studied triphenylmethane dye was crystal violet (CV). The much worse results of the bacterial decolorization of this dye than BG (Fig. 1–3) were probably connected with the differences in chemical structure between them. More structurally complex crystal violet has in molecule six methyl groups bonded with the three phenyl rings, while the brilliant green has four methyl groups bonded with the two phenyl rings (Tab. 1).

It was proved by the other authors, that the dyes' biodegradability decreases with the increasing complexity of their chemical structure, which is connected among others with the presence of a higher number of functional groups in the molecule [17, 25, 28, 31, 32].

The best results of decolorization of crystal violet were achieved by the strains isolated from sewages (Fig. 1) then from rotten wood (Fig. 3) and compost (Fig. 2). After 96 h of incubation the average decolorization of CV was 29.9%, 25.9%, 12.0% respectively. Only 22% of strains isolated from sewages were able to remove more than 50% of CV. It is noteworthy, that all of these strains showed also the highest efficiency of decolorization of BG and additionally they were obtained from cultures on MM medium where the dye was the only source of carbon and energy. The removal of CV higher than 80% was

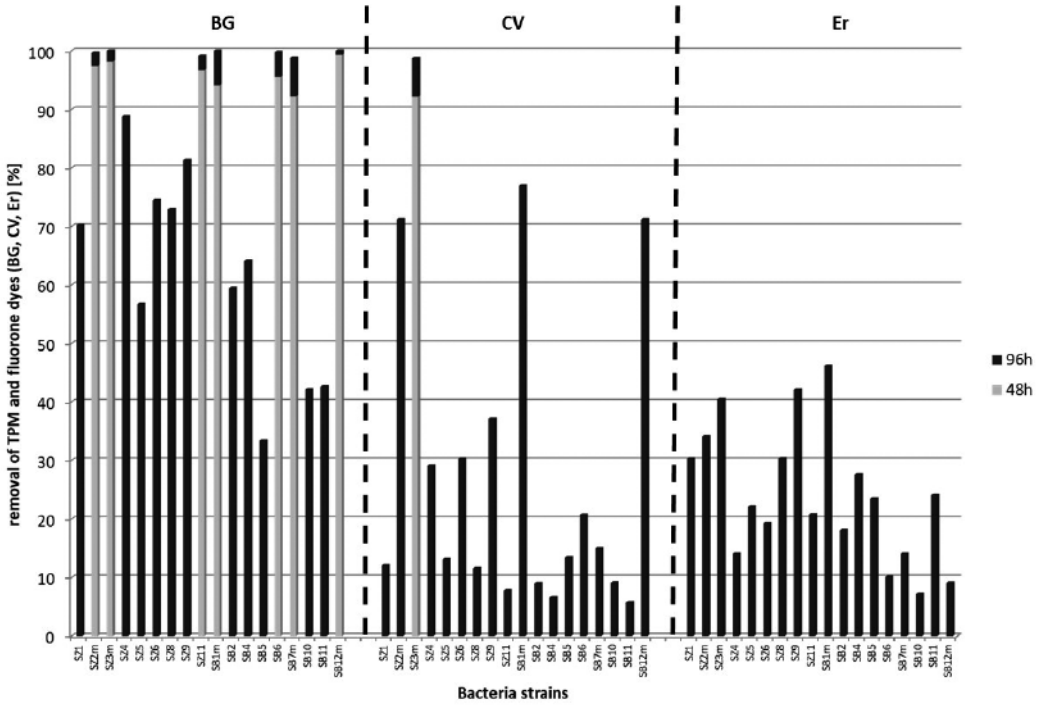


Figure 1.
Decolorization potential of bacteria strains isolated from sewage

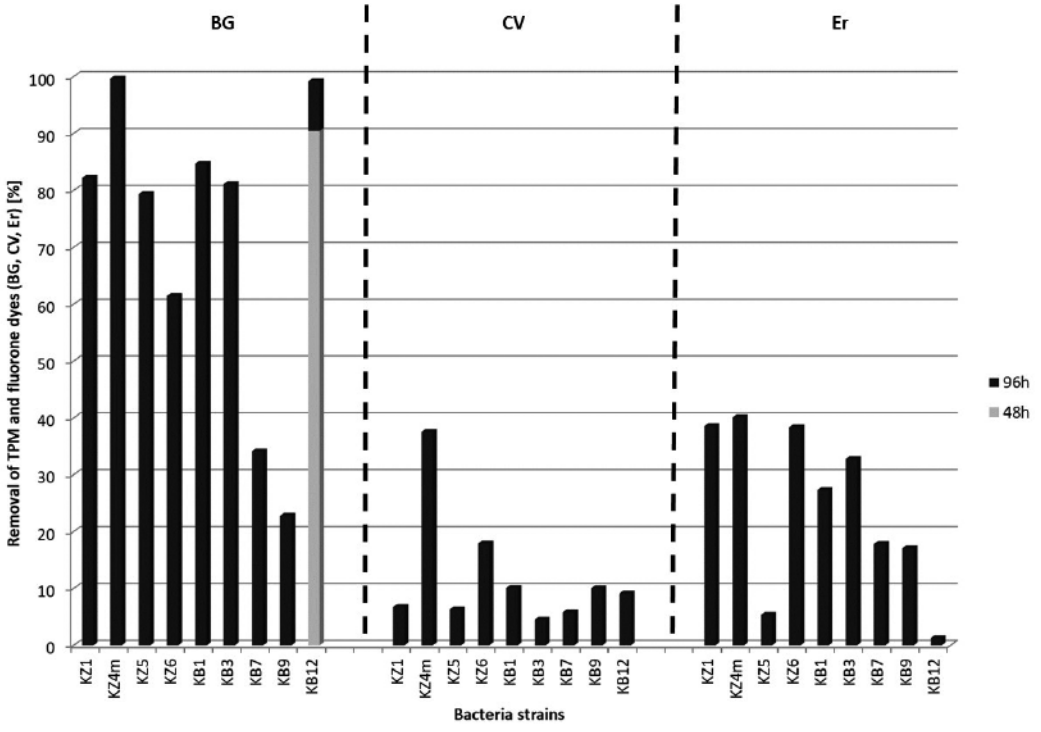


Figure 2.
Decolorization potential of bacteria strains isolated from compost

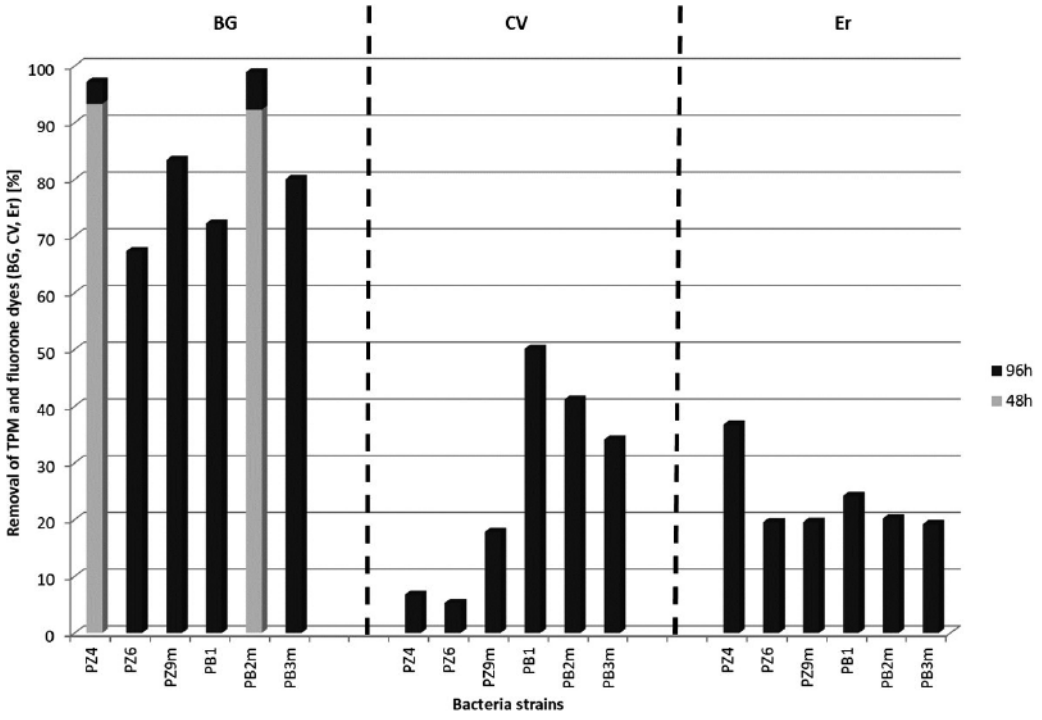


Figure 3.
Decolorization potential of bacteria strains isolated from rotten wood of beech

observed only in the case of one strain (Sz3m) isolated on MM medium, which removed 92% of this dye already after 48 h of incubation (similarly in the case of BG – the removal of 98% after 48 h). None of the strains isolated from the compost and rotten wood removed 50% of CV after 96 h.

The fluorone erythrosine (Er), as well as CV, was more difficult for removal than BG. After 96 h of incubation, the average results of decolorization of Er by the strains isolated from sewage, compost and rotten wood were comparable and were on the level of 24.1%, 24.3%, 23.2% respectively. None of strains removed 50% of this dye from solutions.

3.2. Decolorization potential of bacteria isolated on different growth media

One of the selective factors applied at screening studies was the composition of the growth media used for isolation of the bacteria strains. Both of the used growth media were supplemented with synthetic dyes (BG or EB). One of them was a nutrient agar – rich in easily bioavailable organic ingredients, the other was a mineral medium (MM), free from organic substrates (after supplementation, the only source of carbon and energy was synthetic dye introduced as supplement).

The results of conducted screening depended on the composition of the growth medium used for isolation of the decolorizing bacteria strains (Fig. 4–6). It was observed that from used materials (sewage, compost and rotten wood) much more bacteria strains with the ability for dyes removal were isolated on the nutrient agar (24 strains) then on MM medium (9 strains). But it should be pointed out that the lower number of microorganisms isolated on MM medium (supplemented with the given dye) reached the higher average of decolorization results than a higher number of strains isolated on nutrient agar.

The strains isolated from sewage on the nutrient agar (13 strains) removed dyes with various efficiency (Fig. 4). Only in the case of BG some of them were able to remove more than 50% of this dye. After 96 h of incubation such a result was obtained in the case of 10 strains (77%), amongst them 4 strains (31%) removed more than 80% of BG and 2 of them removed more than 90% already after 48 h. The average efficiency of decolorization of dyes by bacteria isolated from sewage on the nutrient agar was: 68.1% BG, 15.9% CV, 22.3% Er. Whereas the strains isolated from sewage on the MM (5 strains) removed dyes more efficiently (Fig. 4). All of them removed more than 90% of BG after 48 h (about 100% removal after 96 h). The decolorization of CV higher than

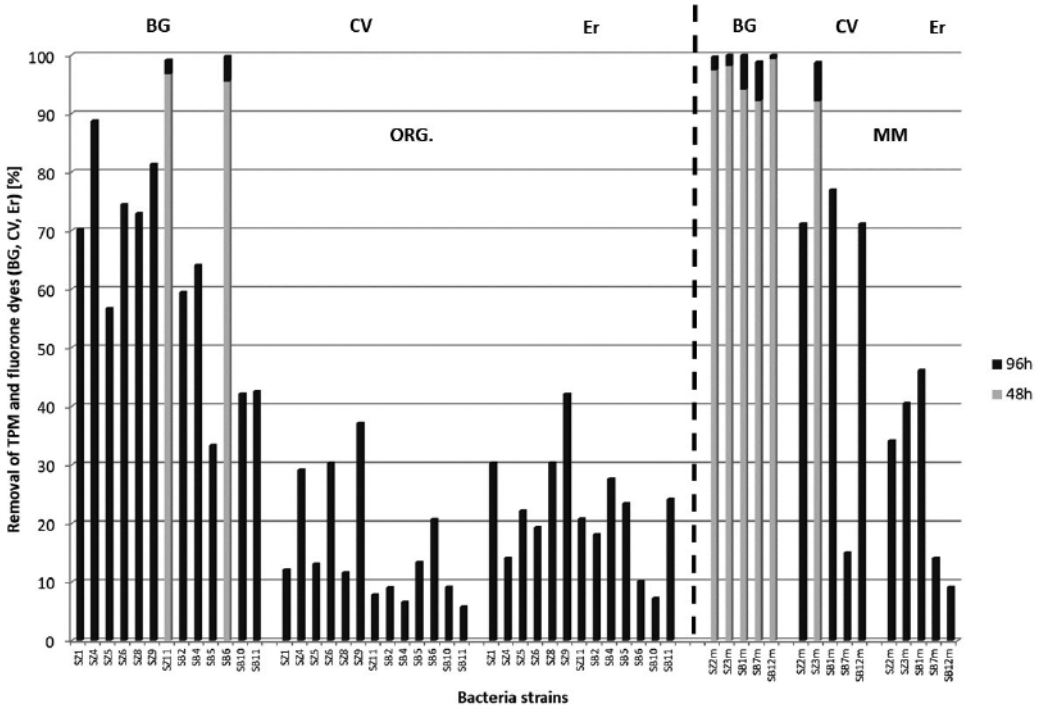


Figure 4.
The impact of usage the organic or mineral medium on decolorization potential of bacteria isolated from sewage

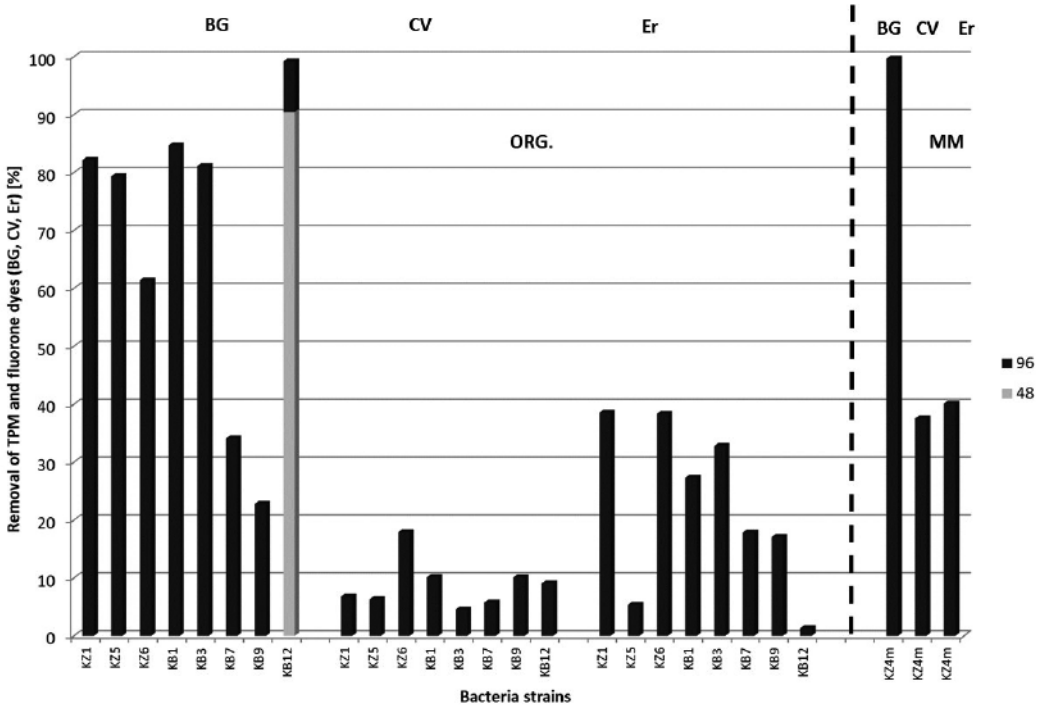


Figure 5.
The impact of usage the organic or mineral medium on decolorization potential of bacteria isolated from compost

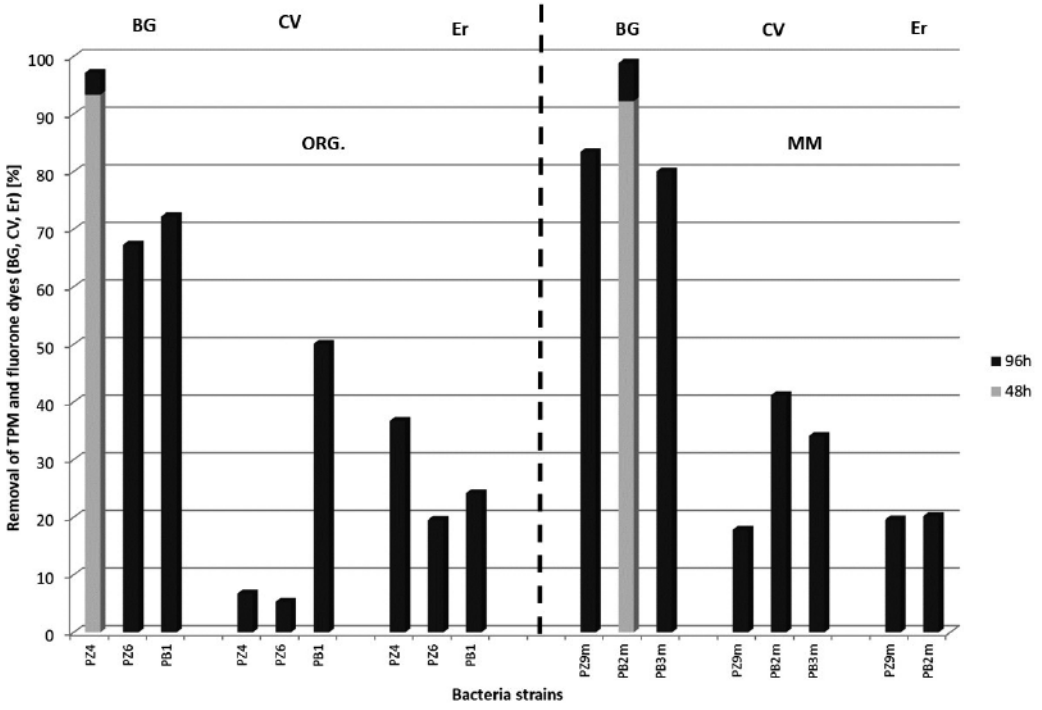


Figure 6.
The impact of usage the organic or mineral medium on decolorization potential of bacteria isolated from rotten wood beech

70% was reached by 80% of strains isolated on MM, and one of them (Sz3m) removed more than 90% of CV already after 48 h. The worst results (removal has not exceeded 50%) were obtained in the case of Er. The average decolorization efficiency of used dyes by bacteria isolated from sewage on the MM was: 99.7% BG, 66.6% CV, 28.8% Er.

Majority of strains isolated from compost were obtained on nutrient agar (8 from 9 strains) (Fig. 5). The best removal results were reached for BG (average removal efficiency 68%), then for Er (average removal 22.3%) and CV (average removal 8.8%). Only in the case of BG the removal efficiency exceeded the level of 50% (75% of strains), and in the case of 50% of strains the results were higher than 80%. From compost on MM medium was obtained only one strain (KZ4m) which was able to decolorize efficiently BG.

Amongst six strains isolated from the rotten wood three of them were obtained on nutrient agar (Fig. 6). The average removal results obtained by these strains depended on the dye and was 78.9% for BG, 20.7% for CV and 26.8% for Er. Strains isolated from rotten wood on MM medium removed BG and CV with higher efficiency (average removal 87.4% and 31% respectively) than these isolated on nutrient agar (average removal 78.9% and 20.7% respectively).

3.3. Impact of dye used at the screening procedure

Two synthetic dyes (triphenylmethane brilliant green (BG) and disazo Evans blue (EB)) were used as selective factors at screening studies. Dyes were used for growth media supplementation.

The influence of used dye on decolorization abilities of the obtained strains was observed (Fig. 7–9).

The presence of the BG in the growth medium allowed to obtain from the sewage the strains with the higher abilities of decolorization of this dye than in the case of using the azo EB for isolation (average removal of BG 82.6% and 71.2% respectively) (Fig. 7). After 96 h of incubation 100% of strains isolated on medium with the BG removed more than 50 % of this dye, whereas in the case of strains isolated on medium supplemented with EB the removal of BG exceeded 50% in case of 71% of strains. Surprisingly after 48 h of incubation, this result was reached by 25% and 36% of strains respectively, which pointed on higher efficiency of removal of BG by strains isolated on medium with EB. It should be emphasized that the majority of these strains were isolated on MM medium, where the only source of carbon and energy was used a given dye.

Similar results were obtained in case of isolation of bacteria strains from compost (Fig. 8). The average

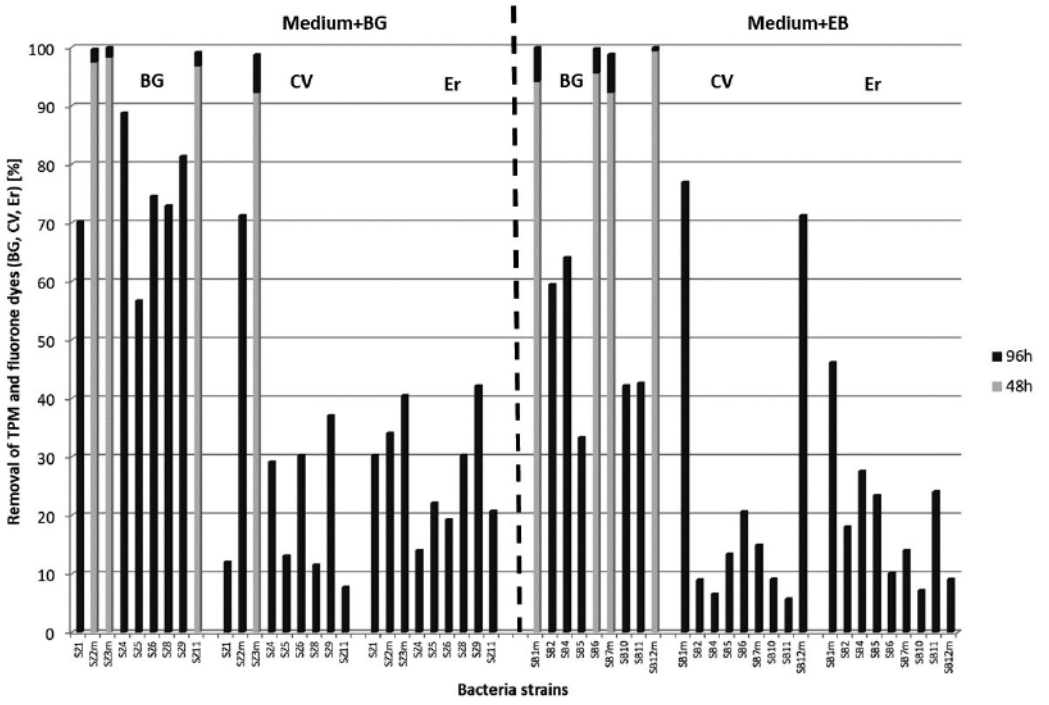


Figure 7.
The impact of dye used for isolation on decolorization potential of bacteria isolated from sewage

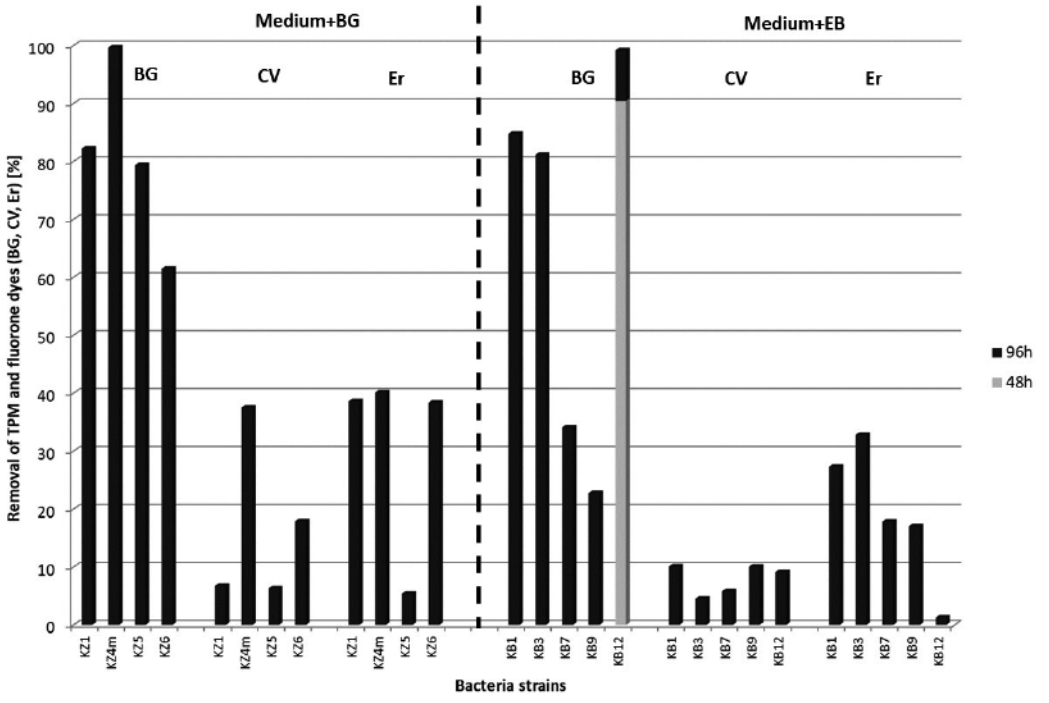


Figure 8.
The impact of dye used for isolation on decolorization potential of bacteria isolated from compost

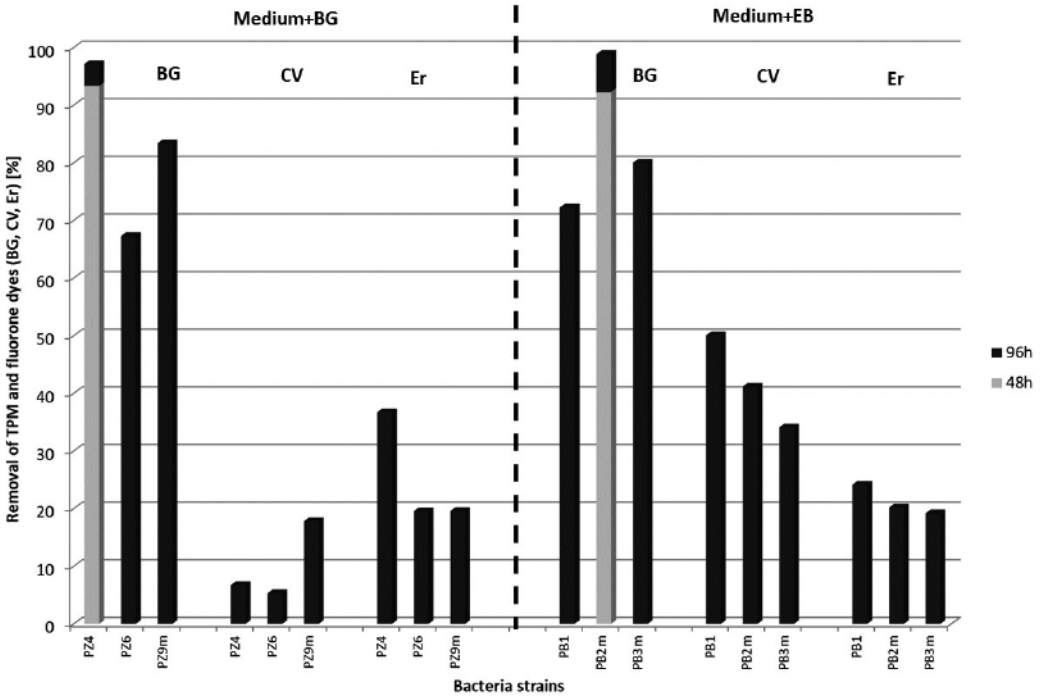


Figure 9.
The impact of dye used for isolation on decolorization potential of bacteria isolated from rotten wood of beech

removal of BG by strains isolated on medium with BG was 80.1% and 64.4% for strains isolated on medium with EB. All strains isolated from rotten wood independently of the dye used in isolation medium had the high abilities of decolorization of BG (average removal after 96 h of incubation was over 80%) (Fig. 9).

The efficiency of removal of CV by strains isolated from sewage and the compost on medium with BG had higher decolorization abilities than in case of strains isolated on medium with EB, but in both cases the average results of CV removal did not exceed 50 % (it was 34.6%, 17.1% and 25.3%, 79% respectively) (Fig. 7–8). In case of strains isolated from rotten wood the higher decolorization potential of CV had strains isolated on medium with azo EB than with BG (41.8% and 10.0% respectively) (Fig. 9).

The abilities of removal of fluorone erythrosine were low and in none case exceeded 50% after 96 h of incubation (Fig. 7–9). However, the strains isolated from sewage, compost and rotten wood on medium with BG reached higher average results of removal of Er than these isolated on medium with azo EB (28.2%, 30.6%, 25.3% and 20.0%, 19.3%, 21.2% respectively).

In spite of isolation of the largest group of decolorizing bacteria strains from sewage, the compost and rotten wood may also be considered as potential sources of obtaining such microorganisms. The usage of the mineral medium at the screening procedure allowed to obtain the lower number but much more efficient bacteria strains. Usage of the certain dye during the isolation process had a lower impact on decolorization abilities of isolated strains, however the usage of the BG allowed to obtain the strains with higher efficiency of removal of studied dyes than in case of using the EB.

Isolated bacteria strains with the highest decolorization potential should be tested on other dyes belonging to various chemical groups and also their mixtures. The selection carried out in this way will allow to obtain of strains with high decolorization potential and create the basis for the creation of biopreparations supporting the removal of dyes from colored wastewater.

4. CONCLUSIONS

Conducted screening tests have shown that municipal sewage, compost and rotten wood of beech may be used as a potential sources of strains of bacteria that have abilities to remove different dyes (triphenylmethane ZB and FK, and fluorone Er). The most numerous group of decolorizing bacteria was isolated from wastewater, followed by compost and rotten wood. The obtained results of triphenylmethane dyes removal showed that even small differences in the chemical structure of dye molecules affect their susceptibility to biodegradation and the results of removal from solutions. Brilliant green with the less complex structure was removed with high efficiency by most of the tested bacterial strains, while the more structurally complex crystal violet was removed with 50% efficiency by only 12% of isolated strains. The ability of removal of Er was low. The selection factors used in screening test had an impact on the decolorization potential of isolated strains. Isolation carried out on a MM medium, where a given dye was the only carbon and energy source, enabled obtaining of the less numerous group of bacteria strains but with higher decolorization efficiency than in case of the organic medium used for isolation. The type of dye used for supplementation of the growth media had an impact on the decolorization abilities of isolated bacteria. The strains with higher decolorization potential were obtained in case of using the BG.

ACKNOWLEDGMENT

This research was supported by the 08/080/BK_19/0067 (BK-258/RIE8/2019) project

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