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Biodegradation of azo and triphenylmethanes dyes: Cytotoxicity of dyes, slime production and enzymatic activities of *Staphylococcus epidermidis* isolated from industrial wastewater

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In view of compliance with increasingly stringent environmental legislation imposed by regional, national and supranational (for example, European Union) authorities, innovative environmental technologies for the treatment of dye-contaminated effluents are necessary in the color industry. In this study, azo (methyl orange (MO) and methyl red (MR)) and triphenylmethanes (malachite green (MG), fushin (F) and crystal violet (CV)) dyes were subjected to distinct treatment strains following an initial qualitative characterization. The effectiveness of their biodegradation using *Staphylococcus epidermidis* strain was compared with respect to parameters such as residual color, and toxicity on human cells. The Hep-2 cells were exposed to three azo and two triphenylmethane dyes at various concentrations. After 72 h exposure, the protein contents of the samples compared to the protein contents of non-exposed cells were measured. The level of protein content indicates the viability of the cells. The IC20 values show the limiting value of toxicity. The IC50 values before biodegradation show whether samples are clearly toxic. The IC50 value for the azo dyes (MO, MR) were 65 and 10 µg/ml, the IC20 value were 10 and 5 µg/ml. The IC50 for the triphenylmethane dyes (MG, F and CV) were 35, 25 and 30 µg/ml, the IC20 value were 5, 5 and 10 µg/ml. The viability of the cells was good, the protein content of the samples being over 80% compared to the non-exposed cells. The Hep-2 cell test indicated the toxicity of pure dyes before biodegradation; the dyes after decolorization had no adverse effect. The human keratinocyte Hep-2 cells seem to be a useful tool for the study of the purity/toxicity of dyes and other substances applied to textiles. In addition, the initial cytotoxicity of dyes was still present after the biodegradation, while it was completely removed through the bacterial treatment. Our results show that *S. epidermidis* was a slime-producer developing almost black colonies on Congo red agar plate. A significant increase in azoreductase, lignin peroxidase and laccase activities in the cells were obtained after complete decolorization.

Key words: Azo dye, triphenylmethane dye, *Staphylococcus epidermidis*, cytotoxicity, slime production.

INTRODUCTION

Large numbers of chemically dyes are used for various industrial applications and significant proportion appears in wastewater and is spilled into the environment. Improper chemical disposal of dyes leads to the reduction in

sunlight penetration that causes decrease in photosynthetic activity. The physical and chemical treatments available have limited use and high operational cost (Alexander, 1994). Azo dyes are the most commonly chemical used in textile, cosmetic, paper-making, and food industry. Azo dyes are synthetic organic compounds widely used in textile dyeing. Over 7 $\times 10^5$ t and approximately 10,000 different dyes and pigments are produced annually worldwide, about 10% of which may be found in wastewater (Ayed et al., 2010a).

This chemical class of dyes, which is characterised by the presence of at least one azo bond (N=N) bearing aromatic rings, dominates the worldwide market of dyestuffs with a share of about 70% (Deveci et al., 2004). They are designed to convey high photolytic stability and resistance towards major oxidising agents (Reife and Othmer, 1993). The release of azo dyes into the environment become a major concern in wastewater treatment, since they are highly recalcitrant to conventional treatment processes which attributed to the presence of sulfonate groups and azo bonds, two features generally considered as xenobiotic (Rieger et al., 2002). In addition, some azo dyes or their metabolites may be mutagens or carcinogens (McCann and Ames, 1976). Currently an extensive research is focusing to find a cheap optimal microbial biomass, which is as cheap as possible for removal of contaminating dyes from polluted water (Jadhav and Govindwar, 2006). Color is usually the first contaminant to be recognised in wastewater and a very small amount of dye in water (10-20 mgl/1) is highly visible and affects water transparency and gas solubility (Chung and Stevens, 1993). The effluents from dye manufacturing and consuming industries are highly colored coupled with high chemical and biochemical oxygen demands (COD and BOD) (Wong and Yu, 1999).

Triphenylmethane dyes are aromatic xenobiotic compounds used extensively in many industrial processes, dye-stuff manufacturing industries, as a biological stain, and textile paper printing (Azmi et al., 1998). Studies of the biodegradation of triphenylmethane dyes have focused on the decolorization of dyes via reaction reduction. Triphenylmethane dye-decolorizing by several microorganisms have been reported elsewhere (Ayed et al., 2010; Azmi et al., 1998).

The information is available concerning the toxicity of textile chemicals, but there is limited data about the overall toxicity of textile chemicals. Although a chemical itself may be toxic, its presence in the finished material may not be harmful. *In vitro* tests can be useful for studying the overall toxicity of textile chemicals on their own or included in fabrics. Textiles manufacturing commonly use reactive dyes for dyeing cotton and other cellulose based fibres. Reactive dyes have complicated chemical structures, including organic ring forms with colour-giving double bonds and form covalent bonds between reactive groups on the cellulose fibres and vinyl sulphonyl groups and chloride atoms on the dye molecules (Ayed et al., 2010b).

However, since by definition reactive dyes are chemically reactive, they may be harmful, especially when in powder form (Trotman, 1984). Many studies have shown that reactive dyes can cause allergic dermatoses and respiratory diseases (Estlander, 1988; Hatch and Maibach, 1995; Manzini et al., 1996; Nilsson et al., 1993; Wilkinson and McGechaen, 1996). Gonzales et al. (1998) stated that workers in the textile industry have a two-fold increased risk of contracting bladder cancer compared to workers in other occupations. The increased risk of contracting colonic and rectal cancers was also noted. However, these cancers related mostly to the synthetic fibre industry (De Roos et al., 2005). Mutagenicity (Przybojewska et al., 1989), genotoxicity (Dogan et al., 2005; Mathur et al., 2005), carcinogenicity (Gonzales et al., 1988; Assefa et al., 1997; Thoren et al., 1980; Worcester Wang et al., 2002; Wilhelm et al., 1994) and teratogenicity (Birhanli and Ozmen, 2005) of textile dyes has been reported.

Kopponen et al. (1997) have used Hepa-1 mouse hepatoma cells to detect common adverse effects of textile substances (Kopponen et al., 1997). Klemola et al. (2007) have studied the toxicity of three reactive dyes and dyed fabrics using boar spermatozoa confirming various degrees of adverse effects. However, more information about the overall toxicity of reactive dyes and dyed fabrics is needed (Gregory, 1993). The human keratinocyte HaCaT cells have been used widely in studying, for instance, skin irritation, skin cancer, genotoxicity, mutagenicity, and cytotoxicity caused by contact with nickel and chromium (Little et al., 1996). HaCaT cells have also used in many investigations for detecting adverse effects of UV radiation (O`Reilly and Mothersill, 1997). In addition to these studies, skin cells have been useful especially for investigating cell signalling pathways (Shimizu et al., 1999).

The aim of this study was to use Hep-2 cells lines for detecting any adverse effects of two azo dyes methyl orange (MO) and methyl red (MR) and three triphenylmethane dyes (malachite green (MG), crystal violet (CV) and fushin (F)) on their own and when present in dyed cotton fabrics. In addition, the effectiveness of using the human epithelial cell line (Hep-2 cells) to give information about the toxicity of azo and triphenylmethanes dyes with them were studied.

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Abbreviations: MO, Methyl orange; **MR,** methyl red; **MG,** malachite green; **F,** fushin; **CV,** crystal violet; **DMSO,** dimethyl sulfoxide; **FBS,** fetal bovine serum; **DMEM,** Dulbecco's modified Eagle's medium; **CRA,** Congo red agar; **Lip,** lignin peroxidase; **ABTS,** 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid.

MATERIALS AND METHODS

Dyes and chemicals

The azo (MO and MR) and triphenylmethanes dyes (MR, fushin and CV), dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich, USA. All chemicals used were of the highest purity available and of analytical grade.

Isolation, screening and identification of the dye degrading microorganisms

The isolation, screening and identification of novel dye degrading microorganisms were carried out as per our earlier report (Ayed et al., 2010a, b, c) with the ability of degrading azo and triphenyl-methanes dyes (Congo red, MO, MR, phenol red, CV, methyl green, fushine and MG).

Culture maintenance and media

Bacterial strain (*Staphylococcus epidermidis*) (stock cultures) were maintained routinely on nutrient agar containing (1 g / l): NaCl 5.0, bacteriological peptone 10.0, yeast extract (2.0), beef extract 1.0 and agar 15.0 and stored at 4°C until used. The organism from stock culture were used for the biodegradation studies after pre-culturing in nutrient broth [(1 g / l): peptone 10, NaCl 5, yeast extract 2 and beef extract 1] at 30 ± 2°C for 24 h under static condition and neutral pH (Ayed et al., 2009, 2010a,b,c).

Cytotoxicity study

Cell culture and treatment

The human epithelial cell line (Hep-2 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% l-glutamine (200 mM), 1% of a mixture penicillin (100 IU/mL) and streptomycin (100 µg/mL). Cells were grown at 37°C in an atmosphere of 5%/CO_2/95% air mixture. The medium was replaced every two days and the maintenance was strictly in accordance with the standard methods. The cells were dissociated with trypsin phosphate versenal glucose in phosphate-buffered saline. The stock cultures were grown in 25-cm^2 tissue culture flasks (Bünger et al., 2004). The experiments were started after confluency was attained.

Cytotoxicity test with human epithelial cells and determination of cell viability

For the examination of cell viability, an MTT assay was performed according to the method of Mosmann (1983). This method assesses the cell ability to convert tetrazolium salts to formazan dye by the succinatetetrazolium reductase, which is a mitochondrial enzyme, active only in viable cells.

Hep-2 cells were seeded in 96-well plastic microplates at a density of 5 x 10^4 cells/cm^2 and incubated for 24 h at 37°C. The dyes samples and its biodegradation products were added at increasing dilutions in DMSO (from 0 to 750µg/ml) and incubated for 24 h. The final concentration of DMSO in the test medium and controls was 1%. Each concentration was tested in quadruplicate together with the control and repeated two times in separate experiments. After incubation, the culture medium was replaced with 200 µL of medium containing 10 µL of MTT solution and the plates were incubated for 4 h at 37°C in humidified 5% CO2. Supernatant was removed and 200 µL of 0.04 N HCl in isopropanol was added to each well before reading optical density at 540 nm with an enzyme linked immunosorbent assay (ELISA) Reader (D.E.E.D Reader, Bio-Rad Instruments). Dose response curves were computer plotted after converting the mean data values to percentages of the control response. The optical density (OD) of formazan formed in untreated cells treated with 1% DMSO was taken as 100% of viability. The inhibitory concentration value, IC50, is the concen-tration (µg/ml) of the dye when the sample contains 50% of the total protein compared to the non-exposed samples. The amount of protein responds to the viability of the cells. The IC50 values for the dye samples were calculated from the curves which describe the percentages of the protein content under different dye concentra-tions (Mosmann, 1983).

When the protein content of the cells was 80%, the IC20 values were measured. The IC50 and IC20 values are the concentrations (µg/ml) on the x-axis when the corresponding value on y-axis is 50 or 80%, respectively. The IC20 values show the lowest toxicity and the IC50 values show the toxic values (Mosmann, 1983). For the fabric extracts, the results were given as percentages of protein content compared to the total protein content of non-exposed cells. The limiting value of the toxicity of the fabric extracts was set as 80% protein in the sample compared to total protein of non-exposed cells. The protein content of 80% represents the lowest concentration/ the limit value of the substance that causes adverse effects. The tests were carried out in triplicate (Mosmann, 1983).

Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation was studied by culturing the strains on Congo red agar (CRA) plates (Bekir et al., 2010). *S. epidermidis* strain was inoculated into the surface of CRA plates, prepared by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, and were incubated for 24 h at 30°C under aerobic conditions and followed overnight at room temperature. Slime producing bacteria appeared as black colonies, whereas non-slime producers remained non pigmented (Bekir et al., 2010).

Enzymatic activities

S. epidermidis cells were grown in the nutrient broth (pH 6.6) incubated at 30°C for 24 h and harvested by centrifugation at 10.000 × *g* for 20 min. These cells (ca. 12 g l^{-1}) were suspended in potassium phosphate buffer (50 mM, pH 7.4) for sonication (Sonics vibracell ultrasonic processor), keeping sonifier output at 40 amp and giving 8 strokes each of 40 s with a 2 min interval at 4°C. This extract was used as source of enzyme without centrifugation. Similar procedures were followed for the obtained cells after complete decolorization (6 h) (Ayed et al., 2010).

Lignin peroxidase (Lip) and laccase activities were assayed in cell-free extract as well as in culture supernatant. Lip activity was determined by monitoring the formation of propanaldehyde at 300 nm in the reaction mixture of 2.5 ml containing 100 mM n-propanol, 250 mM tartaric acid, 10 mM H_2O_2 (Ayed et al., 2010). Laccase activity was determined in the reaction mixture of 2 ml containing 0.1% 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (ABTS; Sigma, St. Louis, USA) in 0.1M acetate buffer (pH 4.9) and monitored by measuring the increase of optical density at 420 nm (Ayed et al., 2010). Enzyme assays were carried in triplicate at 30°C. The blanks contained all components except the enzyme. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg $protein^{-1}$. Azoreductase activity was determined by

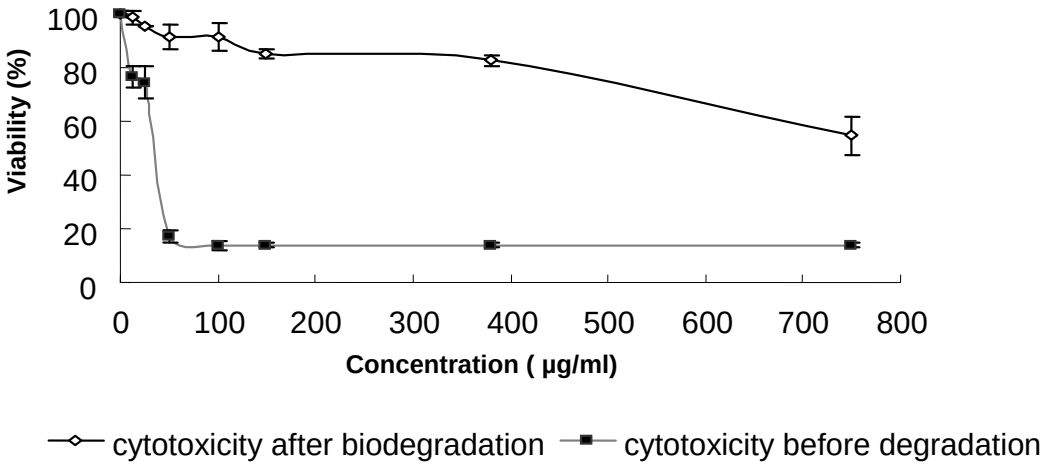

Figure.1. Cytotoxicity of MG before and after biodegradation.

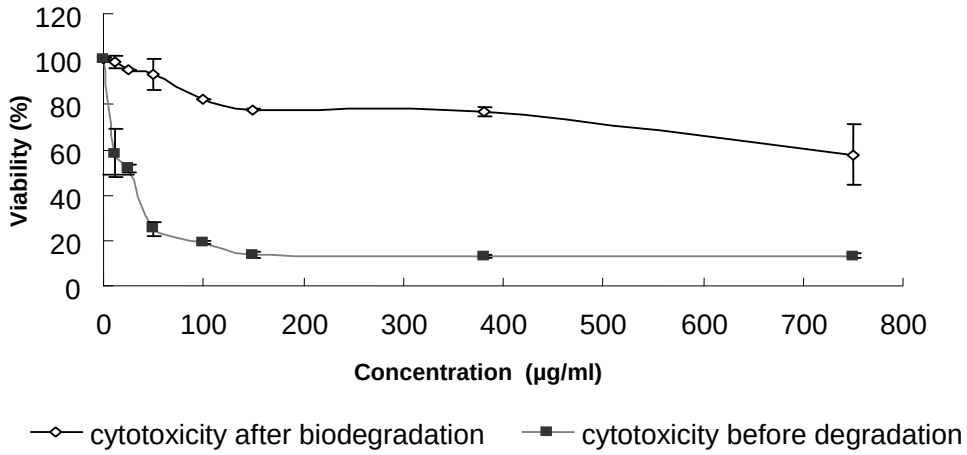

Figure 2. Cytotoxicity of fushin before and after biodegradation.

monitoring the decrease in the MO concentration at 466 nm by the reaction mixture of 2.2 ml containing 152 mM MO, 50 mM sodium phosphate buffer (pH 5.5) and 20 mM NADH. One unit of enzyme activity was defined as a microgram of MO reduced min^{-1} mg $protein^{-1}$ (Ayed et al., 2010). MG reductase activity was determined as described previously by Jadhav and Govindwar (2006). All assays were carried in triplicate.

RESULTS AND DISCUSSION

Azo and triphenylmethanes dyes were toxic in concentrations which were lower than those used in commercial dyeing processes (Trotman, 1984). The azo and triphenylmethane dyes have studied pH values between 4.5 and 6.5; however, the dyes are very reactive in human keratinocyte cells (Merryman, 1999). The textile dyes when used commercially contain many other chemicals in addition to the dye molecules. Our data presented in Figures 1-5 showed that the dyes are toxic. Gohl and Vilensky, (1983) reported that the pure azo and triphenyl-methanes dyes in powder form is very active, but after the dyeing process many of the azo sites on the dye molecules have taken part in the formation of covalent bonds with fibre molecules (Gohl and Vilensky, 1983).

These bonds are very stable and this can explain why the dyed fabric material is not toxic (Gohl and Vilensky, 1983). As pre-sented in Figures 1, 2 and 3, triphenylmethanes dye: MG, F and CV have IC 50 before degradation about 35, 25 and 30 ppm, respectively. After biodegradation the dyes have no toxicity. Before degradation azo dyes MO, MR (Figure 4 and 5) has the IC 50: 65 and 10 ppm, respectively. It is instructive to rank the cellular toxicity as high (<10 ppm), moderate (10 to 100 ppm), low (100 to 1000 ppm), or non detectable (>1000 ppm). This

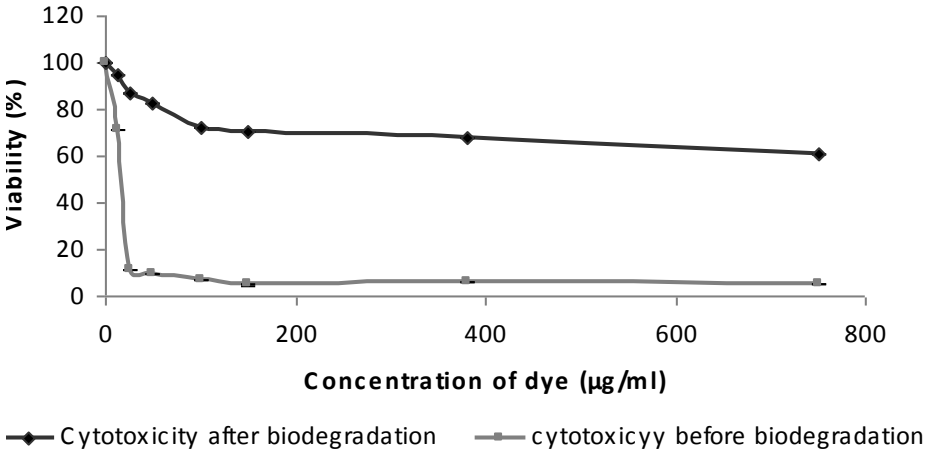

Figure 3. Cytotoxicity of CV before and after biodegradation.

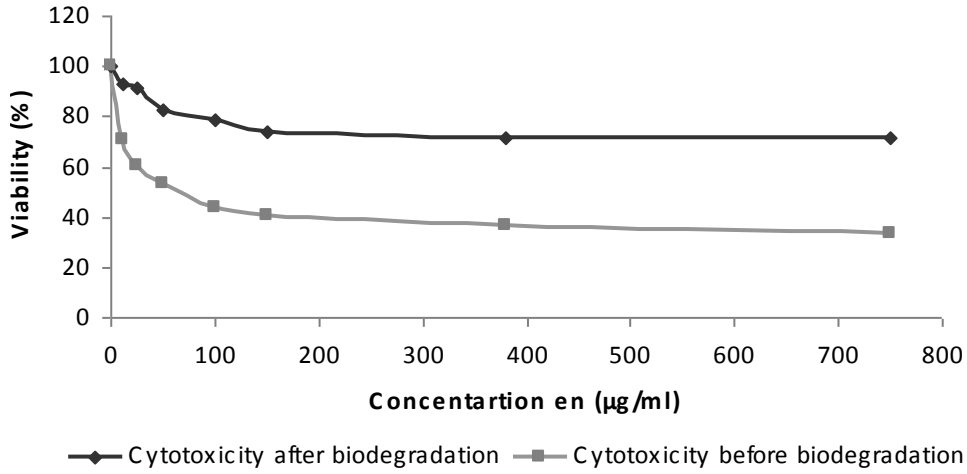

Figure 4. Cytotoxicity of MO before and after biodegradation.

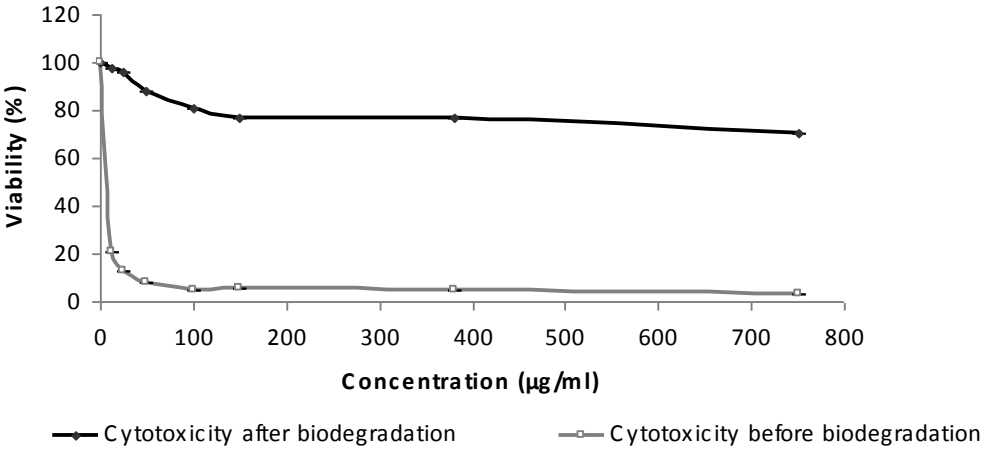

Figure 5. Cytotoxicity of MR before and after biodegradation.

Table 1. Enzymatic activity of control (0 h) and the induced state (48 h decolorization).

Enzyme assay	Control	Induced
		S1
Azo reductase[a]	1.59 ± 0.01	2.48 ± 0.01
Laccase[c]	0.0196± 0.009	0.0206± 0.010
MG reductase[b]	8.91 ± 0.31	15.81 ± 0.31
Lignin peroxidase[c]	10 ± 0.3	13 ± 0.3

[a]µmoles of MO reduced/mg of protein/minute; [b]µg of MG reduced/mg of protein/minute; cEnzyme activity, units /mg of protein/minute; S1, *S. epidermidis*.

ranking scheme is taken from the EPA Level I bioassay procedures (Sandhu, 1979).

After biodegradation the IC50 was > maximal concentration. However these findings suggested the non-toxic nature of the end dyes products. The tested bacteria achieved significantly a higher reduction in color (Ayed et al., 2009). Gohl and Vilensky (1983) showed that the red dye produced adverse effects under the lowest concentration of the dye: IC20 value was 28 µg/ml: the IC50 value (155 µg/ml) was the lowest compared to the values for the other dyes. In this study, the azo dyes (MO and MR) and the triphenylmethanes dyes (MG, F and CV) had IC20 values 5 to 10 µg/ml, showing clear toxicity. Kopponen et al. (1997) have used hepa-1 cells to test the toxicity of textile dyes and dyed fabrics, their results showed similar levels of dye concentration which resulted in toxicity. Klemola et al. (2007) have used the spermatozoa motility test for studying the same textile dyes. In this test the IC50 values were calculated after 24 and 72 h exposure. After 24 h exposure the spermatozoa test showed the red dye to be the most toxic: this is confirmed by this study. The IC20 values from the spermatozoa test after 24 h exposure were higher than those from the HaCaT cell test. The spermatozoa test after 72 h exposure had the most toxic result for the blue dye. The IC50 and the IC20 values from the HaCaT cell test showed the red dye to have the highest toxicity (Klemola et al., 2007).

Klemola et al. (2007) showed that when 80% of the cells are dead IC20 values indicate low toxicity and the results show that the dyes are very toxic even at low concentrations. Although the extracts biodegraded dyes (they were coloured), the results showed that they were not toxic and it can be assumed that the dye in solution had been hydrolyzed. The protein contents of all fabric samples were over 80% compared to the non-exposed cells. However, the mean percentage values for the protein contents of the fabric extracts showed clearly that these were non-toxic. This study therefore supports earlier studies which have shown pure azo and triphenylmethane dyes to have adverse effects. Allergic reactions are commonly known to cause allergic diseases for workers in industry (Docker et al., 1987; Estlander, 1988) Mutagenicity of some textile dyes has been reported (Keneklis, 1981). Wollin and Gorlitz, (2004) showed that several azo are genotoxic on HaCaT cells. In addition Birhanli and Ozmen (2005) *dmonstrated* the teratogenicity of some reactive dyes. The Chemical Safety Data Sheets (2001) showed the LD50 value for the yellow dye to be 5000 mg/kg, higher than the 2000 mg/kg values for the red and blue dyes.

According to toxicity tests using activated sludge, the toxicity of the blue dye measured as EC50 (the molar concentration of an agonist which produces 50% of the maximum possible response for that agonist) was higher than 100 mg/l. Using the OECD 209 method, 1984, the red and yellow dyes had IC50 values higher than 1000 mg/l. Using the OECD 203 method (an acute fish toxicity test using Salmo gairneri and Oncorhynchus mykiss) the values of LC50 (the concentration of a chemical which kills 50% of a sample population) for the red and yellow dyes were found to be higher than 100 mg/l. The cytotoxicity test using HaCaT cells is very sensitive and the IC20 values indicated toxicity in concentrations lower than 100 mg/l; Kopponen et al. (1997) have used hepa-1. The enzymatic activities in *S. epidermidis* cells before and after complete decolorization of dyes in plain distilled water were represented in Table 1. The initial step of the biodegradation of azo compounds is a reductive cleavage of the azo group by azo reductase which cleaves the azo bridge (Ayed et al., 2010c).

The term azo dye reduction may involve different mechanisms or locations like enzymatic, non-enzymatic, mediated, intracellular and various combinations of these mechanisms and locations. Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. The involvement of fungal peroxidases and laccases for the oxidation of sulfonated azo dyes has been reported earlier (Jadhav et al., 2008). Bacterial extracellular azo dyes oxidizing peroxidases have been characterized in *S. epidermidis.* Lignin peroxidase catalyzes the oxidative breakdown of the azo dyes (MO and MR). In this study, the induction of lignin peroxidase, azo reductase, and MG reductase strongly indicated that triphenylmethanes (MG, F and CV) dyes, were degraded and reductively cleaved into the simple metabolites.

Phenotypic slime production was assessed by culturing the investigated strains on CRA plates. Among the *S. epidermidis* was a slime-producer developing almost black colonies on CRA plates (Figure 6). Indeed, slime production play an important role in the pathogenesis of infections caused by different micro-organismes (Bekir et al., 2010) and is considered to be a significant virulence factor for some staphylococci (Bekir et al., 2011) as well as for *Aeromonas* spp which indicates the high-risk source contamination (Sechi et al., 2002). Slimes are generally polysaccharidic materials, although other poly-

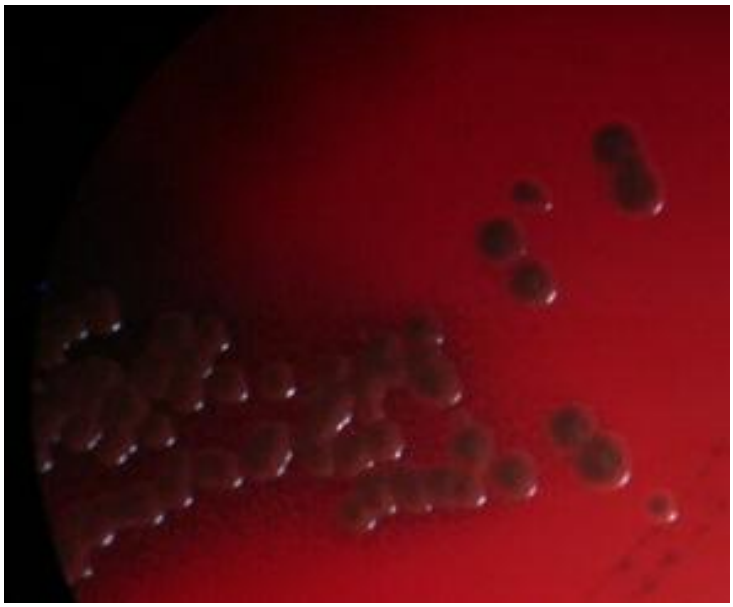

Figure 6. Colourimetric scale for colony analysis of slime production (almost black); by *S.epidermidis* using Congo Red agar assay (G8x).

mers may also be present. They are probably involved in the protection of microbial cells. In addition, microorganisms which produce these exopolymers, such as *Vibrio*, are more resistant to desiccation, predation and toxic chemicals (Ophir and Gutnick, 1994). However, these molecules are also important in the formation of biofilms on surfaces. Indeed, exopolymers have been considered to be involved in the first steps of biofilm formation (Bekir et al., 2011).

Conclusion

Human epithelial cell line (Hep-2 cells) can be used for studying the overall toxicity of six textile dyes before and after biodegradation using the *S. epidermidis* bacterial stain. In addition, the Hep-2 cell line could be used to provide information about the purity of different processes, as well as wastewaters and the environment which could be especially useful when developing textile products for allergic people.

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