Appl Microbiol Biotechnol (2015) 99:8235–8245
DOI 10.1007/s00253-015-6648-4

ENVIRONMENTAL BIOTECHNOLOGY

Decolorization of acid and basic dyes: understanding the metabolic degradation and cell-induced adsorption/precipitation by *Escherichia coli*

Matteo Cerboneschi[1] · Massimo Corsi[2] · Roberto Bianchini[2] · Marco Bonanni[2] · Stefania Tegli[1]

Received: 11 February 2015 / Revised: 16 April 2015 / Accepted: 19 April 2015 / Published online: 11 June 2015

Abstract *Escherichia coli* strain DH5α was successfully employed in the decolorization of commercial anthraquinone and azo dyes, belonging to the general classes of acid or basic dyes. The bacteria showed an aptitude to survive at different pH values on any dye solution tested, and a rapid decolorization was obtained under aerobic conditions for the whole collection of dyes. A deep investigation about the mode of action of *E. coli* was carried out to demonstrate that dye decolorization mainly occurred via three different pathways, specifically bacterial induced precipitation, cell wall adsorption, and metabolism, whose weight was correlated with the chemical nature of the dye. In the case of basic azo dyes, an unexpected fast decolorization was observed after just 2-h postinoculation under aerobic conditions, suggesting that metabolism was the main mechanism involved in basic azo dye degradation, as unequivocally demonstrated by mass spectrometric analysis. The reductive cleavage of the azo group by *E. coli* on basic azo dyes was also further demonstrated by the inhibition of decolorization occurring when glucose was added to the dye solution. Moreover, no residual toxicity was found in the *E. coli*-treated basic azo dye solutions by performing *Daphnia magna* acute toxicity assays. The results of the present study demonstrated that *E. coli* can be simply exploited for its natural metabolic pathways, without applying any recombinant technology. The high versatility and adaptability of this bacterium could encourage its involvement in industrial bioremediation of textile and leather dyeing wastewaters.

Keywords *Escherichia coli* · Bacteria · Dyes · Bioremediation · Azoreductase · Decolorization · Aerobic metabolism · Cell adsorption · Toxicity

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6648-4) contains supplementary material, which is available to authorized users.

✉ Matteo Cerboneschi
matteo.cerboneschi@unifi.it

[1] Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente, Laboratorio di Patologia Vegetale Molecolare, Università degli Studi di Firenze, Sesto Fiorentino (Firenze), Italy

[2] Dipartimento di Chimica "Ugo Schiff", Università degli Studi di Firenze, Sesto Fiorentino (Firenze), Italy

Introduction

Acid and basic dyes are some of the most extensively used colorants within the textile and leather industry (Hunger 2002). Their wide application involves routine dyeing procedures, which may differ among dyers for the number of steps and/or chemical auxiliaries used to favor dyeing homogeneity (Shore 2002). As a result of the large number and different nature of the chemicals used, the wastewaters generated from the dyeing of textile and leather materials are some of the major polluting chemical containing wastes (Claudio 2007; Slater 2003). Although progresses have been made to replace the chemical auxiliaries with natural or semisynthetic substances in a view to generating more environmentally friendly wastewaters (de Sousa et al. 2011), the traditional dyes are still in use and represent one of the main sources of pollution for water streams (Alinsafi et al. 2007). These dyes cause serious environmental and health concerns to aqueous ecosystems and humans (Sarnaik and Kanekar 1995), due to their toxicity (de Campos Ventura-Camargo and Marin-Morales 2013). Typically, dyeing baths contain a nominal percentage of dye between 1 and 15 % by weight of material, but 2 up to 50 % of the original dye is generally lost in the wastewaters, depending on the class of the dye used (Khan et al. 2013; Ogugbue and

Sawidis 2011; Khalid et al. 2008; McMullan et al. 2001). In particular, the global annual production of leather and textile dyes is 7×10^5 T, for an annual trade of these industries that is over 2 and 70 million tons, respectively (The Freedonia Group 2013; Zaharia and Suteu 2012). Therefore, the environmental impact of the effluents from the dyeing industry is a serious concern. In addition, the disposal of dyeing effluents constitutes the largest portion of the costs associated to water management (Slater 2003). Various treatments for dye-containing wastewaters have been developed, to address these environmental and economical issues: mainly chemical and electrochemical oxidation, filtration on micromembranes, precipitation of dyes in the form of insoluble salts, photocatalytic degradation, and adsorption or electrosorption (Singh and Arora 2011; Forgacs et al. 2004; Hao et al. 2000; Avlonitis et al. 2008; Berberidou et al. 2009; Harrelkas et al. 2009; Zhao et al. 2009). Unfortunately, all these methods are highly costly and thus commercially unattractive. Therefore, it is mandatory to develop alternative means of dye decolorization, such as innovative biological methods, to provide more economical cleanup protocols and allow the recycling of industrial treated wastewaters.

In recent years, bioremediation and more generally biotechnologies have gathered growing interest because of their cost-effective and eco-friendly profile (Khan et al. 2013; Ali 2010; Solís et al. 2012). Some algae (Daneshvar et al. 2007; Khataee et al. 2010), bacteria (Ayed et al. 2010; Amoozegar et al. 2011; Saratale et al. 2011), fungi (Kaushik and Malik 2009; Novotný et al. 2011; Verma et al. 2012; Ali et al. 2008; Jin et al. 2007; Xian-Chun et al. 2007; Fu and Viraraghavan 2001; Marimuthu et al. 2013; Tegli et al. 2014), and yeasts (Vitor and Corso 2008; Qu et al. 2012) were proved to achieve acceptable and efficient dye removal. The vast majority of the research on biological decolorization has been carried out on fungi and mainly on their ligninolytic enzymes, discovered to degrade azo dyes aerobically (Erkurt et al. 2010). Conversely, studies on dye removal operated by bacteria are relatively less reported (Pokharia and Ahluwalia 2013; Satheesh Babu et al. 2013; Pandey et al. 2007), although the bacterial decolorization is normally faster than the fungal systems (Shobana and Thangam 2012). Several bacteria have been shown to degrade azo dyes (Saratale et al. 2011), and it has been documented that decolorization occurs via bacterial azoreductases, followed by the aerobic mineralization of colorless amines (Pandey et al. 2007; Van der Zee and Villaverde 2005). Generally, this two-step process may require a bacterial consortium to successfully complete the azo dye biodegradation task, but the reproducibility profile is often low undermining potential industrial applications (Phugare et al. 2011). The most extensively studied bacterial species belong to the genus *Pseudomonas* (e.g., *P. luteola*) (Chang et al. 2001; Chen and Lin 2007). Surprisingly, the degrading abilities of enteric bacteria have been by far less investigated (Chen et al. 2004; Mate and Pathade 2012; Chan et al. 2012) including *Escherichia coli* frequently used to produce recombinant proteins and overexpress foreign bacterial genes coding for azoreductase enzymes (Lončar et al. 2013; Işık and Sponza 2003; Sandhya et al. 2008; Chang and Lin 2001; Chang et al. 2000).

In this paper, we explored for the first time the aerobic dye degrading performances of *E. coli* DH5α, whose metabolic potential in dye bioremediation has been rarely explored to the best of our knowledge (Rau et al. 2002). This strain is highly adaptable, it has the aptitude to switch between aerobic and anaerobic lifestyles (Dougan et al. 2001), and it is characterized by fast growth kinetic even on very simple nutrient media (Sezonov et al. 2007). *E. coli* DH5α was tested on several azo and anthraquinone dyes among those most commonly used within the dyeing industry. The kinetics of decolorization and the influence of operational parameters were also studied on a representative dye for each chemical group. A significant abiotic dye removal was also revealed, which rarely has been considered in previous studies (Sarioglu et al. 2007).

Materials and methods

Dyes

The 15 dyes used in this study were kindly provided by Tintoria Cometa Srl (Prato, Italy) and were of commercial quality. These dyes were grouped according to their chemical nature: acid anthraquinone (group A), acid azo (group B), basic anthraquinone (group C), basic azo (group D). The name, CAS number, and the wavelength of maximum absorption (λ_{max}) of each dye are reported in Table 1. Experiments were carried out with an initial concentration of 0.3 mg/ml of each dye dissolved in double distilled water.

E. coli growth conditions and culture media

The bacterial strain used in this study was *E. coli* DH5α (DSM No. 6897 at Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures), grown aerobically at 37 °C on Luria–Bertani broth (LB) (BD Biosciences, Milan, Italy, supplemented with 15 mg/l of nalidixic acid (Sigma Aldrich Co, Milan, Italy)), under orbital shaking (150 rpm). Overnight bacterial cultures having an $OD_{600}=1$ were harvested by centrifugation (5000*g*, 10 min at room temperature) and washed twice with sterile physiological solution (NaCl 8.5 % *w*/*w* in water). Subsequently, the bacterial pellets were used for decolorization experiments. The average wet weight biomass of *E. coli* culture sampled from an early stationary phase was approximately 1.0 mg/ml, corresponding to about 5×10^9 cells/ml.

Table 1 Commercial dyes

Type of dye	Lab code	Name	CAS[39]	λ_{max} (nm)
Group A acid anthraquinone	A277	Acid Blue 277	25797-81-3	605
	A260	Acid Blue 260	62168-86-9	590
	A40	Acid Blue 40	6424-85-7	620
	A25	Acid Blue 25	6408-78-2	605
	A43	Acid Violet 43	4430-18-6	580
	A324	Acid Blue 324	88264-80-6	610
Group B acid azo	A361	Acid Red 361	61931-22-4	525
	A118	Acid Red 118	12217-35-5	505
	A97	Acid Red 97	10169-02-5	495
	A357	Acid Red 357	61951-36-8	490
	A151	Acid Yellow 151	12715-61-6	435
Group C basic anthraquinone	B47	Basic Blue 47	12217-43-5	575
Group D basic azo	B46	Basic Red 46	12221-69-1	530
	B41	Basic Blue 41	12270-13-2	605
	B51	Basic Red51	12270-25-6	520

Decolorization assays: experimental and analytical procedures

Batch decolorization assays were carried out by inoculating *E. coli* in 50-ml Falcon tubes (BD Biosciences), containing 20 ml of LB medium amended separately with each of the dyes reported in Table 1, at known concentrations. The decolorization performances were systematically analyzed as a function of the following parameters: static or shaken (150 rpm) cultures, incubation temperature (25 and 37 °C), initial dye concentration, and 15-mg wet weight biomass. The effect of glucose, ammonium chloride, and ammonium sulfate on *E. coli* decolorizing activity was also investigated, by adding separately these nutrients (500, 250, and 250 mg/l, respectively) to the decolorizing medium. Negative controls with uninoculated dye medium and heat-killed *E. coli* cells (10 min at 100 °C) were also performed. Treatments and controls were run in triplicate, in three independent experiments.

Incubation of *E. coli* on decolorizing medium was kept up to a maximum of 48 h. Aliquots were periodically and aseptically sampled, centrifuged (5000*g*, 5 min at room temperature) to collect the biomass as a pellet, and filtered on 0.2-μm sterile cellulose acetate Nalgene® filter (VWR, Milan, Italy).

A UV–Vis spectrophotometer (Infinite 200Pro, Tecan, Seetrasse, Switzerland) was used to measure the λ_{max} of the supernatants of each dye. The percentage of dye reduction (DR%) was calculated according to the following:

$$DR = \left[(A_0 - A_1)/A_0\right] \times 100$$

where DR=decolorization as percentage; A_0 and A_1, initial and final absorbance at the λ_{max} of each dye in the visible region. The pH of the supernatants was also measured, using a Basic 20-Crison pH meter (Carpi, Italy). Bacterial growth was measured as cell biomass.

Experimental data were subjected to statistical analysis to determine means and the corresponding standard deviations. Significant differences between means of experiments were determined by analysis of variance (ANOVA) with a significance level of 0.05.

Mass spectrometry

A 50.0-μl aliquot of each dye solution subjected to decolorization was diluted with 2 ml of HPLC grade methanol (Fisher Scientific, Leicestershire, UK) and then filtered on Titan2® Syringe Filter (ThermoFisher Scientific, Austin TX, USA). The filtered sample was analyzed using a ThermoScientific LCQ-Fleet mass spectrometer under electrospray ionization (ESI) (Thermo Scientific, Austin TX, USA) by direct infusion with a 500 KL Hamilton microsyringe and using a 10-μl/min flow. Data were acquired over hundred scans, after stabilization of the ionic current for 5 min at the above mentioned sample flow. The mass spectrometer parameters used were as follows: sheet gas flow=10, auxiliary gas flow=0, sweep gas flow=0, spray voltage=5.00 kV, capillary tube temperature=220 °C, capillary voltage=12.0 V, and tube lens=77.8 V.

Toxicity assay

The short-term tests to determine acute toxicity were carried out using the water flea *Daphnia magna*, a standardized test organism for toxicity assays and proposed as a representative

invertebrate in the EU Guidance Document on Aquatic Ecotoxicological and among EU criteria for classification of dangerous substances (Verma 2008; Crosby et al. 1966; Brock and Van Wijngaarden 2012). Young organisms (6–24-h life) were used for toxicity bioassay and were not fed during the test period. For each sample and each experimental condition, 25 organisms were tested in triplicate, along with negative (water) and positive (untreated dye) controls. The number of immobile individuals was evaluated after 48-h incubation at 21±1 °C, with a 16-h light/8-h dark photoperiod. *D. magna* individuals are officially accepted as dead if they do not show any mobility within 30 s of observation.

Results

Preliminary screening on *E. coli* dye decolorization ability

Initially, a general screening about the *E. coli* decolorization abilities was carried out for the whole selection of dyes, to evaluate the bacterial activity on a wide range of colorants. The solutions were inoculated with an overnight starter culture of *E. coli* in shaken tubes at constant temperature (25 and 37 °C). The pH of each sample was monitored at the beginning and at the end of the experiment. *E. coli* modified the initial pH of each sample to a value oscillating between 6 and 7 regardless of the incubation temperature. This behavior was coherent with the bacterial growth, which was recorded positive for each sample and generally higher at 37 °C, as expected (Fig. 1a). Therefore, it was not surprising that the visible spectrophotometric analysis of the supernatants showed a sensible decrease in dye concentration (Fig. 1b).

The decolorization was statistically similar at 37 and 25 °C, except for dye A40. Conversely, a stronger decolorization was obtained for compounds A357, B41, B46, and B51 at 37 °C. Also, it was interesting to note that when the bacterial biomass of each experiment was separated from the treated dye solutions and visually examined, the bacterial pellets retained some of the original color in the case of dyes of groups A, B, and C. On the other hand, no coloration was observed for dyes of group D (data not shown), since the original color had completely vanished from both the supernatant and the pellet. It is known that some bacteria may degrade azo dyes, here represented by groups B and D, through specific azoreductase enzymes and during anaerobic growth (Liu et al. 2009). In our case, this event may have happened in aerobic growth conditions. However, the data did not allow to understand which mechanisms were involved in the decolorization of dyes. Therefore, A324, A361, B47, and B46 were selected as representative dyes of each group, in order to carry out further investigation to clarify the mode of action of *E. coli*.

Analysis of decolorization behavior of *E. coli*

The decolorization of dyes may take place according to very different mechanisms, such as adsorption and degradation (Saratale et al. 2011). In the first case, dyes are adsorbed onto the bacterial cell surface, while when dyes are going to be degraded by bacterial enzymes, they are first compartmentalized inside the bacterial cells. The representative colorants were treated with *E. coli* for 16 h at 37 °C. Afterward, the biomasses were separated from the supernatants (hereafter named "first generation supernatants" for the total decolorization), and the resulting bacterial pellets were washed with water. This step was repeated until the absorbance of the supernatant washing water was completely clear at naked eye. These supernatants (hereafter named "second generation supernatants") corresponded to the washing of the dye probably precipitated and adsorbed on the bacterial cell surface. Subsequently, each pellet was subjected to thermal lysis in water at 100 °C for 10 min and separated from the supernatant (hereafter named "third generation supernatants") which contained the intracellular dye portion (Fig. 2). The spectrophotometric analysis in the visible region of the supernatants thus obtained from A324, A361, and B47 confirmed that their λ_{max} was consistent with that of the untreated dye (Table 2). These results were also consistent with the data derived from the mass spectrometric analysis of the supernatants (Fig. 3). The *E. coli* pellets derived from the decolorization on A324 and A361 released the color both during the washing and the lysis procedure, suggesting the presence of the dyes both on the bacterial surface and inside the bacterial cell wall, respectively. The same also occurred for B47, although to a lesser extent. However, data obtained from second generation supernatants could not exclude a potential dye precipitation induced by bacterial addition and cell growth. Visible spectra of B46 supernatants did not exhibit any λ_{max} consistent with this dye. The differences between the absorbance of the first generation supernatants and those combined from the second and third generation supernatants were correlated to the metabolic contribution of *E. coli* to the overall decolorization. Metabolism was 27, 36, and 45 % for A324, A361, and B47, respectively. In the case of B46, this percentage reached 98 %, clearly indicating that metabolism was the only mechanism involved in decolorization. In addition, mass spectrometric analysis confirmed (Table 2 and Fig. 3) the presence of *N*′-benzyl-*N*′-methylbenzene-1,4-diamine 1, having a molecular weight of 212 (Fig. S1) which would originate from the reductive cleavage of the azo group of B46, promoted by *E. coli* (Fig. 3d and Fig. S1). This transformation may have been driven also by the cationic nature of this dye, as represented by its resonance hybrid structure 3 (Fig. S2) during its interaction with the degrading bacteria.

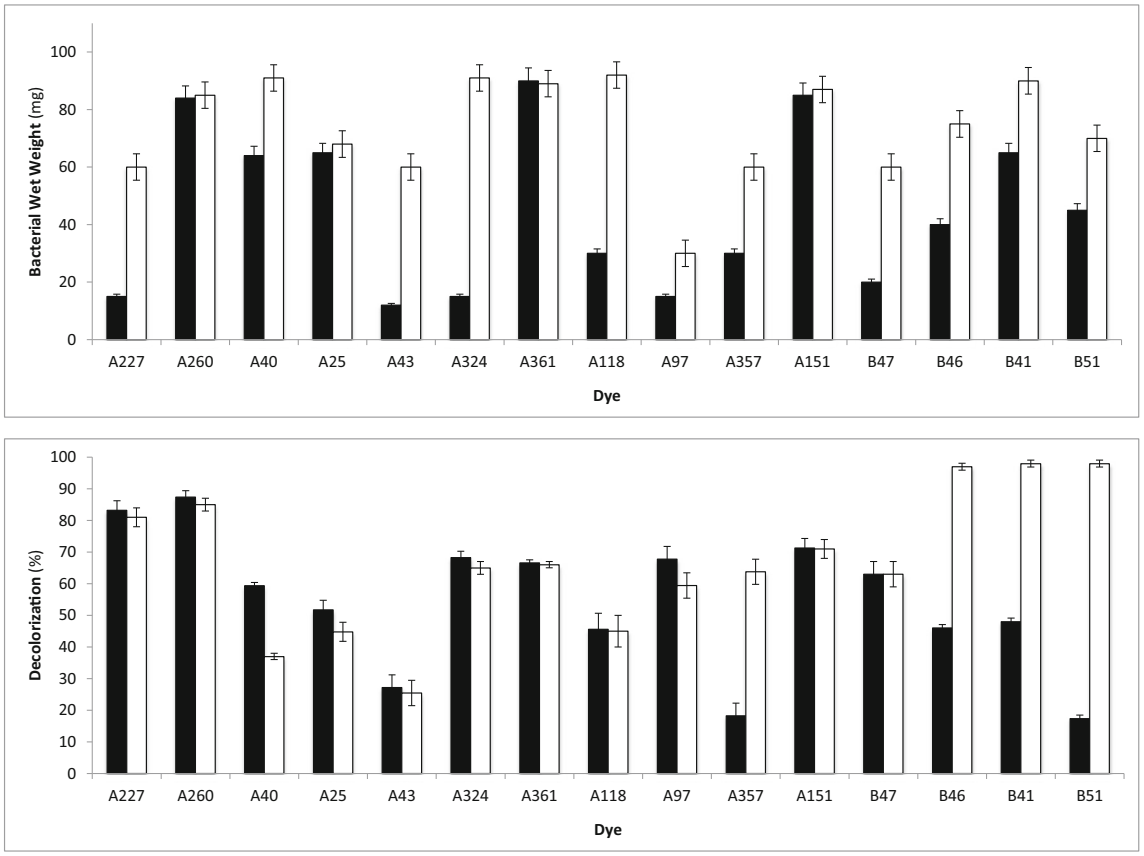

Fig. 1 **a** *E. coli* growth at 25 (*black*) and 37 °C (*white*) after 16 h of incubation at 0.3 mg/ml of dye concentration. **b** Dye decolorization reported in this figure was related to the same trial in which bacterial growth had been monitored

Effect of bacterial biomass on *E. coli* decolorization activity

Different amounts of *E. coli* biomass (1.5, 7.5, 15, and 30 mg of bacterial wet weight) were inoculated separately into each representative dye solution, and the cultures were homogenized by mechanical stirring. An aliquot (1 ml) was taken from each sample and centrifuged at time 0 (t_0) to assess whether precipitation of the dye may have been induced by bacterial biomass. Subsequently, the cultures were incubated at 37 °C for 48 h on an orbital shaker, sampled at 2-h interval from t_0 to 8 h, then at 24 and 48 h. The decolorization results obtained for A324, A361, and B47 showed a nonproportional increase in dye reduction related to the different biomass amounts. In Fig. 4, it was reported the decolorization trend for experiments containing the maximum amount of bacterial biomass. Dye reduction occurred even at t_0 for those three dyes (46, 17, and 38 %, respectively) remaining practically constant for 8 h, a time which implicates at least 24 *E. coli* generations (Kutsu 2007). Finally, the decolorization percentage increased to a threshold value at 48 h regardless of the dye (Fig. 4). The overall profile of the experiments appeared to remain constant, suggesting that decolorization of A324, A361, and B47 had been occurring with a combination of metabolism and cell-induced dye precipitation. Control experiments were also prepared in the absence of bacterial biomass. After mechanical homogenization, each solution was centrifuged without observing any precipitation, leaving the samples on standing for 24 h.

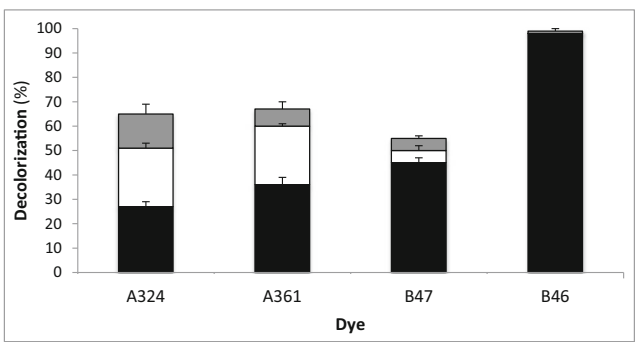

Fig. 2 Decolorization rate after 16 h of *E. coli* activity at 0.3 mg/ml of dye concentration: precipitation, adsorption, and metabolism. *Black portion* metabolic contribution; *white portion* precipitation and adsorption on bacterial cell wall; *gray* intracellular accumulation

Table 2 Data from the visible spectroscopic (λ_{max}) and mass spectrometric ($^{m}/_{z}$) analyses of the supernatants after decolorization of A324, A361, B47, and B46

Dye	Reference		Supernatants					
Lab code	λ_{max} (nm)	mass ion ($^{m}/_{z}$)	1st generation		2nd generation		3rd generation	
A324	610	450.44[a]	615	450.34	614	450.34	615	450.33
A361	525	517.60[a]	530	517.34	530	517.34	528	517.40
B47	575	371.43[b]	585	372.15	587	372.13	588	372.15
B46	530	321.18[b]	n.d.	213.06	n.d.	213.12	n.d.	213.12

[a] Anion, analysis under ESI-*c* method

[b] Cation, analysis under ESI+*c* method

n.d not detected

The experiments carried out on the degradation of B46 showed the typical trend of a metabolic decolorizing activity (Fig. 4): the biomass increase induced a proportional reduction of time to reach the plateau of bacterial decolorization at

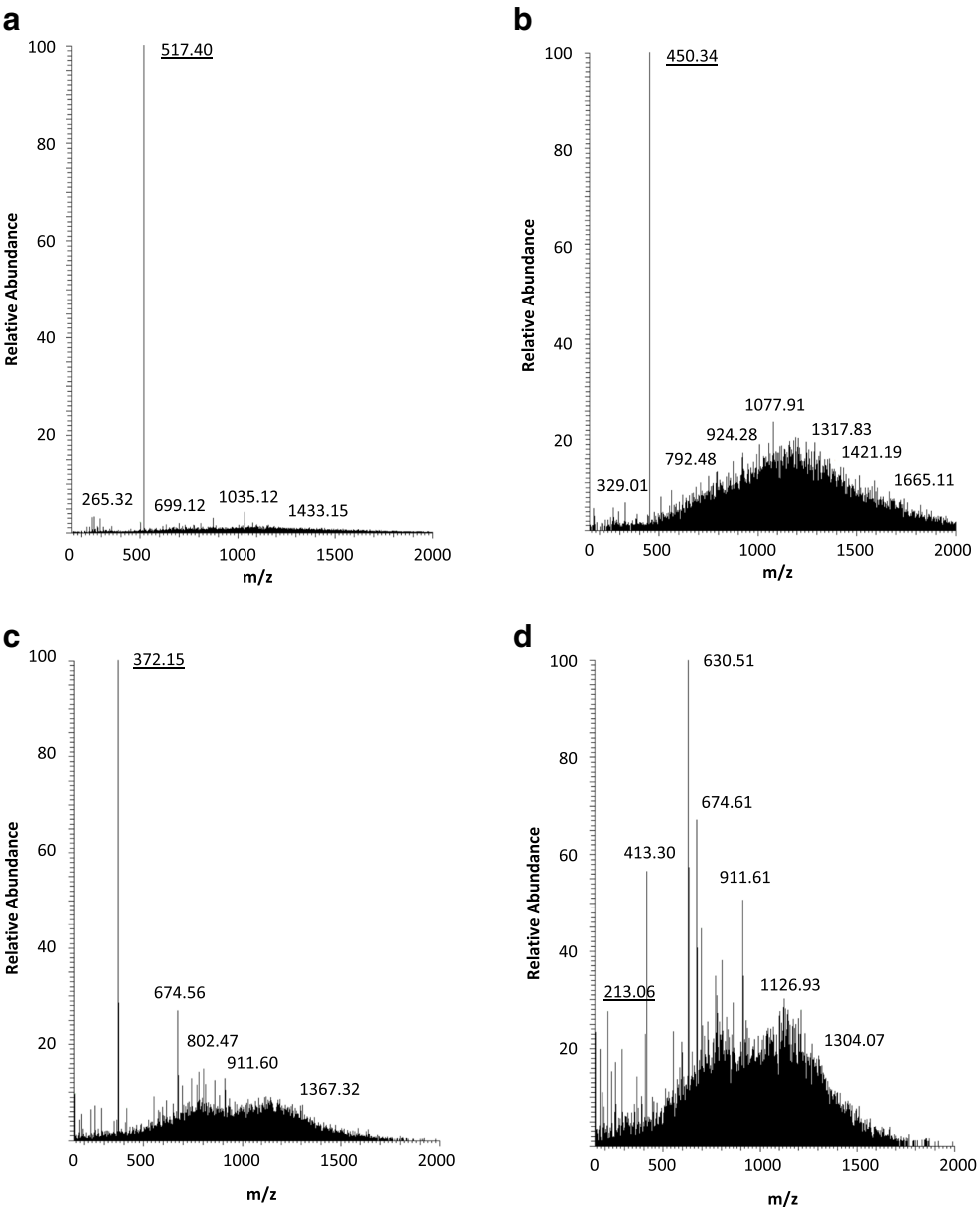

Fig. 3 Mass spectra of the first generation supernatants from decolorization assay. **a** A324; **b** A361; **c** B47; **d** B46. Mass spectra were carried out on the supernatants collected after centrifugation and without further purification. The presence of other peaks was related to chemical species of the LB medium after bacterial treatment

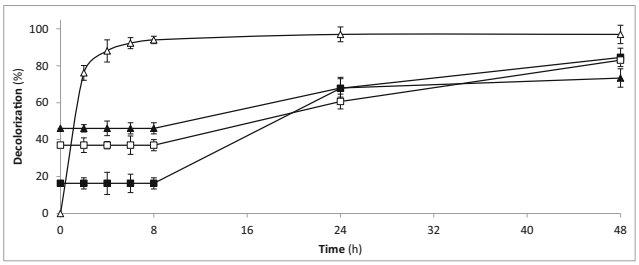

Fig. 4 Effect of maximum *E. coli* biomass (30 mg) on the decolorization of A324 (*black triangle*), B47 (*white square*), A361 (*black square*), and B46 (*white triangle*) as a function of time

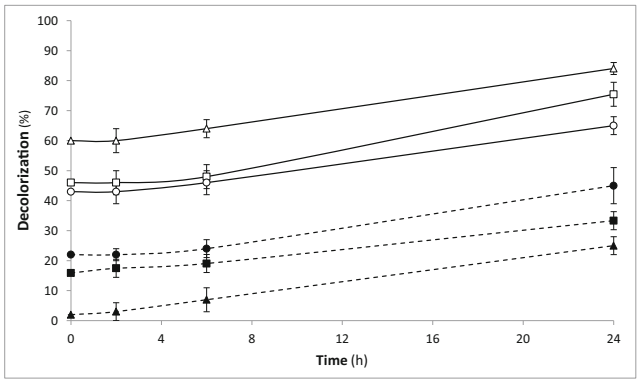

Fig. 5 Decolorization at the lowest and the highest concentrations of dye. A324 (*triangle*); A361 (*square*); B47 (*circle*). *White indicators* were related to maximum concentration of dye (*continuous lines*), *black indicators* to the minimum concentration of dye (*dashed lines*)

24 h (data not shown). This evidence was supportive for a specific metabolic activity, induced by the presence of azoreductase enzymes produced by *E. coli* (Pandey et al. 2007).

A comparative experiment between alive and dead bacterial cells shed light on the contribution of both metabolism and adsorption to the decolorizing activity of *E. coli. S*olutions of A324, A361, B47, and B46 were inoculated and evaluated after 16 h of incubation. As reported in Fig. S3, only for A361, it was possible to observe a dye reduction (over 10 %) caused by the inoculation of dead cells, while for A324 and B47, the decolorization level was less than 5 %. Conversely, there was no evidence of *E. coli* absorption activity on the B46 solution.

Using the same experimental conditions, we decided to verify whether the decolorization performances of *E. coli* were affected by the oxygenation of the cultures (steady or shaken). The dye removal rate decreased in a range between 10 and 20 % for A324, A361, and B47, when the cultures were not under agitation (Fig. S3). On the other hand, the experiment of B46 did not display any significant difference related to the decolorizing performances of *E. coli* cells in steady or shaken condition.

Effect of dye concentration on *E. coli* decolorization activity

Culture samples were prepared at 0.06-, 0.15-, 0.3-, 0.4-, and 0.6-mg/ml dye concentration, and the solutions were inoculated with *E. coli* biomass. Aliquots (1 ml) were sampled at 2, 6, 8, and 24 h. As reported in Fig. 5, the analysis of the supernatants by visible spectrophotometry indicated that the highest percentages of color removal for dyes A324, A361, and B47 were obtained at 0.6 mg/ml. Decolorization also showed similar patterns from the lowest to the highest dye concentration regardless of the initial amount of color, highlighting an almost linear trend. The largest difference of decolorization was observed for dye A324 from the lowest to the highest concentration, whereas in the case of A361 and B47, this phenomenon was reduced of about 50 %.

Concerning compound B46, the highest percentages of decolorization were obtained from 0.06- to 0.3-mg/ml concentration (Fig. 6a), and surprisingly, the decolorization at t_4 and t_{24} was somehow similar. This result indicated that as the concentration of dye increased, it would not be necessary to prolong the experiment beyond the 4 h: or at least to extend it necessarily to t_{24} or longer. A marked difference was observed for the 0.4- and 0.6-mg/ml concentrations in the same interval of time. At the maximum concentration tested, we could assume that the relative high concentration of B46 had induced *E. coli* to adapt its membrane transport systems. The decolorization after 4 h was lower than 10 % of the initial dye concentration as indicated by the speed plot: the maximum speed of dye reduction could be observed just after 2 h at lower concentrations (Fig. 6b).

Inhibition of *E. coli* azoreductase activity

The decolorization of azo dyes by bacterial strains is typically initiated by azoreductase enzymes in anaerobic condition (Pandey et al. 2007). On the contrary, as previously shown, the reduction of B46 was due to the aerobic metabolism of *E. coli*. Therefore, we decided to verify the kinetic of dye degradation in relation to the azoreductase activity. It is known that the reduction of azo dyes is linked to the presence and availability of cosubstrates as carbon and nitrogen sources, and the degradation rate of bacterial cells is correlated to the chemical structures of these molecules.

The assay for the azoreductase inhibition was carried out for 6 h in aerobic condition at 0.15 mg/ml of B46. For each replicate, a specific amount of glucose (carbon source), ammonium chloride, and ammonium sulfate (nitrogen sources) was added, at 1, 0.5, and 0.5 % (*w*/*v*), respectively, which had already demonstrated to be able to decrease dye decolorization (Modi et al. 2010). There was a decrease of B46 dye reduction up to 4 % in the case of glucose supplementation and moderate scores for nitrogen substrates (Fig. S4). Thus, these results

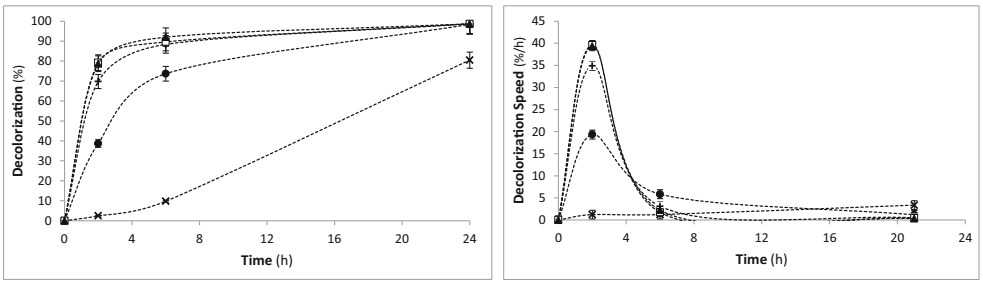

Fig. 6 Rate (**a**) and speed (**b**) decolorization with 15-mg wet weight of *E. coli* at different dye concentrations of B46. Dye concentration (mg/ml): *triangle* 0.06; *square* 0.15; *plus* 0.30; *circle* 0.40; *cross* 0.60

showed that the decolorization of B46 had been occurred in aerobic conditions and included azoreductase activity too.

Toxicity assay

The assessment of the toxicity of the residual dye mixtures treated with *E. coli* was a very important aspect for bioremediation applications, to demonstrate that the solutions thus obtained were not toxic, as well as visually clear. Acute toxicity tests were carried out on the representative dyes treated with *E. coli* after 16 h of incubation. As shown in Table S1, the toxicity rates of B46-treated solutions were absent after 6 h. Excellent scores were achieved by A324 and A361 after 12 h (after 6 h ca. 2 and 4 %, respectively), while the worst performances were obtained for B47 (after 12 h ca. 5 %). According to these results, it is reasonable to hypothesize that the differences here observed in the acute toxicity between the different dyes might be mainly related to putatively different mechanisms adopted by this bacteria for decolorization.

Discussion

In general, the adaptability of *E. coli* to xenobiotic species such as dyes was proved by the cell growth and by the adjustment of the initial pH of the dye solutions to nearly neutral values. This finding meant that the cells were inclined to make their surrounding environment favorable to survive, although the decolorization performance was found not always strictly correlated to the bacterial growth.

Here, we demonstrated that the *E. coli* type strain DH5α worked well on acid and basic dyes under aerobic conditions, showing different mechanisms of decolorization. Both acid anthraquinone (group A) and acid azo (group B) dyes were removed from effluents by the combined action of precipitation, cell adsorption, and metabolism. These three events have been rarely considered as acting altogether in previous studies (Saratale et al. 2011; Giwa et al. 2012) while claiming the metabolic activity of the bacterial biomasses as the only decolorizing mechanism. It is important to underline that those phenomena occurred during cell culture growth regardless of the amount of biomass and the type of acid dyes. It is reasonable to assume that groups A and B dyes were likely to precipitate in the presence of *E. coli* already at t_0 as demonstrated by A324 and A361. The phenomenon can be certainly described by a biotic event, since control experiments in the absence of *E. coli* did not show any dye precipitation induced by the nutrient medium or by mechanical action such as centrifugation. The effect of biomass growth resulted in an increase of decolorization mainly due to precipitation, cell wall adsorption, and intracellular accumulation, although a significant fraction could be also associated to metabolic events, related to the activity of bacterial azo reductases (Liu et al. 2009). Similar findings were also described for a basic anthraquinone (group C) dye, which has never been reported in previous papers to the best of our knowledge. Even in this particular case, the presence of bacterial biomass induced a strong precipitation effect at t_0, causing a reduction of metabolism contribution. It was interesting to notice that the different chemical nature of A324 and B47 anthraquinone dyes caused their opposite contribution between metabolism (27 and 45 %, respectively) and precipitation effect (46 and 38 %, respectively). This phenomenon is related to the different chemical affinity of acid or basic anthraquinone dyes to the cell wall of the Gram-negative *E. coli*.

Degradation of basic azo dyes (group D) occurred efficiently under aerobic conditions, confirming the metabolic pathway of *E. coli* involving oxygen-resistant azo reductases. This result was also consistent with recent findings, which demonstrated the prominent role of *E. coli* AzoR-purified enzyme under aerobic conditions (Mercier et al. 2013). The remarkably high rate of decolorization for B46 was found to be dependent on the amount of biomass and the concentration of dye. Conversely, metabolic contribution to total dye reduction on A324, A361, and B47 was even lower than 50 %, probably because of the absence of redox mediators as recently reported (Cui et al. 2015).

Comparative trials with steady and shaken alive cells confirmed that precipitation and absorption phenomena contributed in a different way to the total dye reduction for A324, A361, and B47, whereas B46 underwent enzymatic degradation. This concept may be indirectly confirmed by the decolorization of B46, which resulted marginally affected by steady and shaken biomass. In this case, it could be remarked that

metabolism was the principle, if not the unique, mode of dye degradation activated by *E. coli*, demonstrated by the absence of any degradation with dead cells.

Experiments carried out on A324, A361, and B47 at variable amount of color demonstrated that *E. coli* was able to keep a similar behavior across the whole incubation time and regardless of the initial dye concentration. These findings may have important implications at industrial level: particularly for the textile industry, which exploits the vast majority of the global production of dyes. The variation of dye concentration in effluents is quite often unpredictable and fluctuating, due to dye reactivity with water, dye fixation, and variable exhaustion of the dyeing bath (Ghaly et al. 2014). In the case of B46, *E. coli* showed a stronger ability to metabolize basic azo dyes at the typical concentration founded in effluents after a dyeing processes. Only 2 h was sufficient to completely remove B46 from trial solution. Mass spectrometric analysis demonstrated that metabolite 1 was originated by reductive cleavage (Pandey et al. 2007) of B46 and further degraded by *E. coli* in aerobic conditions. Also, compound 1 resulted compatible with the toxicity prescriptions of REACH (EC 2006). The cationic nature of B46 was crucial for its quantitative uptake and degradation by *E. coli*, due to the high affinity of this dye for the bacterial cell wall and membrane structure. This assumption could be explained taking into account the Hard-Soft-Acid-base model (Ho 1975). The lone pair of the *N*′-benzyl-*N*′-methyl moiety of B46 is delocalized through the azophenyl fragment into the triazole ring, as shown by the resonance hybrid structure 3, defined a soft cation because of the distribution of the positive charge onto multiple centers. It follows that compounds such as 3 interact quite well with lipids and phospholipids of the bacterial cell wall and membrane, since the negatively charged polar heads are diffused soft anions. Consequently, electrostatic interactions between the dye and the cell anions are supposed being driven entropically to establish a soft-cation-soft-anion couple (the B46 cation and the phospholipid anion) and a hard-cation-hard-anion couple (sodium cation and bromide anion).

The inhibition of bacterial decolorization by glucose on B46 further demonstrated that the reductive cleavage of the azo group took place under aerobic conditions. In addition, no residual toxicity was found in the *E. coli*-treated basic azo dye solutions. Thus, the observed differences in the acute toxicity could be related to a complete process of dye metabolism, excluding the formation of undesirable aromatic amines (EC 2006).

The results of the present study have important implications from an industrial point of view, since margins to implement such a bioremediation technology would be flexible to achieve the decolorization of textile and leather dyeing wastewaters in short time spells. Moreover, interesting recent results have opened the opportunity to select *E. coli* DH5α populations, by bacterial biomass recycle, in order to increase their decolorization abilities (Corsi, unpublished observations). So far, this approach has been applied to the new class of "naturalized" dyes (Pellegrini et al. 2015), water soluble and eco-friendly, synthesized from chromophores of commercial disperse colorants. Overall, these perspectives will represent further attractive improvements for *E. coli* dye decolorization in textile and leather industries, beyond the general classes of acid and basic dyes here studied.

Acknowledgments We gratefully acknowledge Tintoria Cometa Srl (Prato, Italy) for providing the commercial dyes and "Ente Cassa di Risparmio di Firenze" for financially supporting this research.

Ethical statement M. Cerboneschi states that there is no conflicts of interest between the authors of the present manuscript. This article does not contain any studies carried out on human or animal subjects.

References

Ali H (2010) Biodegradation of synthetic dyes – a review. Water Air Soil Pollut 213:251–273. doi:10.1007/s11270-010-0382-4

Ali N, Hameed A, Ahmed S, Khan AG (2008) Decolorization of structurally different textile dyes by *Aspergillus niger* SA1. World J Microbiol Biotechnol 24:1067–1072. doi:10.1007/s11274-007-9577-2

Alinsafi A, Evenou F, Abdulkarim EM, Pons MN, Zahraa O, Benhammou A, Yaacoubi A, Nejmeddine A (2007) Treatment of textile industry wastewater by supported photocatalysis. Dyes Pigments 74:439–445. doi:10.1016/j.dyepig.2006.02.024

Amoozegar MA, Hajighasemi M, Hamedi J, Asad S, Ventosa A (2011) Azo dye decolorization by halophilic and halotolerant microorganisms. Ann Microbiol 61:217–230. doi:10.1007/s13213-010-0144-y

Avlonitis SA, Poulios I, Sotiriou D, Pappas M, Moutesidis K (2008) Simulated cotton dye effluents treatment and reuse by nanofiltration. Desalination 221:259–267. doi:10.1016/j.desal.2007.01.082

Ayed L, Achour S, Khelifi E, Cheref A, Bakhrouf A (2010) Use of active consortia of constructed ternary bacterial cultures via mixture design for Congo Red decolorization enhancement. Chem Eng J 162:495–502. doi:10.1016/j.cej.2010.05.050

Berberidou C, Avlonitis S, Poulios I (2009) Dyestuff effluent treatment by integrated sequential photocatalytic oxidation and membrane filtration. Desalination 249:1099–1106. doi:10.1016/j.desal.2009.06.045

Brock TCM, Van Wijngaarden RPA (2012) Acute toxicity tests with *Daphnia magna*, *Americamysis bahia*, *Chironomus riparius* and *Gammarus pulex* and implications of new EU requirements for the aquatic effect assessment of insecticides. Environ Sci Pollut Res Int 19:3610–3618. doi:10.1007/s11356-012-0930-0

Chan GF, Gan HM, Rashid NAA (2012) Genome sequence of *Enterococcus* sp. Strain C1, an Azo Dye decolorizer. J Bacteriol 194:5716–5717. doi:10.1128/JB.01372-12

Chang JS, Lin CY (2001) Decolorization kinetics of recombinant *Escherichia coli* strain harboring azo dye decolorization determinants for *Rhodococcus* sp. Biotechnol Lett 23:631–636. doi:10.1023/A:1010306114286

Chang JS, Kuo TS, Chao YP, Ho JY, Lin PJ (2000) Azo dye decolorization with a mutant *Escherichia coli* strain. Biotechnol Lett 22:807–812. doi:10.1023/A:1005624707777

Chang JS, Chou C, Lin YC, Lin PJ, Ho JY, Hu TL (2001) Kinetic characteristics of bacterial azo-dye decolorization by *Pseudomonas*

luteola. Water Res 35:2841–2850. doi:10.1016/S0043-1354(00)00581-9

Chen JP, Lin YS (2007) Decolorization of azo dye by immobilized *Pseudomonas luteola* entrapped in alginate–silicate sol–gel beads. Process Biochem 42:934–942. doi:10.1016/j.procbio.2007.03.001

Chen H, Wang RF, Cerniglia CE (2004) Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from *Enterococcus faecalis*. Protein Expr Purif 34:302–310. doi:10.1016/j.pep.2003.12.016

Claudio L (2007) Waste couture: environmental impact of the clothing industry. Environ Health Perspect 115:A449–A454, PMCID: PMC1964887

Crosby DG, Tucker RK, Aharonson N (1966) The detection of acute toxicity with *Daphnia magna*. Food Cosmet Toxicol 4:503–514. doi:10.1016/S0015-6264(66)80637-5

Cui D, Li G, Zhao D, Zhao M (2015) Effect of quinoid redox mediators on the aerobic decolorization of azo dyes by cells and cell extracts from *Escherichia coli*. Environ Sci Pollut Res 22:4621–4630. doi:10.1007/s11356-014-3698-6

Daneshvar N, Ayazloo M, Khataee AR, Pourhassan M (2007) Biological decolorization of dye solution containing malachite green by microalgae *Cosmarium* sp. Bioresour Technol 98:1176–1182. doi:10.1016/j.biortech.2006.05.025

de Campos Ventura-Camargo B and Marin-Morales MA (2013) Azo dyes: characterization and toxicity– A review. Textiles and light industrial science and technology (TLIST) 2:85–103. http://www.tlist-journal.org/paperInfo.aspx?ID=5405

de Sousa IS, Castanheira EM, Rocha Gomes JI, Real Oliveira ME (2011) Study of the release of a microencapsulated acid dye in polyamide dyeing using mixed cationic liposomes. J Liposome Res 21(2):151–157. doi:10.3109/08982104.2010.492478

Dougan G, Haque A, Pickard D, Frankel G, O'Goara P, Wain J (2001) The *Escherichia coli* gene pool. Curr Opin Microbiol 4:90–94. doi:10.1016/S1369-5274(00)00170-3

EC (2006) The European Parliament and the Council. Regulation No 1907/2006. http://ec.europa.eu/enterprise/sectors/chemicals/documents/reach/index_en.htm#h2-1

Erkurt EA, Atacag Erkurt H, Unyayar A (2010) Decolorization of azo dyes by white rot fungi. In: atacag erkurt H (ed) biodegradation of azo dyes, Handb Environ Chem 9:157–167

Forgacs E, Cserhati T, Oros G (2004) Removal of synthetic dyes from wastewaters: a review. Environ Int 30:953–971. doi:10.1016/j.envint.2004.02.001

Fu Y, Viraraghavan T (2001) Fungal decolorization of dye wastewaters: a review. Bioresour Technol 79:251–262. doi:10.1016/S0960-8524(01)00028-1

Ghaly AE, Ananthashankar R, Alhattab M, Ramakrishnan VV (2014) Production, characterization and treatment of textile effluents: a critical review. J Chem Eng Process Technol 5:182. doi:10.4172/2157-7048.1000182

Giwa A, Giwa FJ, Ifu BJ (2012) Microbial decolourization of an anthraquinone dye C.I reactive blue 19 using *Bacillus cereus*. ACSj 2:60–68. doi:10.9734/ACSJ/2012/1519

Hao OJ, Kim H, Chiang PC (2000) Decolorization of wastewater. Crit Rev Environ Sci Technol 30:449–505. doi:10.1080/10643380091184237

Harrelkas F, Azizi A, Yaacoubi A, Benhammou A, Pons MN (2009) Treatment of textile dye effluents using coagulation–flocculation coupled with membrane processes or adsorption on powdered activated carbon. Desalination 235:330–339. doi:10.1016/j.desal.2008.02.012

Ho TL (1975) Hard soft acids bases (HSAB) principle and organic chemistry. Chem Rev 75:1–20. doi:10.1021/cr60293a001

Hunger H (2002) Industrial dyes. Wiley, London

Işık M, Sponza DT (2003) Effect of oxygen on decolorization of azo dyes by *Escherichia coli* and *Pseudomonas* sp. and fate of aromatic amines. Process Biochem 38:1183–1192. doi:10.1016/S0032-9592(02)00282-0

Jin XC, Liu GQ, Xu ZH, Tao WY (2007) Decolorization of a dye industry effluent by *Aspergillus fumigatus* XC6. Appl Microbiol Biotechnol 74:239–243. doi:10.1007/s00253-006-0658-1

Kaushik P, Malik A (2009) Fungal dye decolorization: recent advances and future potential. Environ Int 35:127–141. doi:10.1016/j.envint.2008.05.010

Khalid A, Arshad M, Crowley DE (2008) Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains. Appl Microbiol Biotechnol 78:361–369. doi:10.1007/s00253-007-1302-4

Khan R, Bhawana P, Fulekar MH (2013) Microbial decolorization and degradation of synthetic dyes: a review. Rev Environ Sci Biotechnol 12:75–97. doi:10.1007/s11157-012-9287-6

Khataee AR, Dehghan G, Ebadi A, Zarei M, Pourhassan M (2010) Biological treatment of a dye solution by macroalgae *Chara* sp.: effect of operational parameters, intermediates identification and artificial neural network modeling. Bioresour Technol 101:2252–2258. doi:10.1016/j.biortech.2009.11.079

Kutsu S (2007) Growth of *E. coli* and determination of its doubling times on enriched and minimal media. PMB C112L. http://coli.berkeley.edu/kustu/lab4.pdf

Liu G, Zhou J, Fu QS, Wang J (2009) The *Escherichia coli* azoreductase AzoR is involved in resistance to thiol-specific stress caused by electrophilic quinines. J Bacteriol 191:6394–6400. doi:10.1128/JB.00552-09

Lončar N, Božić N, Lopez-Santin J, Vujčić Z (2013) *Bacillus amyloliquefaciens* laccase – from soil bacteria to recombinant enzyme for wastewater decolorization. Bioresour Technol 147:177–183. doi:10.1016/j.biortech.2013.08.056

Marimuthu T, Rajendran S, Manivannan M (2013) Fungal decolorization of dye water. Int J Sci Eng Res 4:1635–1645, ISSN 2229–5518

Mate MS, Pathade G (2012) Biodegradation of C.I. Reactive Red 195 by *Enterococcus faecalis* strain YZ66. World J Microbiol Biotechnol 28:815–826. doi:10.1007/s11274-011-0874-4

McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat I, Marchant R, Smyth W (2001) Microbial decolourization and degradation of textile dyes. Appl Microbiol Biotechnol 56:81–87. doi:10.1007/s002530000587

Mercier C, Chalansonnet V, Orenga S, Gilbert C (2013) Characteristics of major *Escherichia coli* reductases involved in aerobic nitro and azo reduction. J Appl Microbiol 115:1012–1022. doi:10.1111/jam.12294

Modi HA, Rajput G, Ambasana C (2010) Decolorization of water soluble azo dyes by bacterial cultures, isolated from dye house effluent. Bioresour Technol 101:6580–6583. doi:10.1016/j.biortech.2010.03.067

Novotný C, Svobodová K, Benada O, Kofroňová O, Heissenberger A, Fuchs W (2011) Potential of combined fungal and bacterial treatment for color removal in textile wastewater. Bioresour Technol 102:879–888. doi:10.1016/j.biortech.2010.09.014

Ogugbue CJ, Sawidis T (2011) Bioremediation and detoxification of synthetic wastewater containing triarylmethane dyes by *Aeromonas hydrophila* isolated from industrial effluent. Biotechnol Res Int 2011:11. doi:10.4061/2011/967925

Pandey A, Singh P, Iyengar L (2007) Bacterial decolorization and degradation of azo dyes. Int Biodeterior Biodegrad 59:73–84. doi:10.1016/j.ibiod.2006.08.006

Pellegrini D, Corsi M, Bonanni M, Bianchini R, D'Ulivo A, Bramanti E (2015) Study of the interaction between collagen and naturalized and commercial dyes by fourier transform infrared spectroscopy and thermogravimetric analysis. Dyes Pigments 116:65–73. doi:10.1016/j.dyepig.2015.01.012

Phugare SS, Kalyani DC, Patil AV, Jadhav JP (2011) Textile dye degradation by bacterial consortium and subsequent toxicological

analysis of dye and dye metabolites using cytotoxicity, genotoxicity and oxidative stress studies. J Hazard Mater 186:713–723. doi:10.1016/j.jhazmat.2010.11.049

Pokharia A and Ahluwalia SS (2013) Isolation and screening of dye decolorizing bacterial isolates from contaminated sites. Textiles and light industrial science and technology (TLIST) 2:54–61. http://www.tlist-journal.org/paperInfo.aspx?ID=5410

Qu Y, Cao X, Ma Q, Shi S, Tan L, Li X, Zhou H, Zhang X, Zhou J (2012) Aerobic decolorization and degradation of acid Red B by a newly isolated *Pichia* sp. TCL. J Hazard Mater 223–224:31–38. doi:10.1016/j.jhazmat.2012.04.034

Rau J, Knackmuss HJ, Stolz A (2002) Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria. Environ Sci Technol 36:1497–1504. doi:10.1021/es010227+

Sandhya S, Sarayu K, Uma B, Swaminathan K (2008) Decolorizing kinetics of a recombinant *Escherichia coli* SS125 strain harboring azoreductase gene from *Bacillus latrosporus* RRK1. Bioresour Technol 99:2187–2191. doi:10.1016/j.biortech.2007.05.027

Saratale RG, Saratale GD, Chang JS, Govindwar SP (2011) Bacterial decolorization and degradation of azo dyes: a review. J Taiwan Inst Chem Eng 42:138–157. doi:10.1016/j.jtice.2010.06.006

Sarioglu M, Bali U, Bisgin T (2007) The removal of C.I. Basic Red 46 in a mixed methanogenic anaerobic culture. Dyes Pigments 74:223–229. doi:10.1016/j.dyepig.2006.02.001

Sarnaik S, Kanekar P (1995) Bioremediation of colour of methyl violet and phenol from a dye-industry waste effluent using *Pseudomonas* spp. Isolated from factory soil. J Appl Bacteriol 79:459–469. doi:10.1111/j.1365-2672.1995.tb03162.x

Satheesh Babu S, Mohandass C, Vijay Raj AS, Rajasabapathy R, Dhale MA (2013) Multiple approaches towards decolorization and reuse of a textile dye (VB-B) by a marine bacterium *Shewanella decolorationis*. Water Air Soil Pollut 224:1500. doi:10.1007/s11270-013-1500-x

Sezonov G, Joseleau-Petit D, D'Ari R (2007) *Escherichia coli* physiology in luria-bertani broth. J Bacteriol 189:8746–8749. doi:10.1128/JB.01368-07

Shobana S, Thangam EB (2012) Biodegradation and decolorization of reactive orange 16 by *Nocardiopsis alba* soil isolate. J Bioremed Biodeg 3:155. doi:10.4172/2155-6199.1000155

Shore J (2002) Colorants and auxiliaries, 2nd edn. Society of Dyers and Colorists, Bradford

Singh K, Arora S (2011) Removal of synthetic textile dyes from wastewaters: a critical review on present treatment technologies. Crit Rev Environ Sci Technol 41:807–878. doi:10.1080/10643380903218376

Slater K (2003) Environmental impact of textiles: production, processes and protection. Woodhead publishing limited, Cambridge. https://www.elsevier.com/books/environmental-impact-of-textiles/slater/978-1-85573-541-5

Solís M, Solís A, Pérez HI, Manjarrez N, Flores M (2012) Microbial decolouration of azo dyes: a review. Process Biochem 47:1723–1748. doi:10.1016/j.procbio.2012.08.014

Tegli S, Cerboneschi M, Corsi M, Bonnanni M, Bianchini R (2014) Water recycle as a must: decolorization of textile wastewaters by plant-associated fungi. J Basic Microbiol 54:120–132. doi:10.1002/jobm.201200401

The Freedonia Group (2013) World dyes & organic pigments, industry study with forecasts for 2013 and 2018

Van Der Zee FP, Villaverde S (2005) Combined anaerobic-aerobic treatment of azo dyes - a short review of bioreactor studies. Water Res 39:1425–1440. doi:10.1016/j.watres.2005.03.007

Verma Y (2008) Acute toxicity assessment of textile dyes and textile and dyes industrial effluents using *Daphnia magna* bioassay. Toxicol Ind Health 24:491–500. doi:10.1177/0748233708095769

Verma AK, Raghukumar C, Parvatkar RR, Naik CG (2012) A rapid two-step bioremediation of the anthraquinone dye reactive blue 4 by a marine derived fungus. Water Air Soil Pollut 223:3499–3509. doi:10.1007/s11270-012-1127-3

Vitor V, Corso CR (2008) Decolorization of textile dye by *Candida albicans* isolated from industrial effluents. J Ind Microbiol Biotechnol 35:1353–1357. doi:10.1007/s10295-008-0435-5

Xian-Chun J, Gao-Qiang L, Zheng-Hong X, Wen-Yi T (2007) Decolorization of a dye industry effluent by *Aspergillus fumigatus* XC6. Appl Microbiol Biotechnol 74:239–243

Zaharia C and Suteu D (2012) Textile organic dyes – characteristics, polluting effects and separation/elimination procedures from industrial effluents – a critical overview. Organic pollutants 10 years after the Stockholm convention. Environ Anal Updat. Dr Tomasz Puzyn, ISBN:978-953-307-917-2. doi:10.5772/1381

Zhao G, Gao J, Shi W, Liu M, Li D (2009) Electrochemical incineration of high concentration azo dye wastewater on the in situ activated platinum electrode with sustained microwave radiation. Chemosphere 77:188–193. doi:10.1016/j. chemosphere .2009.07.044