J Ind Microbiol Biotechnol (2011) 38:1729–1738
DOI 10.1007/s10295-011-0962-3

ORIGINAL PAPER

Effects of Orange II and Sudan III azo dyes and their metabolites on *Staphylococcus aureus*

Hongmiao Pan · Jinhui Feng · Carl E. Cerniglia · Huizhong Chen

Received: 28 January 2011 / Accepted: 12 March 2011 / Published online: 31 March 2011
© Springer-Verlag(outside the USA) 2011

Abstract Azo dyes are widely used in the plastic, paper, cosmetics, food, and pharmaceutical industries. Some metabolites of these dyes are potentially genotoxic. The toxic effects of azo dyes and their potential reduction metabolites on *Staphylococcus aureus* ATCC BAA 1556 were studied. When the cultures were incubated with 6, 18, and 36 μg/ml of Orange II and Sudan III for 48 h, 76.3, 68.5, and 61.7% of Orange II and 97.8, 93.9, and 75.8% of Sudan III were reduced by the bacterium, respectively. In the presence of 36 μg/ml Sudan III, the cell viability of the bacterium decreased to 61.9% after 48 h of incubation, whereas the cell viability of the control culture without the dye was 71.5%. Moreover, the optical density of the bacterial cultures at 10 h decreased from 0.74 to 0.55, indicating that Sudan III is able to inhibit growth of the bacterium. However, Orange II had no significant effects on either cell growth or cell viability of the bacterium at the tested concentrations. 1-Amino-2-naphthol, a metabolite common to Orange II and Sudan III, was capable of inhibiting cell growth of the bacterium at 1 μg/ml and completely stopped bacterial cell growth at 24–48 μg/ml. On the other hand, the other metabolites of Orange II and Sudan III, namely sulfanilic acid, *p*-phenylenediamine, and aniline, showed no significant effects on cell growth. *p*-Phenylenediamine exhibited a synergistic effect with 1-amino-2-naphthol on cell growth inhibition. All of the dye metabolites had no significant effects on cell viability of the bacterium.

H. Pan · J. Feng · C. E. Cerniglia · H. Chen (✉)
Division of Microbiology, National Center for Toxicological Research, US FDA, 3900 NCTR Rd., Jefferson, AR 72079-9502, USA
e-mail: huizhong.chen@fda.hhs.gov

Keywords *Staphylococcus aureus* · Orange II · Sudan III · Metabolite · Cell growth and viability

Introduction

Azo dyes consist of a diazotized amine coupled to an amine or a phenol, and contain one or more azo linkages (R–N=N–R) [12]. Thousands of different varieties of azo dyes are extensively used in the textile, paper, food, cosmetics, and pharmaceutical industries [12, 14]. Many color additives used in cosmetics are azo colorants [46]. Through ingestion, inhalation, or skin contact, humans are exposed to these compounds. Previous reports have shown that some aromatic amines, as metabolites formed from mammalian and microbial enzymatic reduction of azo dyes, are potentially genotoxic to humans, fish, and animals [1, 13, 28]. The initial step of degradation of azo dyes is reduction of the azo bond catalyzed by azoreductase. Azoreductases are commonly found in bacteria and have been purified and characterized from *Bacillus subtilis* [39], *Escherichia coli* [25, 26], *Enterococcus faecalis* [11, 34, 35], *Pseudomonas aeruginosa* [45, 47, 48], *Saccharomyces cereviaeae* [32], and *Staphylococcus aureus* [9]. The enzymes can be classified into three groups, flavin-dependent NADH-preferred azoreductase, flavin-dependent NADPH-preferred azoreductase, and flavin-free NADPH-preferred azoreductase [8].

Orange II (D&C Orange No. 4) is a sulfonated dye that is approved for use in drug and cosmetic products as a colorant [6, 37]. Sudan dyes are a family of industrial red dyes, normally used for coloring plastics and other synthetic materials [2, 5, 10]. These dyes are classified by the International Agency for Research on Cancer (IARC) as category 3 carcinogens to humans. Although Sudan dyes

are banned for food usage in most countries, they are illegally used to enhance or maintain the colored appearance of food products [49, 50]. Some Sudan dyes are also pigments of cosmetics. For example, Sudan I is in the Indian cosmetic "kumkum" and is frequently applied to the forehead [29]. Sudan III is also permitted for use in drug and cosmetic products and classified as colorant D&C Red No. 17. Under microaerobic conditions, many human commensal bacteria are able to reduce azo dyes to produce their respective aromatic compounds [46, 49, 50]. The bacterial reduction pathway of the dyes is well established: 1-amino-2-naphthol and sulfanilic acid are the metabolites of Orange II; and 1-amino-2-naphthol, *p*-phenylenediamine, and aniline are the reduction products of Sudan III (Table 1) [24, 36, 49].

The human skin harbors a complex and diverse microbiota comprised of at least several hundred species, which are vastly different from, but just as complex as, those of the intestine [21]. The skin microbiota are a dynamic population that is influenced by its host and environment. Interactions between the microbiota and human host have implications for nutrition, infection, metabolism, toxicity, and cancer. The skin microbiota play a very important role in preventing pathogens from establishing in the skin and causing disease [16]. This process is known as colonization resistance and is a major beneficial effect of the normal skin microbiota. Externally applied cosmetics containing azo dyes could potentially impact the microbial ecology of the skin, which may affect human health by breaking the permeability barrier, which could encourage pathogen colonization. To our understanding, the effects of azo dyes used in cosmetics and their potential metabolites on human skin microbiota have not been addressed. Concerns about the safety of azo colorants in cosmetics have been raised because metabolic cleavage by skin bacteria yielded aromatic amines [15, 30, 43]. For human safety evaluation of the dermal exposure to azo dyes in cosmetics, the potential toxic effects on skin microbiota and the human body should be considered.

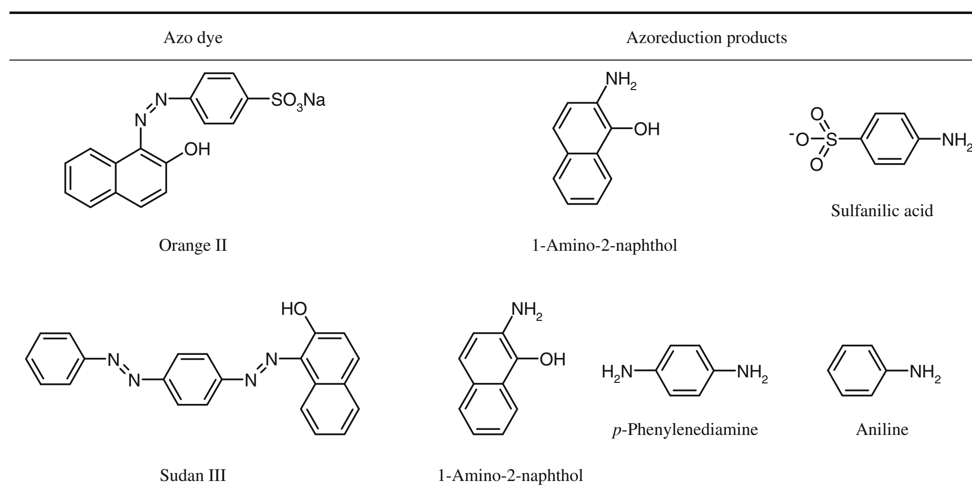
Staphylococcus aureus is a part of normal flora colonizing humans asymptomatically [7, 38]. *S. aureus* may be found widely over the surface of both normal and diseased skin. Some strains of *S. aureus* have been demonstrated to produce bacteriocins [16]. *S. aureus* is the most common cause of hospital-acquired infections; about 2% of all patients admitted are infected [33]. *S. aureus* is also one of the commonly identified foodborne etiologic agents [17]. It has been suggested that *S. aureus* may serve as a model strain for measuring the ability of human skin microbiota to metabolize azo dyes [43]. Our previous studies have shown that at least a portion of human skin microbiota is able to metabolize azo dyes [9, 46]. This study examines the effects of Orange II and Sudan III azo dyes used in cosmetics and their metabolites on the cell growth and viability of *S. aureus*.

Materials and methods

Materials

Orange II (4-(2-hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt), Sudan III (1-[4-(phenylazo)phenylazo]-2-naphthol), 1-amino-2-naphthol (hydrochloride), sulfanilic acid, aniline, *p*-phenylenediamine, dimethyl sulfoxide (DMSO), and absolute ethanol were purchased from Sigma Chemical Co. The LIVE/DEAD BacLight bacterial viability and counting kit containing solutions of 3.34 mM SYTO9 in DMSO (200 µl), 20 mM propidium iodide (PI) in DMSO (200 µl), and a calibrated suspension of microspheres (diameter 6 µm, 1 ml; concentration 1.0×10^8 beads/ml) was purchased from Molecular Probes. Stock solutions of

Table 1 Chemical structures of Orange II and Sudan III and their azoreduction products



Sudan III, sulfanilic acid, 1-amino-2-naphthol, aniline, and *p*-phenylenediamine were prepared by dissolving the chemicals in DMSO.

Bacterial strain and culture conditions

Because of the availability of genome sequence and our unpublished microarray data, *S. aureus* ATCC BAA 1556 was chosen for the experiments. The strain was routinely cultured on Brain Heart Infusion (BHI) agar. After 16–18 h of incubation at 37°C, one colony was picked by a loop and inoculated into a 50-ml centrifuge tube containing 50 ml BHI medium. The culture was incubated under static conditions at 37°C overnight for use as seed culture. The bacterial seed culture was inoculated into BHI medium with an inoculation ratio of 0.5% (v/v), and then the medium was transferred to a centrifuge tube containing 50 ml BHI medium. Orange II or Sudan III was added to the BHI medium at final concentrations of 6, 18, and 36 μg/ml (each in triplicate, all experiments were in triplicate unless otherwise stated) according to Muzzall and Cook [40]. The cultures were incubated at 37°C without agitation. After 24 and 48 h incubation, the cultures were collected for measurement of Orange II and Sudan III degradation and cell viability of the bacterium as described below. To determine the effect of the azo dyes and their metabolites on growth of the bacterium, 200 μl culture per well with various concentrations of the dyes or metabolites was added to a 96-well plate (BD Falcon). Each individual metabolite or a combination of the metabolites was added to the cultures to study their effects on the cell growth of the bacterium. If needed, the metabolites were added at a ratio of approximately 12:11 in weight for 1-amino-2-naphthol/sulfanilic acid and 6:12:7 for aniline/1-amino-2-naphthol/*p*-phenylenediamine to match the ratio of products from the reduction of Orange II and Sudan III, respectively. Bacterial growth of the cultures was monitored at 660 nm in a SpectraMax M2 plate reader (Molecular Devices) at 37°C for 12 h, with 10 s of shaking before readings. For examining the effect of metabolites on cell viability, bacterial cells were inoculated at a ratio of 0.5% (v/v) into BHI medium and 50-ml aliquots of the cultures were transferred to centrifuge tubes. The cultures were allowed to grow to the stationary phase at 37°C for 12 h. Thereafter, dye metabolites at various concentrations were added to the cultures. After exposure of the bacterial cells to the metabolites for 12 h, the bacterium was collected for cell viability measurement.

Assay of the degradation of azo dyes by *S. aureus*

To determine degradation of Orange II, 1-ml samples were collected from the cultures at 0, 24, and 48 h, and centrifuged at 10,000×*g* for 3 min. Supernatants of the cultures were assayed by measuring the absorption in the plate reader at 483 nm [46]. To determine degradation of Sudan III, two volumes of absolute ethanol were added to 0.5 ml of the samples collected from the cultures at 0, 24, and 48 h, respectively. The mixtures were briefly vortexed, and then the samples were assayed at 500 nm following the procedure described above for Orange II [49]. Simultaneously, the bacterial cell viability of the samples was determined as described below.

Bacterial cell viability assay

One-milliliter samples collected from the cultures were stained according to the manufacturer's instruction using the BacLight LIVE/DEAD bacterial viability and counting kit. The bacterial cell viability assay was analyzed by flow cytometry (FCM) on an Accuri C6 FCM (Accuri Cytometers), with a 488-nm excitation from a blue solid-state laser at 50 mW. Fluorescence filters and detectors were all standardized with green fluorescence collected in the FL1 channel (530 ± 15 nm) and red fluorescence collected in the FL3 channel (>670 nm). All parameters were collected as logarithmic signals, including the forward scatter (FSC) and side scatter (SSC). Fluorescence (FL1) trigger threshold of 2,000 and secondary FL3 threshold of 500 were used in order to remove noise. Flow rate of the samples was adjusted to keep the event rate below 5,000 events per second. At least 20,000 cells for each sample were counted. Data were analyzed using CFlow Plus software (Accuri Cytometers). In density plots of light-scattering properties, bacterial cells were gated from irrelevant counts for fluorescence analyses. In density plots of fluorescence, the distinct bacterial populations (P1, live cells; P2, dead cells) were gated on the basis of the different viability stages. Cell viability (%) = percent of P1 (green cells)/(percent of P1 + percent of P2 (red cells)) × 100.

Results

Degradation of Orange II and Sudan III by *S. aureus*

We used *S. aureus* as a model strain of human skin microbiota to evaluate effects of two cosmetic dyes, Orange II and Sudan III, at various concentrations on skin bacteria. The disappearance of the dyes, indicative of reduction, was measured spectrophotometrically in the presence of the bacterium (Table 2). When the cultures were incubated for 48 h, 76.3, 68.5, and 61.7% of Orange II added at 6, 18, and 36 μg/ml were reduced, respectively; whereas 97.8, 93.9, and 75.8% of Sudan III added at the same concentrations were metabolized, respectively. The degradation

Table 2 Reduction of Orange II and Sudan III at various concentrations by *S. aureus*

Dye	Concentration (μg/ml)	Dye degradation (%)[a]	
		Culture time 24 h	Culture time 48 h
Orange II	6	60.3 ± 1.9	76.3 ± 1.4
	18	48.5 ± 0.9	68.5 ± 0.5
	36	40.6 ± 0.8	61.7 ± 0.6
Sudan III	6	76.2 ± 1.0	97.8 ± 1.0
	18	49.6 ± 0.4	93.9 ± 0.9
	36	34.9 ± 0.3	75.8 ± 0.9

Data are presented in mean ± SD. The mean was from triplicate incubations with standard deviations (SDs)

[a] Sterile control with dyes was used as control. The stock solution of the dyes was freshly prepared and there was no detectable degradation of the dyes in DMSO or medium for 24 and 48 h

efficiency decreased with the increase in concentrations of the dyes and *S. aureus* degraded Sudan III more effectively than Orange II. The azoreductase activity in the control cultures without dye was 0.84 U × 10^{-4}/ml culture and there were no apparent changes in the enzyme activities of the cultures with various concentrations of Orange II or Sudan III, indicating that constitutive azoreductases are produced in *S. aureus*.

Effects of Orange II and Sudan III on the cell growth and viability of *S. aureus*

As shown in Fig. 1a, the optical densities (ODs) of the bacterial cultures with Orange II at 6 and 18 μg/ml were 0.74 and 0.73, respectively, at 10 h. Even when the concentration of Orange II was 36 μg/ml, the cultures grew to an OD of 0.74, which is very similar to that of the cultures without azo dyes (0.72). Orange II had no effect on the growth of *S. aureus* in the presence of different concentrations of the dye. However, the observed behavior was different for Sudan III. As shown in Fig. 1b, when the concentration of Sudan III increased from 6 to 36 μg/ml, an inhibition on the bacterial cell growth was observed.

The effects of Orange II and Sudan III on the bacterial cell viability were determined by flow cytometric measurements using the BacLight LIVE/DEAD bacterial viability and counting kit. As shown in Fig. 2, two distinctive groups are formed. Group P1 is the population of living bacterial cells, whereas group P2 is the population of dead bacterial cells. The cell viabilities of *S. aureus* with various concentrations of dyes are illustrated in Table 3. At 24 h of incubation, the percentages of living bacterial cells with Orange II at concentrations of 6, 18, and 36 μg/ml were between 90 and 95%, similar to that of the cultures without dye. When the incubation time was prolonged to 48 h, bacterial cell viabilities were similar in the presence and absence of Orange II. Therefore, the addition of Orange II to the cultures did not affect the cell viability of *S. aureus*. As for Sudan III, the percentage of the living bacterial cells with increased dye concentrations from 6 to 36 μg/ml decreased from 95.5 to 89.3% for 24-h cultures and from 74.2 to 61.9% for 48-h cultures. Therefore, with increasing Sudan III (36 μg/ml) concentration in the cultures, more dead bacterial cells were observed.

Effects of dye metabolites on the cell growth and viability of *S. aureus*

The proposed metabolites of Orange II formed by bacteria are 1-amino-2-naphthol and sulfanilic acid. The three metabolites of the bacterial reduction of Sudan III are 1-amino-2-naphthol, *p*-phenylenediamine, and aniline. As shown in Fig. 3, sulfanilic acid and aniline had no significant effects on the cell growth of *S. aureus*. Even at the highest tested concentrations of sulfanilic acid (66 μg/ml) and aniline (36 μg/ml), both growth curves of the bacterium were

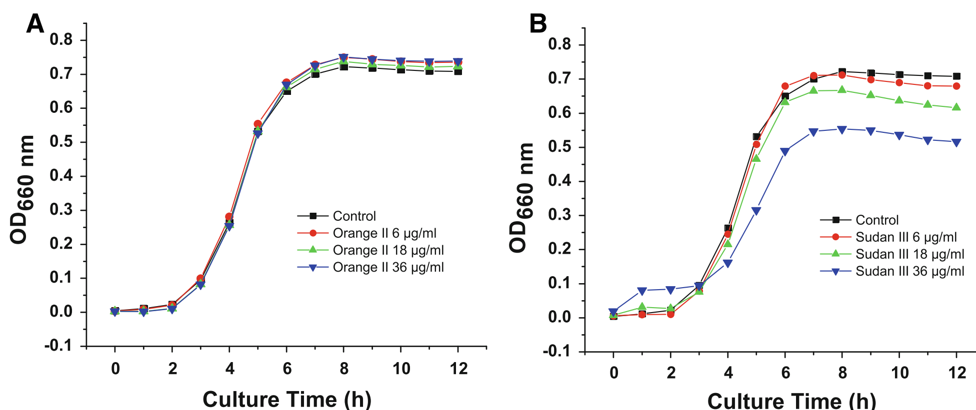


Fig. 1 Effects of various concentrations of **a** Orange II and **b** Sudan III on the cell growth of *S. aureus*. Each data point is presented as mean ± SD. The mean was from triplicate incubations with SDs < 5%

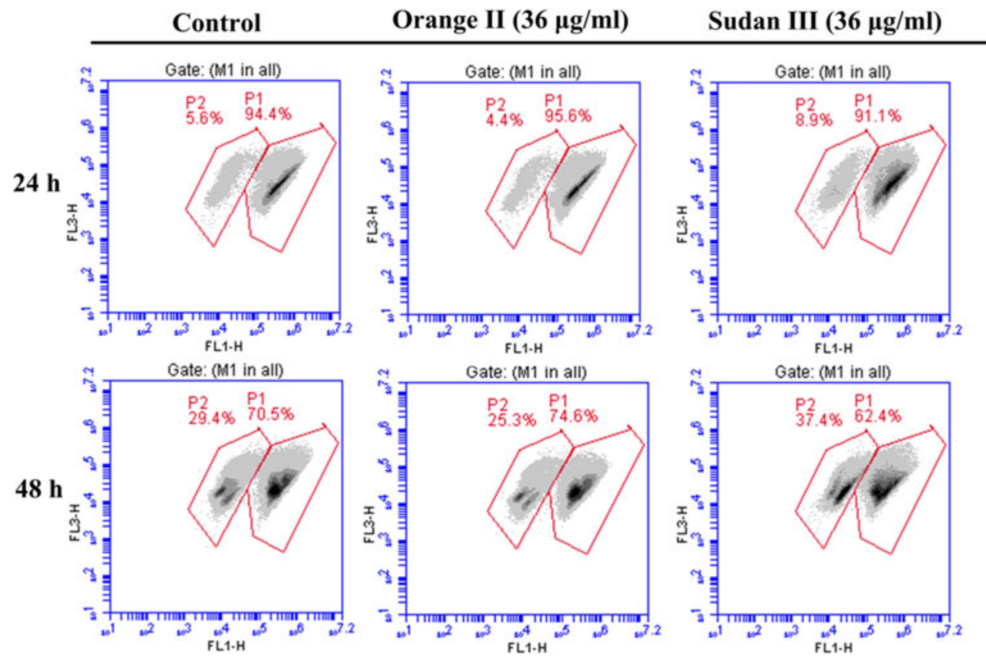


Fig. 2 Effects of Orange II and Sudan III on the cell viability of *S. aureus*. The samples were stained using SYTO9 plus PI. For all FL1-H/FL3-H density plots, *horizontal axes* (FL1-H) represent green fluorescence and *vertical axes* (FL3-H) represent red fluorescence (FL1, 530 ± 15 nm; FL3, >670 nm). Gate P1 and P2 represent those used to define populations designated as live and dead cells, respectively

similar to that of the control (Fig. 3f). At the lowest tested concentration of *p*-phenylenediamine, much less than 7 μg/ml, the metabolite had no significant effect on the cell growth of the bacterium. Similar results were also found when aniline and *p*-phenylenediamine were added to culture as a mixture, as shown in Fig. 3a, b, c. For example, the ODs at 10 h were 0.78, 0.75, and 0.76 for the cultures with 6 μg/ml aniline, 7 μg/ml *p*-phenylenediamine, and the mixture of these two metabolites, respectively, similar to the control (OD = 0.76). When the concentration of *p*-phenylenediamine increased both with and without the aniline, a slight inhibition on the cell growth of the bacterium was observed, as shown in Fig. 3d, e, f. The inhibition on the bacterial cell growth by 1-amino-2-naphthol occurred at a concentration of 1 μg/ml, as shown in Fig. 3a. A stronger inhibition was observed with increasing concentrations of the metabolite. For example, the OD of the cultures with 48 μg/ml of 1-amino-2-naphthol was only 0.12 at 10 h (Fig. 3e). In addition, any mixture that contained 1-amino-2-naphthol inhibited the cell growth. In Fig. 3c, d, the strongest inhibition belonged to the mixtures containing both *p*-phenylenediamine and 1-amino-2-naphthol. ODs of the cultures at 10 h were 0.32, 0.34, and 0.61 for the mixture of 1-amino-2-naphthol, *p*-phenylenediamine, and aniline, of 1-amino-2-naphthol and *p*-phenylenediamine, and of 1-amino-2-naphthol and aniline, respectively (Fig. 3c). High concentrations of 1-amino-2-naphthol or any mixture containing this metabolite can completely inhibit the cell growth of the bacterium, as shown in Fig. 3e, f.

The cell viabilities of *S. aureus* with the individual metabolites of Orange II and Sudan III or combinations of the metabolites were measured, as shown in Table 4. Each individual metabolite or mixture of them had no apparent effect on the cell viability even when the concentration was as high as 1 mg/ml for sulfanilic acid, aniline, and *p*-phenylenediamine or 100 μg/ml for 1-amino-2-naphthol. After another 12 h, the cell viabilities were 90.5 90.8, 91.5, and 90.3% for cultures with 1 mg/ml sulfanilic acid, 1 mg/ml aniline, 1 mg/ml *p*-phenylenediamine, and 100 μg/ml 1-amino-2-naphthol, respectively. The cell viability of the cultures with 110 μg/ml sulfanilic acid and 120 μg/ml 1-amino-2-naphthol, or 60 μg/ml aniline, 120 μg/ml 1-amino-2-naphthol, and 70 μg/ml *p*-phenylenediamine for 12 h were 87.5% or 89.9%, whereas that of the culture without metabolite was 90.0%. The metabolites of the azo dyes had no significant effects on the cell viability of *S. aureus*.

Table 3 Effect of Orange II and Sudan III on the cell viability of *S. aureus*

Item	Concentration of the dyes (μg/ml)	Cell viability (%)	
		Culture time 24 h	Culture time 48 h
Control (no dyes)		92.7 ± 2.3	71.5 ± 0.5
Orange II	6	92.8 ± 2.4	71.6 ± 3.1
	18	94.4 ± 0.2	75.2 ± 1.7
	36	94.7 ± 1.7	74.2 ± 0.8
Sudan III	6	95.5 ± 1.2	74.2 ± 2.7
	18	92.5 ± 1.5	74.7 ± 0.8
	36	89.3 ± 2.2	61.9 ± 3.6

Data are presented in mean ± SD. The mean was from triplicate incubations with SDs

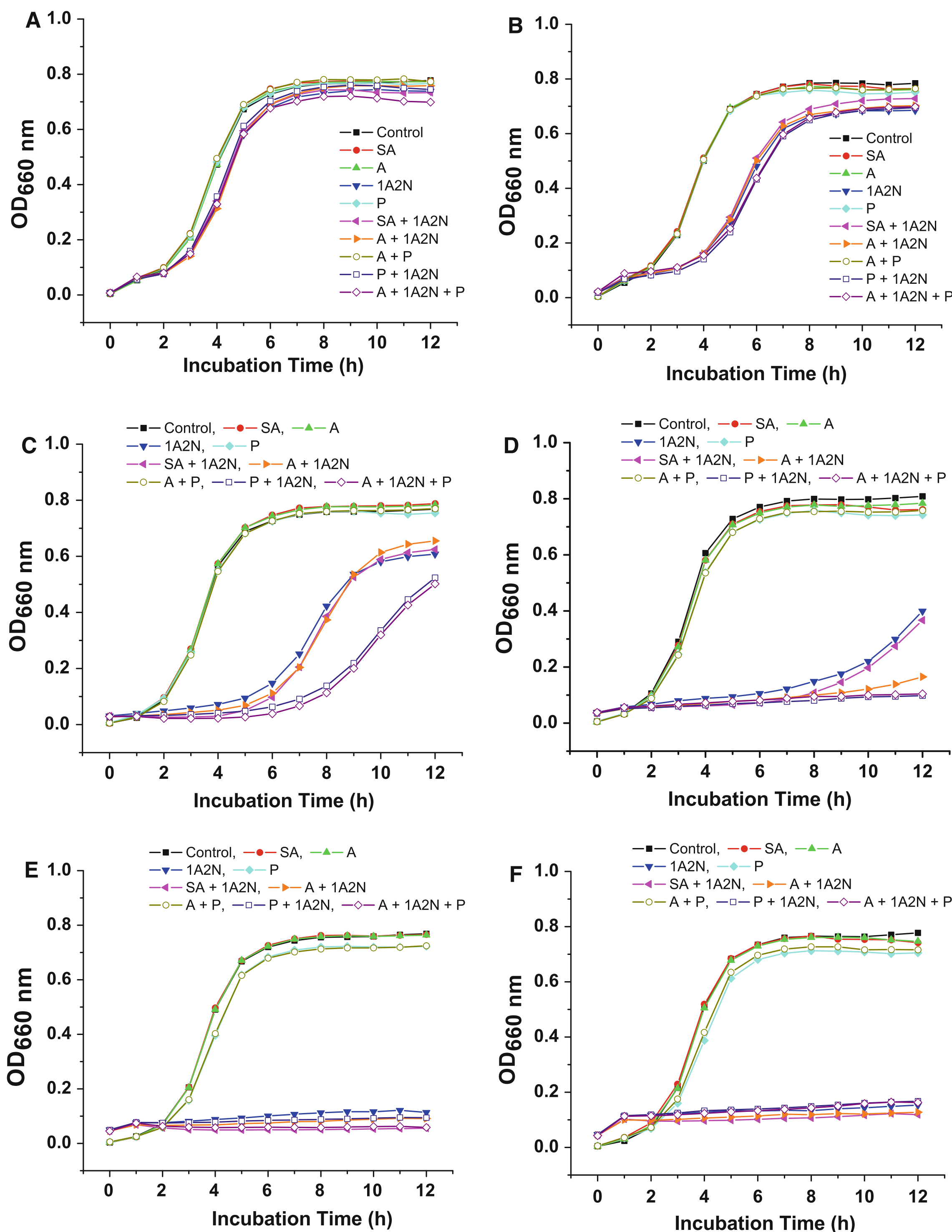


Fig. 3 Effects of metabolites on *S. aureus* cell growth. Aniline (A), 1-amino-2-naphthol (1A2N), *p*-phenylendiamine (P), sulfanilic acid (SA). *Filled squares*, control; *filled circles*, cultured with SA; *filled triangles*, cultured with A; *filled down triangles*, cultured with 1A2N; *filled diamonds*, cultured with P; *filled left triangles*, cultured with the mixture of SA and 1A2N; *filled right triangles*, cultured with the mixture A and 1A2N; *open circles*, cultured with the mixture of A and P; *open squares*, cultured with the mixture of P and 1A2N; *open diamonds*, cultured with the mixture of A, P, and 1A2N. Concentrations of SA, A, 1A2N, and P were respectively **a** 0.9, 0.5, 1, and 0.6 μg/ml; **b** 5.5, 3, 6, and 3.5 μg/ml; **c** 11, 6, 12, and 7 μg/ml; **d** 22, 12, 24, and 14 μg/ml; **e** 44, 24, 48, and 28 μg/ml; **f** 66, 36, 72, and 42 μg/ml. Each data point is presented as mean ± SD. The mean was from triplicate incubations with SDs < 5%

Table 4 Effects of different metabolites on the cell viability of *S. aureus*

Concentrations of metabolites	Cell viability (%)[a]
Control (no metabolites)	90.0 ± 3.2
Sulfanilic acid (11 μg/ml)	91.2 ± 1.1
Sulfanilic acid (1 mg/ml)	90.5 ± 0.7
1-Amino-2-naphthol (1 μg/ml)	87.8 ± 4.0
1-Amino-2-naphthol (12 μg/ml)	89.8 ± 2.2
1-Amino-2-naphthol (100 μg/ml)	90.3 ± 1.7
1-Amino-2-naphthol (120 μg/ml)	86.3 ± 3.1
Sulfanilic acid (11 μg/ml) + 1-amino-2-naphthol (12 μg/ml)	88.6 ± 1.6
Sulfanilic acid (110 μg/ml) + 1-amino-2-naphthol (120 μg/ml)	87.5 ± 0.1
Aniline (6 μg/ml)	88.7 ± 0.4
Aniline (1 mg/ml)	90.8 ± 1.3
p-Phenylenediamine (7 μg/ml)	88.7 ± 1.9
p-Phenylenediamine (1 mg/ml)	91.5 ± 0.4
Aniline (6 μg/ml) + *p*-phenylenediamine (7 μg/ml) + 1-amino-2-naphthol (12 μg/ml)	89.0 ± 1.8
Aniline (60 μg/ml) + *p*-phenylenediamine (70 μg/ml) + 1-amino-2-naphthol (120 μg/ml)	89.9 ± 1.5

The dye metabolites were added after incubating the cultures at 37°C for 12 h. After exposure of the bacterial cells to the metabolites for 12 h, the bacterium was collected for cell viability measurement

[a] Data are presented as mean ± SD. The mean was from triplicate incubations with SDs

Discussion

Azo dyes are mainly reduced by bacteria to colorless aromatic amines and the resulting amines can be further degraded aerobically by bacteria. Some bacteria have the capacity to metabolize azo dyes both aerobically and anaerobically. White rot fungi can efficiently break down azo dyes through oxidation by utilizing ligninolytic enzymes [51]. Simultaneous heavy metal reduction and azo dye decolorization have been described in bacteria [41]. Azo dyes can be converted into colorless aromatic amines, some of which may become less toxic than the original dye, whereas others, such as arylamines and free radicals, are potentially carcinogenic. It has been demonstrated that Sudan Orange G has a significant cytotoxic effect on yeast cell growth and that CotA-laccase enzymatic treatment of the dye can effectively reduce the toxicity of Sudan Orange G [42]. *Staphylococcus arlettae* is able to form aromatic amines by reductive breakdown of the azo bond and to oxidize them into non-toxic metabolites by sequential microaerophilic/aerobic stages [18]. A bacterial consortium is capable of decolorizing different azo dyes under microaerophilic conditions [27]. On the other hand, the genotoxicity of the metabolites of Acid Orange 52 produced by *Pseudomonas putida* mt-2 has been connected to azoreductive conversion of the dye into corresponding aromatic amines. The metabolites produced in static incubation with the bacterium showed a higher pro-oxidant effect in comparison to those of shaken cultures [3].

Maintaining a normal skin microbiota is vital for human skin health. Azo dyes are important colorants in the cosmetics industry. Metabolism of azo dyes in cosmetics by human skin microbiota and potential effects of the metabolism on human health have not been vigorously studied in comparison to the intestinal microbiota [46]. Previously a few studies showed that some skin bacteria are able to reduce water-soluble azo dyes resulting in the formation of aromatic compounds, which may easily penetrate skin [43, 46].

In the present study, we have demonstrated that *S. aureus* can efficiently metabolize water-soluble Orange II or water-insoluble Sudan III. About 70% of Orange II or 90% of Sudan III at 18 μg/ml were metabolized at 48 h, although the degradation effectiveness decreased with increasing concentration of the dyes. Previous studies suggested that mechanisms of the bacterial degradation of water-soluble and water-insoluble azo dyes might be different [50]. The water-soluble dyes pass through the bacterial cell membrane, and are reduced in the cytoplasm by azoreductases [20]. However, the particles of water-insoluble dyes were bound to the bacteria, and reduced on the membrane [50]. A study to investigate the interactions of Sudan dyes with *E. coli* membranes showed that over 60% of the dyes (Sudan II or Sudan IV) penetrated into *E. coli*, and 90% of these dyes remained in the membrane of the bacterium [31]. Another study on the membrane transport of the bacterium also proved that Sudan dyes would accumulate on the membrane of *E. coli* [44]. Therefore, the water-insoluble Sudan III should remain in the membrane of *S. aureus* until being reduced by the enzymes. Previously it has been demonstrated that both cytosolic and membrane-associated azoreductases are present in *E. faecalis*, which is able to metabolize both water-soluble and -insoluble azo dyes. However, no membrane-associated azoreductase activity has been found in *E. coli*, which is not able to metabolize water-insoluble Sudan azo dyes [50]. The inhibition of the bacterial cell growth and the decrease of viability of the bacterial cells may result from the dye accumulated on the membrane which is likely to perturb the structure and function of the membrane [31, 50]. Therefore, it is reasonable that Sudan III can inhibit the cell growth and decrease the cell viability of the bacterium, whereas Orange II can not cause obvious negative effects on the bacterial cell growth and cell viability at the tested concentrations.

Among the metabolites of Orange II and Sudan III reduced by *S. aureus*, 1-amino-2-naphthol was the only

metabolite that significantly inhibited the bacterial cell growth, which agreed with a study on the toxicity of 1-amino-2-naphthol [23]. In their toxicity assay of 1-amino-2-naphthol on a bioluminescent organism *Vibrio fischeri*, Gottlieb et al. [23] determined the sample concentration that inhibited 50% of the light output after a 5-min exposure period (EC_{50}). On the basis of this study, 1-amino-2-naphthol was very toxic, with an EC_{50} of only 0.1 mg/l. In a genotoxicity assay comparing the survival of a repair deficient mutant *E. coli* to that of the repair proficient wild-type strain, 1-amino-2-naphthol was genotoxic only at a much higher concentration (100 mg/l) [23]. Therefore, 1-amino-2-naphthol is more toxic than genotoxic at low concentrations, which may explain why it inhibited cell growth while not affecting cell viability in our study. In addition, *p*-phenylenediamine enhanced the inhibitory effect of 1-amino-2-naphthol on the bacterial cell growth (Fig. 3c). However, none of the dye metabolites had a significant effect on the bacterial cell viability. Previously, we showed that Orange II reduction by *S. aureus* ATCC 25923 began around the late exponential or early stationary phase of the culture, and the dye was not completely reduced within 24 h [46]. This resulted in no accumulation of large quantities of metabolites formed before the stationary phase. Therefore, no effect on the cell growth and viability was found when Orange II was used as substrate, though both Orange II and Sudan III are reduced to 1-amino-2-naphthol by *S. aureus*. The results that sulfanilic acid, aniline, and *p*-phenylenediamine had no effect on the cell growth and viability also confirm other genotoxcity studies. The mutagenicity of sulfanilic acid was evaluated by using *Salmonella* Typhimurium TA 102 and TA 104 and no mutagenicity was observed in the presence or absence of a metabolic activation system (S9), which is the post-mitochondrial fraction prepared from the livers of rats [36]. Aniline was proved to be non-mutagenic in *Salmonella* Typhimurium and *E. coli* systems [4]. Even at concentrations over 1,860 μg/ml, aniline did not induce mutations [19]. In our study, aniline did not affect the cell viability of the bacterium at a concentration as high as 1,000 μg/ml. The results that *p*-phenylenediamine alone did not affect the cell growth and viability also supported a study which suggested that *p*-phenylenediamine only possess slight genotoxicity [22]. In addition, although degradation of Sudan III by *S. aureus* started in the early exponential phase, only 20% of the dye (18 μg/ml) was degraded at 10 h, which means less than 2 μg/ml of 1-amino-2-naphthol was produced (data not shown). Hence, the inhibition on the cell growth as shown in Fig. 1 may be mostly contributed by Sudan III.

In summary, our results demonstrated that *S. aureus* metabolizes water-soluble Orange II and water-insoluble Sudan III azo dyes. Sudan III inhibits cell growth and viability of the bacterium. Among the products of Sudan III reduction, only 1-amino-2-naphthol can inhibit the cell growth and the inhibition can be enhanced by *p*-phenylenediamine. However, none of the metabolites from both of the dyes reduced by the bacterium had effects on the cell viability. This investigation provides data examining the potential effects of azo dyes permitted for use in cosmetics. This information will be useful in the risk assessment process when evaluating azo dye ingredients in cosmetics. On the basis of the current results, further studies on the toxicological effects of other cosmetic dyes on various human skin bacteria in vivo and studies on the inhibition mechanisms of azo dyes and their metabolites at the gene and protein levels are warranted.

Acknowledgments We thank Drs. Huanli Liu, Rajesh Nayak, and Robin Stingley for their critical review of the manuscript, Dr. Mark Hart for strain ATCC BAA 1556, and Ohgew Kweon for helpful discussions. This study was funded by the Office of Women's Health and the National Center for Toxicological Research, United States Food and Drug Administration, and supported in part by appointments (HP and JF) to the Postgraduate Research Fellowship Program by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the US Food and Drug Administration. The views presented in this article do not necessarily reflect those of the Food and Drug Administration.

References

1. Abram FSH, Sims IR (1982) The toxicity of aniline to rainbow trout. Water Res 16:1309–1312
2. An Y, Jiang L, Cao J, Geng C, Zhong L (2007) Sudan I induces genotoxic effects and oxidative DNA damage in HepG2 cells. Mutat Res 627:164–170
3. Ben Mansour H, Corroler D, Barillier D, Ghedira K, Chekir L, Mosrati R (2007) Evaluation of genotoxicity and pro-oxidant effect of the azo dyes: acids yellow 17, violet 7 and orange 52, and of their degradation products by Pseudomonas putida mt-2. Food Chem Toxicol 45:1670–1677
4. Bomhard EM, Herbold BA (2005) Genotoxic activities of aniline and its metabolites and their relationship to the carcinogenicity of aniline in the spleen of rats. Crit Rev Toxicol 35:783–835
5. Calbiani F, Careri M, Elviri L, Mangia A, Pistara L, Zagnoni I (2004) Development and in-house validation of a liquid chromatography-electrospray-tandem mass spectrometry method for the simultaneous determination of Sudan I, Sudan II, Sudan III and Sudan IV in hot chilli products. J Chromatogr A 1042:123–130
6. Cerniglia CE, Zhuo Z, Manning BW, Federle TW, Heflich RH (1986) Mutagenic activation of the benzidine-based dye direct black 38 by human intestinal microflora. Mutat Res 175:11–16
7. Chambers HF, Deleo FR (2009) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 7:629–641
8. Chen H, Feng J, Kweon O, Xu H, Cerniglia C (2010) Identification and molecular characterization of a novel flavin-free NADPH preferred azoreductase encoded by azoB in Pigmentiphaga kullae K24. BMC Biochemistry 11:13
9. Chen H, Hopper SL, Cerniglia CE (2005) Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. Microbiology 151:1433–1441

10. Chen H, Xu H, Heinze TM, Cerniglia CE (2009) Decolorization of water and oil-soluble azo dyes by Lactobacillus acidophilus and Lactobacillus fermentum. J Ind Microbiol Biotechnol 36: 1459–1466
11. Chen H, Xu H, Kweon O, Chen S, Cerniglia CE (2008) Functional role of Trp-105 of *Enterococcus faecalis* azoreductase (AzoA) as resolved by structural and mutational analysis. Microbiology 154:2659–2667
12. Chen KC, Huang WT, Wu JY, Houng JY (1999) Microbial decolorization of azo dyes by Proteus mirabilis. J Ind Microbiol Biotechnol 23:686–690
13. Chung KT (1983) The significance of azo-reduction in the mutagenesis and carcinogenesis of azo dyes. Mutat Res 114: 269–281
14. Chung KT, Fulk GE, Egan M (1978) Reduction of azo dyes by intestinal anaerobes. Appl Environ Microbiol 35:558–562
15. Chung KT, Stevens SE Jr, Cerniglia CE (1992) The reduction of azo dyes by the intestinal microflora. Crit Rev Microbiol 18: 175–190
16. Cogen AL, Nizet V, Gallo RL (2008) Skin microbiota: a source of disease or defence? Br J Dermatol 158:442–455
17. Daniels NA, MacKinnon L, Rowe SM, Bean NH, Griffin PM, Mead PS (2002) Foodborne disease outbreaks in United States schools. Pediatr Infect Dis J 21:623–628
18. Elisangela F, Andrea Z, Fabio DG, Menezes Cristiano R, Regina DL, Artur C-P (2009) Biodegradation of textile azo dyes by a facultative Staphylococcus arlettae strain VN-11 using a sequential microaerophilic/aerobic process. Int Biodeter Biodegr 63:280–288
19. Fassina G, Abbondandolo A, Mariani L, Taningher M, Parodi S (1990) Mutagenicity in V79 cells does not correlate with carcinogenity in small rodents for 12 aromatic amines. J Toxicol Environ Health 29:109–130
20. Feng J, Heinze TM, Xu H, Cerniglia CE, Chen H (2010) Evidence for significantly enhancing reduction of Azo dyes in *Escherichia coli* by expressed cytoplasmic Azoreductase (AzoA) of *Enterococcus faecalis*. Protein Pept Lett 17:578–584
21. Gao Z, Tseng CH, Pei Z, Blaser MJ (2007) Molecular analysis of human forearm superficial skin bacterial biota. Proc Natl Acad Sci U S A 104:2927–2932
22. Garrigue JL, Ballantyne M, Kumaravel T, Lloyd M, Nohynek GJ, Kirkland D, Toutain H (2006) In vitro genotoxicity of para-phenylenediamine and its N-monoacetyl or N,N′-diacetyl metabolites. Mutat Res 608:58–71
23. Gottlieb A, Shaw C, Smith A, Wheatley A, Forsythe S (2003) The toxicity of textile reactive azo dyes after hydrolysis and decolourisation. J Biotechnol 101:49–56
24. Hou M, Li F, Liu X, Wang X, Wan H (2007) The effect of substituent groups on the reductive degradation of azo dyes by zerovalent iron. J Hazard Mater 145:305–314
25. Ito K, Nakanishi M, Lee WC, Sasaki H, Zenno S, Saigo K, Kitade Y, Tanokura M (2006) Three-dimensional structure of AzoR from *Escherichia coli*. An oxidereductase conserved in microorganisms. J Biol Chem 281:20567–20576
26. Ito K, Nakanishi M, Lee WC, Zhi Y, Sasaki H, Zenno S, Saigo K, Kitade Y, Tanokura M (2008) Expansion of substrate specificity and catalytic mechanism of azoreductase by X-ray crystallography and site-directed mutagenesis. J Biol Chem 283:13889–13896
27. Joshi T, Iyengar L, Singh K, Garg S (2008) Isolation, identification and application of novel bacterial consortium TJ-1 for the decolourization of structurally different azo dyes. Bioresour Technol 99:7115–7121
28. Khan MF, Wu X, Kaphalia BS, Boor PJ, Ansari GAS (1997) Acute hematopoietic toxicity of aniline in rats. Toxicol Lett 92:31–37
29. Kozuka I, Goh CL, Doi T, Yioshikawa K (1988) Sudan I as a cause of pigmented contact dermatitis in “kumkum” (an Indian cosmetic). Ann Acad Med Singapore 17:492–494
30. Levine WG (1991) Metabolism of azo dyes: implication for detoxication and activation. Drug Metab Rev 23:253–309
31. Li L, Gao HW, Ren JR, Chen L, Li YC, Zhao JF, Zhao HP, Yuan Y (2007) Binding of Sudan II and IV to lecithin liposomes and *E. coli* membranes: insights into the toxicity of hydrophobic azo dyes. BMC Struct Biol 7:16
32. Liger D, Graille M, Zhou CZ, Leulliot N, Quevillon-Cheruel S, Blondeau K, Janin J, van Tilbeurgh H (2004) Crystal structure and functional characterization of yeast YLR011wp, an enzyme with NAD(P)H-FMN and ferric iron reductase activities. J Biol Chem 279:34890–34897
33. Lindsay JA, Holden MT (2004) *Staphylococcus aureus*: superbug, super genome? Trends Microbiol 12:378–385
34. Liu ZJ, Chen H, Shaw N, Hopper SL, Chen L, Chen S, Cerniglia CE, Wang BC (2007) Crystal structure of an aerobic FMN-dependent azoreductase (AzoA) from *Enterococcus faecalis*. Arch Biochem Biophys 463:68–77
35. Macwana SR, Punj S, Cooper J, Schwenk E, John GH (2009) Identification and Isolation of an Azoreductase from Enterococcus faecium. Curr Issues Mol Biol 12:43–48
36. Mansour HB, Mosrati R, Corroler D, Ghedira K, Barillier D, Chekir-Ghedira L (2009) Mutagenicity and genotoxicity of acid yellow 17 and its biodegradation products. Drug Chem Toxicol 32:222–229
37. Marmion DM (1991) Handbook of US colorants: foods, drugs, cosmetics, and medical devices, 3rd edn. Wiley-Interscience, London
38. Massey RC, Horsburgh MJ, Lina G, Hook M, Recker M (2006) The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? Nat Rev Microbiol 4:953–958
39. Morokutti A, Lyskowski A, Sollner S, Pointner E, Fitzpatrick TB, Kratky C, Gruber K, Macheroux P (2005) Structure and function of YcnD from Bacillus subtilis, a flavin-containing oxidoreductase. Biochemistry 44:13724–13733
40. Muzzall JM, Cook WL (1979) Mutagenicity test of dyes used in cosmetics with the Salmonella/mammalian-microsome test. Mutat Res 67:1–8
41. Ng TW, Cai Q, Wong C-K, Chow AT, Wong P-K (2010) Simultaneous chromate reduction and azo dye decolourization by Brevibacterium casei: azo dye as electron donor for chromate reduction. J Hazard Mater 182:792–800
42. Pereira L, Coelho AV, Viegas CA, Santos MM, Robalo MP, Martins LO (2009) Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase. J Biotechnol 139:68–77
43. Platzek T, Lang C, Grohmann G, Gi US, Baltes W (1999) Formation of a carcinogenic aromatic amine from an azo dye by human skin bacteria in vitro. Hum Exp Toxicol 18:552–559
44. Ren JR, Zhao HP, Song C, Wang SL, Li L, Xu YT, Gao HW (2010) Comparative transmembrane transports of four typical lipophilic organic chemicals. Bioresour Technol 101:8632–8638
45. Ryan A, Laurieri N, Westwood I, Wang CJ, Lowe E, Sim E (2010) A novel mechanism for azoreduction. J Mol Biol 400:24–37
46. Stingley RL, Zou W, Heinze TM, Chen HZ, Cerniglia CE (2010) Metabolism of azo dyes by human skin microbiota. J Med Microbiol 59:108–114
47. Wang CJ, Hagemeier C, Rahman N, Lowe E, Noble M, Coughtrie M, Sim E, Westwood I (2007) Molecular cloning, characterisation and ligand-bound structure of an azoreductase from Pseudomonas aeruginosa. J Mol Biol 373:1213–1228

48. Wang CJ, Laurieri N, Abuhammad A, Lowe E, Westwood I, Ryan A, Sim E (2010) Role of tyrosine 131 in the active site of paAzoR1, an azoreductase with specificity for the inflammatory bowel disease prodrug balsalazide. Acta Crystallogr Sect F Struct Biol Cryst Commun 66:2–7
49. Xu H, Heinze TM, Chen S, Cerniglia CE, Chen H (2007) Anaerobic metabolism of 1-amino-2-naphthol-based azo dyes (Sudan dyes) by human intestinal microflora. Appl Environ Microbiol 73:7759–7762
50. Xu H, Heinze TM, Paine DD, Cerniglia CE, Chen H (2010) Sudan azo dyes and Para Red degradation by prevalent bacteria of the human gastrointestinal tract. Anaerobe 16:114–119
51. Zhao X, Lu Y, Phillips DR, Hwang HM, Hardin IR (2007) Study of biodegradation products from azo dyes in fungal degradation by capillary electrophoresis/electrospray mass spectrometry. J Chromatogr A 1159:217–224