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Isolation and screening of bacterial isolates from wastewater treatment plants to decolorize azo dyes

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The discharge of dye-contaminated wastewater into natural waterways presents a substantial risk to human and environmental health, therefore necessitating the treatment and removal of toxic dyes from colored wastewaters before their release into the ecosystem. The aim of this study was to isolate and characterize bacterial strains capable of decolorizing and/or degrading azo dyes commonly applied in textile production (monoazo dye Reactive Orange 16 and diazo dye Reactive Green 19) from activated sludge systems used in the treatment of (textile) wastewater. Following a prescreening of 125 isolates for their decolorization potential five strains were retained for further evaluation of decolorization rate and effects of physicochemical parameters using a microtiter plate method. Of those five strains, one strain belonging to the genus *Acinetobacter* (ST16.16/164) and another belonging to *Klebsiella* (ST16.16/034) outperformed the other tested strains. Both strains exhibited strong decolorization ability (>80%) within a wide temperature range (20 °C–40 °C) and retained good decolorization activity at temperatures as low as 10 °C (especially strain ST16.16/034). Among the different pH values tested (pH 4, 7 and 10), highest dye removal for both strains occurred at pH 7, with decolorization efficiency remaining relatively high under alkaline conditions (pH 10), and neither isolates decolorization efficiency was negatively impacted by high salt or high dye concentration. Furthermore, both strains displayed the highest rate of decolorization and were able to completely (ST16.16/034) or partly (ST16.16/164) degrade the azo dyes. Altogether, our results support the use of these bacteria in the treatment of industrial wastewaters containing azo dyes.

[**Key words:** Activated sludge; Decolorization; Dye degradation; Reactive azo dye; Textile wastewater]

Environmental pollution caused by the release of dye-containing wastewaters, e.g., from textile and printing processes, dry cleaning and tanneries, the food industry, and paint and varnish industries, is a serious problem in present days (1). Reduced water clarity not only makes the water aesthetically less pleasing, it also leads to a reduction in gas solubility and decreases the amount of sunlight penetrating the water, thereby affecting photosynthesis and entire aquatic ecosystems (2). In addition, the discharge of dye-containing effluents into water resources like rivers, ponds and lakes alters the pH and increases biological oxygen demand (BOD), chemical oxygen demand (COD) and total organic carbon (TOC) (3). Further, some synthetic dyes such as azo dyes, i.e., dyes characterized by at least one nitrogen to nitrogen double bond (–N=N–), and their degradation products are toxic, carcinogenic or mutagenic, leading to potential hazards to human and environmental health (4). Azo dyes are commonly used in the food, pharmaceutical and textile industry, and account for approximately 50% of all known dyes produced annually (5–7), and are therefore also the most common synthetic colorants massively released into the environment (8–10).

Various methods have been proposed for dye removal from wastewater effluents, including adsorption, coagulation, precipitation, filtration, electrochemical oxidation and chemical oxidation (11,12). However, many of these methods have important drawbacks of being economically unfeasible or are unable to adequately remove or degrade the dyes and/or their degradation products (13). Additionally, excessive use of chemicals produces large amounts of sludge causing additional waste streams, or may lead to secondary pollution (14). Therefore, there is a need for more effective and cheaper ways of treating colored wastewaters. Biological treatment methods based on microbial or enzymatic activities are a promising alternative to decolorize dye-contaminated wastewaters (15), and may take place by an anaerobic and/or aerobic process (16). They are relatively cheap, effective and less energy intensive. Additionally, these methods are environmentally-friendly and can yield end products that are stable and non-toxic. Furthermore, compared to physicochemical methods, less amounts of sludge are produced (13,15).

The effectiveness of microbial decolorization strongly depends on the adaptability and activity of the selected microorganisms. As a result, a large number of microbial species has been tested for the decolorization and mineralization of various dyes in recent years,

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including bacteria, fungi, yeasts and algae (12,17–19). Decolorization by fungi is mainly attributed to adsorption rather than degradation, which, together with slow fungal growth, often results in low decolorization efficiency (20). In contrast, bacteria can achieve a higher degree of degradation and even complete mineralization of dyes under optimum conditions (21,22). Azo dye decolorizing bacteria have been isolated from diverse habitats, including soil, water, colored effluents, human/animal excreta, and contaminated food materials (17,23–25). Additionally, azo dye reducing bacteria have been isolated from activated sludge wastewater treatment plants (WWTPs) (26–28). Their abundant presence in activated sludge systems has recently been confirmed through culture-independent microbial community analysis of activated sludge used for the biological treatment of dye-contaminated wastewater (29,30). In these habitats, a highly adapted bacterial community harboring several taxa shown to possess azo dye degrading activity was found, illustrating the potential to find novel, effective azo dye decolorizing bacteria within such systems. Therefore, the aim of this study was to isolate and characterize bacterial strains that are capable of decolorizing and/or degrading azo dyes from activated sludge systems treating (textile) wastewater. More specifically, 125 bacteria were isolated and screened for their ability to decolorize azo dyes, of which the five most promising strains were used to investigate the influence of various environmental parameters on decolorization ability. Furthermore, UV–VIS spectrophotometry was used to investigate whether decolorization was due to biodegradation.

MATERIALS AND METHODS

Sampling, bacterial isolation and identification Activated sludge samples were gathered in August 2016 from two well-operating WWTPs located in Flanders, Belgium. These included a textile WWTP specifically treating colored textile wastewaters and a municipal WWTP that predominantly treats domestic wastewater. Following aeration of 1 h, 10 ml of the activated sludge samples were inoculated in duplicate in 250 ml Erlenmeyer flasks containing 100 ml Tryptic Soy Broth (Sigma, Saint Louis, MO, USA) with increased salt concentration (composition final medium: 15.0 g l^{-1} pancreatic digest of casein, 5.0 g l^{-1} enzymatic digest of soybean, 40.5 g l^{-1} NaCl, 9.8 g l^{-1} $MgSO_4 \cdot 7H_2O$, 3.5 g l^{-1} $MgCl_2$ and 1.0 g l^{-1} KCl) or 100 ml minimal medium containing reactive azo dye as sole carbon source (1.0 g l^{-1} $(NH_4)_2SO_4$, 1.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g l^{-1} $CaCl_2 \cdot 2H_2O$ and 0.1 g l^{-1} of a mixture of the monoazo dye Reactive Orange 16 and the diazo dye Reactive Green 19 (Sigma)). Flasks were incubated at 30 °C for 48 h under shaking conditions (120 rpm). Subsequently, for each flask, a 10-fold serial dilution (from 10^{-1} to 10^{-4}) was plated in duplicate on Tryptic Soy Agar (Sigma), and incubated at 30 °C for 48 h. Subsequently, for each countable plate (<300 colonies), bacterial colonies with distinct morphology were picked up and purified by subculturing on the same medium. In total, 125 colonies were selected for purification. Isolates were identified by amplifying and sequencing part of the small subunit ribosomal RNA (rRNA) gene as described previously (31). Obtained sequences were compared with available sequence data in the Silva database (v. 123), manually curated to include organisms previously observed in activated sludge (Midas 2.0; http://www.midasfieldguide.org/) (32). Furthermore, for the five strains that were retained after screening the collection's decolorizing ability (see further), identifications were refined through sequence comparison with available sequences in the EZ-taxon database (http://www.ezbiocloud.net/identify) and the nt database in GenBank (excluding uncultured bacteria, unclassified sequences and environmental samples) (http://www.ncbi.nlm.nih.gov/genbank/index.html). All isolates were preserved at −80 °C in Nutrient Broth (Sigma) containing 25% glycerol.

Screening azo dye decolorizing bacterial isolates All isolates were screened for their ability to decolorize azo dyes. More particularly, strains were subjected to one of two azo dyes that are widely used in the dyeing industry, including a monoazo dye, i.e., Reactive Orange 16 (RO16; λ_{max} 494 nm), and a diazo dye, i.e., Reactive Green 19 (RG19; λ_{max} 630 nm) (dyes obtained from Sigma) (Fig. S1). Further, *Paenibacillus azoreducens* LMG 21668, which is known for its azo dye reducing abilities (33), was included as a reference. Prior to screening, stored isolates were recultured twice on Nutrient Agar (Sigma) for 48 h at 30 °C, and subsequently inoculated in Nutrient Broth and incubated for 24 h at 30 °C. Next, a 10% (v/v) aliquot (20 µl) of each isolate (OD of 0.6 at 600 nm) was inoculated in duplicate in the wells of a 96 well microtiter plate (34), each containing 180 µl sterile test solution (pH 7.0), consisting of Mineral Salt Medium (MSM; 1.6 g l^{-1} K_2HPO_4, 0.2 g l^{-1} KH_2PO_4, 1.0 g l^{-1} $(NH_4)_2SO_4$, 0.1 g l^{-1} NaCl, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g l^{-1} $FeSO_4 \cdot 7H_2O$ and 0.02 g l^{-1} $CaCl_2 \cdot 2H_2O$) supplemented with 100 mg l^{-1} filter-sterilized azo dye, 3.0 g l^{-1} glucose and 1.0 g l^{-1} yeast extract as described previously (35). In order to prevent evaporation outer wells were filled with sterile water (200 µl/well) and remained uninoculated. Furthermore, each plate contained two wells that were not inoculated but contained only test solution as a control. Plates were covered with gas-permeable sealing films (Diversified Biotech, Dedham, MA, USA), incubated under static conditions for 72 h at 30 °C and subsequently visually scored for decolorization.

For the five strains that most effectively removed color, Erlenmeyer experiments (in duplicate) were performed to confirm their azo dye decolorizing ability in a larger volume as well as to assess differences in their rate of decolorization. To this end, 250 ml Erlenmeyer flasks filled with 47.5 ml of the same test medium mentioned above were inoculated with 2.5 ml bacterial inoculum of an overnight NB culture (OD of 0.6 at 600 nm), and incubated at 30 °C under static conditions for 72 h. At the start of the experiment, as well as after 6 h, 24 h, 48 h and 72 h, 1.2 ml subsamples were taken from each Erlenmeyer and centrifuged (6000 rpm; 5 min) to remove cells and determine color removal by using spectrophotometry. Absorbance readings on the cell-free supernatants were performed at the visual maximum peak wavelength of the dyes tested (494 nm for RO16; 630 nm for RG19) in semi-micro polystyrene cuvettes (Brand, Köln, Germany) using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Uninoculated medium (MSM medium supplemented with 3.0 g l^{-1} glucose and 1.0 g l^{-1} yeast extract) without azo dye was used as a blank solution to calibrate the spectrophotometer. Decolorization capacity (%) was then calculated as follows:

$$\text{Decolorization capacity (\%)} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100 \quad (1)$$

Again, *P. azoreducens* LMG 21668 was included as a reference. Furthermore, uninoculated media were incubated under the same conditions to check for potential contamination or abiotic decolorization (which was never observed throughout our experiments). In order to assess strain differences, first data were analyzed using a repeated measures analysis of variance (ANOVA) with the bacterial strain as fixed factor and decolorization in time as dependent variable. Next, a Tukey HSD (honest significant difference) post hoc test was performed to investigate which strains performed significantly better than others in terms of decolorization capacity in a 3-day incubation period. While ANOVA tells us whether there are statistically significant differences between groups (in this case "strains"), the procedure does not, in and of itself, tell us which specific groups are actually different when there are more than two groups. Therefore, to determine which groups significantly differ from each other, post hoc comparisons such as the Tukey HSD post hoc test are used.

Impact of physicochemical parameters The potential of the five retained bacterial isolates to decolorize azo dyes was further investigated using the microplate-based assay described earlier by varying different environmental factors. First, the effect of different carbon sources was investigated by supplementing the azo dye-containing (100 mg l^{-1}) MSM medium with 1.0 g l^{-1} yeast extract and one of six different carbon sources at a concentration of 3.0 g l^{-1} (glucose, lactose, maltose, mannitol, sucrose and xylose). To study the effect of different nitrogen sources, the dye-containing MSM medium was supplemented with 3.0 g l^{-1} glucose and one of three organic (yeast extract, peptone and ureum) or one of three inorganic nitrogen sources (NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$) at a concentration of 1.0 g l^{-1} (all tested C- and N-sources were obtained from Sigma). Further, the effect of temperature (10 °C, 20 °C, 30 °C and 40 °C), pH (pH 4, 7 and 10), dye concentration (100, 250 and 500 mg l^{-1}) and additional salt concentration (2%, 4% and 6% NaCl) on the decolorization ability of the isolates was evaluated using the conditions mentioned above with exception of the investigated parameter. For all treatments, after 72 h of static incubation decolorization percentage was determined as described above. To this end, plates were centrifuged (2500 rpm; 2 min) to settle down the cultures, and dye decolorization capacity was determined by measuring the supernatant absorbance at the visual maximum peak wavelengths for both tested dyes (see above). All experiments were performed in duplicate, and in all experiments non-inoculated negative controls as well as a positive control (*P. azoreducens* LMG 21668) were included. Differences in decolorization were analyzed using ANOVA with environmental parameter or strain as fixed factor and decolorization as dependent variable. Additionally, a Tukey HSD post hoc test was performed to investigate differences between strains and between the different conditions tested.

Analysis of dye decolorization and degradation In order to study the decolorization and degradation process, degradation products formed by the selected isolates (including reference isolate *P. azoreducens* LMG 21668) were examined by following the change in the UV–VIS spectra (300–800 nm) using a UV–VIS spectrophotometer (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific). Experiments were performed in larger test volumes of 50 ml in 250 ml Erlenmeyer flasks (see above). Uninoculated media were incubated under similar conditions to check for potential abiotic decolorization of the dyes. UV–VIS spectra were recorded at the start of the experiment, after 6 h and every 24 h for a total of 3 days.