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DNA damage induced by hydroquinone can be prevented by fungal detoxification

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ABSTRACT

Hydroquinone is a benzene metabolite with a wide range of industrial applications, which has potential for widespread human exposure; however, the toxicity of hydroquinone on human cells remains unclear. The aims of this study are to investigate the cytotoxicity and genotoxicity of hydroquinone in human primary fibroblasts and human colon cancer cells (HCT116). Low doses of hydroquinone (227-454 μM) reduce the viability of fibroblasts and HCT116 cells, determined by resazurin conversion, and induce genotoxic damage (DNA strand breaks), as assessed by alkaline comet assays. Bioremediation may provide an excellent alternative to promote the degradation of hydroquinone, however few microorganisms are known that efficiently degrade it. Here we also investigate the capacity of a halotolerant fungus, *Penicillium chrysogenum* var. *halophenolicum*, to remove hydroquinone toxicity under hypersaline condition. The fungus is able to tolerate high concentrations of hydroquinone and can reverse these noxious effects via degradation of hydroquinone to completion, even when the initial concentration of this compound is as high as 7265 μM. Our findings reveal that *P. chrysogenum* var. *halophenolicum* efficiently degrade hydroquinone under hypersaline conditions, placing this fungus among the best candidates for the detoxification of habitats contaminated with this aromatic compound.

1. Introduction

Human exposure to hydroquinone, a phenolic compound also known as the major benzene metabolite, can occur by dietary, smoke, occupational and environmental sources. Due to the rapid industrialization and urbanization, the number of hydroquinone sources has increased and consequently its discharge into the environment, leading to serious toxic effects on fauna and flora. Hydroquinone is commonly used as a photographic developer, dye intermediate, stabilizer in paints, varnishes oils and motor fuels as well as in the rubber, antioxidant and food industry. Moreover, hydroquinone can be the product of several phenolic biotransformations, such as benzaldehyde, benzoic acid, 4-ethylphenol, 4-hydroxyacetophenone, phenol and substituted phenols, including 4-chloro, 4-fluoro, 4-bromo, 4-iodo and 4-nitrophenol [3,11,18,20,22,31]. It is known that phenolic compounds can negatively influence the organoleptic properties of fish and shellfish when present at concentrations of part-per-billion [14]. In fact, phenolic compounds are one of the priority pollutants of the United States Environmental Protection Agency (USEPA) list.

Although there are already some studies on the hydroquinone potential hazard to aquatic organisms, its genotoxic capacity and mechanism remain largely

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unknown. Most of the attention has been focused on acute toxicity. Bahrs and coworkers [4] determined 48-h EC_{50} values of 1.5 mg/l, 0.68 mg/l, 0.21 mg/l and 0.054 mg/l for *Desmodesmus armatus, Synechocystis* sp., *Nostoc* sp. and *Microcystis aeruginosa*, respectively, showing that hydroquinone can be highly toxic to aquatic organisms at concentrations of parts-per-million. Green algal species were found to be relatively less sensitive to hydroquinone than cyanobacterial species [4]. Meanwhile, 48-h EC_{50} value of 0.15 mg/l for *Daphnia magna* and 24-h LC_{50} values ranging from 0.22 to 0.28 mg/l for *Brachionus plicatilis* have been reported [14]. Hydroquinone was also toxic to marine bacteria as well as to fishes like rainbow trout and fathead minnows [9]. Indeed, hydroquinone can be a thousand times more toxic to *Vibrio fischeri* NRRL B-11177 than its isomers [19]. In epidemiological studies, correlations between the genotoxic concern of aquatic ecosystems and carcinogenic effects in human have been detected [7,12,15].

Despite the fact that hydroquinone seems to be one of the benzene metabolites implicated as causative agent of benzene-associated disease, there is no consensus among researchers regarding the relevance of the severity of hydroquinone on human cell viability and DNA damage. Some researchers proposed that hydroquinone could induce DNA damage by a combination of damage to the mitotic spindle, inhibition of topoisomerase II and the formation of DNA strand breaks via generation of reactive oxygen species [1,32,34], however others considered hydroquinone to be inactive by analyzing the frequency of DNA breaks using comet assay [21]. For the above reason, in the present study, we evaluated the cytotoxic effects of hydroquinone on the viability of human primary fibroblasts and human colon cancer cells (HCT116) using a commercial cell health indicator assay, and for assessment of the genotoxicity, alkaline comet assay was performed. In addition, the potential of a *Penicillium chrysogenum* strain for reducing hydroquinone concentrations and reversing its noxious effects via degradation of hydroquinone was evaluated. Cyto/genotoxic studies were conducted to determine the effect of exposure to medium conditioned by the metabolic activity of this fungal strain.

2. Materials and methods

2.1. Design of fungal experiments

P. chrysogenum var. *halophenolicum* was used throughout this study; this strain was isolated from a salt mine in Algarve, Portugal, and previously characterized [22,23]. The fungal strain was maintained at 4 °C on nutrient agar plates with 5.9% (w/v) NaCl. Precultures of cells were routinely aerobically cultivated in MC medium as described by [13].

To study the utilization of hydroquinone, the strain was cultivated in 500-ml flasks containing 100 ml of MC medium for 68 h at 160 rpm in an orbital shaker (Certomat® BS-T Incubator, Sartorius Stedim Biotech, Goettingen, Germany). Cells were centrifuged for 10 min at 10,000 × *g* and washed three times in 0.85% (w/v) of NaCl. Then, a 10% aliquot was inoculated in MMFe medium (50 ml in 250-ml flasks) [13] with different concentrations of hydroquinone (Sigma–Aldrich, ReagentPlus™, ≥99%, Batch#: 114K2623) (see Section 3). Three replicates were used per test for each hydroquinone concentration. Uninoculated control flasks (duplicates) were incubated and aerated in parallel as negative controls of the experiment. Hydroquinone concentration was monitored up to an incubation time of 96 h.

Biosorption by dead biomass was determined by batch adsorption equilibrium experiments as follows. The strain *P. chrysogenum* var. *halophenolicum* was grown in the MC liquid medium at 25 °C in a shaker incubator at 160 rpm for 68 h. Mycelium pellets were separated from the growth medium by centrifugation and washed twice with NaCl solution (0.85% (w/v)). The biomass was sterilized for 15 min at 121 °C and 124 kPa to kill the fungus, preventing biodegradation and bioaccumulation of hydroquinone in the subsequent adsorption experiments. The biomass was then rewashed with NaCl solution (0.85% (w/v)), centrifuged and approximately 50 ml of MMFe with 300 mg/l of hydroquinone were mixed with 0.10 g biomass (dry weight). The suspension was shaken at 25 °C in a rotary shaker at 160 rpm for 56 h, before the residual aqueous concentration of hydroquinone was measured by HPLC.

2.2. Analytical methods

Hydroquinone concentrations were quantified by High Performance Liquid Chromatography apparatus L-7100 (LaChrom HPLC System, Merck), equipped with a quaternary pump system, and L-7400 UV detector according to a previously published method [22]. Hydroquinone could be separated and concentrations estimated within 10 min, using standard (Sigma–Aldrich, ReagentPlus™, ≥99%).

The OxiTop® respirometric system (WTW, Germany) was used for assessing the biodegradability of hydroquinone over 5 days. The principle of the operation was based on the measurement of the pressure difference in the closed system. During hydroquinone biodegradation the respiration increases, the produced CO_2 was captured by an alkaline solution, and microbial oxygen consumption resulted in the subsequent pressure drop. All experiments were performed in reactors consisting of headspace and glass bottles (510 ml nominal volume) with a carbon dioxide trap (approximately 0.5 g of NaOH was added in each trap) with 97 ml of sample volume (MMFe with 5% of inoculum supplemented with 4541 and 7265 μM of hydroquinone). Fungal blanks were analyzed in parallel to correct for endogenous respiration. Respirometric analyses were conducted for 120 h in a temperature controlled chamber at 20 ± 1 °C and in the darkness. Decrease in headspace pressure inside the reactor was continuously and automatically recorded. Three experiments were performed, samples were done in triplicate and controls in duplicate. The quantity of oxygen consumption was calculated according to the manufacturer instructions.

2.3. Culture of human cells and cell viability assay

Colon cancer HCT116 cells (ATCC number CCL-247) and human primary fibroblasts (Coriell Institute, Candem,

NJ, Ref. GM05565) were cultured in McCoy's 5a Modified medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 1% MEM non-essential amino acids and 100 U/ml penicillin/streptomycin (Gibco, Life Technologies), and maintained at 37 °C in a humidified incubator under 6% CO_2. Cells were cultured in 24-well plates for 24 h before initiation of experiments using McCoy's supplemented with either (1) MMFe medium originating from cultures of *P. chrysogenum* var. *halophenolicum* (conditioned composite medium), (2) freshly prepared MMFe medium (plain composite medium), or (3) either hydroquinone, etoposide or drug solvent (controls).

Cell viability was assessed using Alamar Blue® (Molecular Probes, Life Technologies), a commercial assay which is based on the reduction of the cell permeable redox indicator resazurin (deep blue) into resorufin (pink and fluorescent) by viable, metabolically active cells. At the end of specified incubation times, 50 μl of Alamar Blue® solution was added per 1 ml of culture medium and incubated for an additional 2 h. Plates were then analysed for fluorescence emission in a Tecan Infinite M200 plate reader, using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Results were read using Tecan i-Control v. 1.4.5.0 plate reader software. Each experiment was performed as a triplicate.

2.4. Alkaline single cell gel electrophoresis

DNA strand breaks were evaluated using Trevigen Comet Assay® kit (Trevigen Inc., Gaithersburg, MD, USA). Briefly, cells were resuspended in ice cold PBS (Ca^{2+} and Mg^{2+} free) to a concentration of 1×10^5 cells/ml. An aliquot of 5 μl of cells was added to 50 μl of molten LM Agarose (1% low-melting agarose) kept at 37 °C. 50 μl were pipetted immediately and evenly spread onto the comet slides. Slides were incubated at 4 °C in the dark for 10 min to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X-100, pH 10) for 30 min at 4 °C. A denaturation step was performed in alkali solution (300 mM NaOH, 1 mM EDTA, pH > 13) at room temperature for 30 min, in the dark. Slides were then transferred to prechilled alkaline electrophoresis solution pH > 13 (300 mM NaOH, 1 mM EDTA) and subjected to electrophoresis at 1 V/cm, 300 mA for 30 min in the dark at 4 °C. The slides were then washed with deionized water and immersed in 70% ethanol at room temperature for 5 min and air dried. DNA was stained with 100 μl of SYBR Green I dye (Trevigen, 1:10,000 in Tris–EDTA buffer, pH 7.5) for 10 min at 4 °C in the dark and immediately analyzed using a CCD camera (Roper Scientific Coolsnap HQ CCD) attached to a Zeiss Axiovert 200M widefield fluorescence microscope. Comets were visualized with an excitation filter of 450–490 nm and an emission filter of 515 nm and fluorescent images of single cells were captured at 200× magnification. A minimum of 100 randomly chosen cells per experimental group were scored for comet parameters such as tail length and percentage of DNA in tail [28] using the Tritek CometScore Freeware v1.5 image analysis software.

3. Results

3.1. Cytotoxicity effects of hydroquinone

Results from the Alamar Blue® assay showed that hydroquinone treatment reduced the viability of human primary fibroblasts and colon cancer HCT116 cells in a dose-dependent manner. As shown in Fig. 1, high concentrations of hydroquinone (227 μM, 454 μM, 908 μM, 2270 μM and 4541 μM) greatly decreased cell viability. Compared to control, metabolic activity drastically dropped after exposure to any concentration equal or above 227 μM of hydroquinone. This negative effect on metabolic activity is more effective in HCT116 cells (11.25%) than fibroblasts cells (43.22%). EC_{50} for cytotoxicity in fibroblasts and HCT116 cells was 329.2 ± 4.8 μM and 132.3 ± 10.7 μM, respectively. There is a good fit between the dose response curve and the data points for cytotoxic effects on HCT116 cells and fibroblasts cells after 24 h ($r^2 = 0.9175$ and $r^2 = 0.9773$, respectively).

3.2. Genotoxicity of hydroquinone in cancer cells

One of the possible ways by which hydroquinone reduces cell survival could be through induction of DNA damage. We then addressed whether hydroquinone induced DNA damage in primary human skin fibroblasts and HCT116 cells, using the same range of concentrations previously demonstrated to reduce survival of both cells. To this end, we exposed HCT116 cells to increasing concentrations of hydroquinone (9.08, 45.4, 90.8, 227.0 and 454.1 μM; Table 1) for 24 h using as controls cells exposed to either no drug (solvent alone; negative control), or to etoposide for 15 min (50 μM; positive control), a well-known potent inducer of DNA breaks [10]. Since fibroblasts cells were less sensitive to hydroquinone as shown by the Alamar Blue® assay, we exposed fibroblasts cells to concentrations of 454.1 and 908.2 μM of hydroquinone (Table 1). DNA breaks were detected using the highly sensitive alkaline comet assay, an electrophoresis-based assay that allows detection of both single and double-stranded DNA breaks at the single cell level. As expected, etoposide induced significant DNA damage on fibroblasts and HCT116 cells with ~50% and 80%, respectively, of the DNA leaving the nucleus and migrating as the comet tail (Table 1). Importantly, treatment of HCT116 cells with 227 or 454 μM hydroquinone induced DNA damage similar to that caused by sub-apoptotic levels of etoposide in the same cell line. In fibroblasts, however, exposure to 454.1 μM of hydroquinone induced a much higher % of tail DNA in comets compared to etoposide (Table 1).

3.3. Genotoxicity of hydroquinone in cancer cells can be abolished by fungal treatment

To investigate if the presence of a fungal strain capable of degrading phenols, *P. chrysogenum* var. *halophenolicum*, reduces the toxicity of hydroquinone in fibroblasts and human colon cancer cells (HCT116), new experiments were done. Fungal cultures in minimal medium containing hydroquinone were incubated at several times to ensure

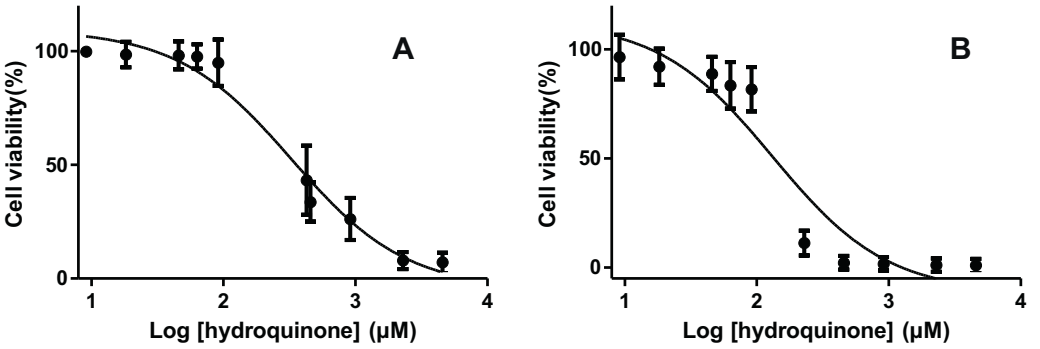

Fig. 1. Dose response curves of hydroquinone in fibroblasts cells (A) and HCT 116 cells (B).

Table 1
Evaluation of primary DNA damage measured in HCT 116 and fibroblasts cells following exposure to hydroquinone.

Cell treatment	Conc. (μM)	Tail intensity (%DNA)	Tail length (pixels)	Tail moment
HCT116				
Etop 15 min	50.0[a]	85.58 ± 5.30	161.23 ± 7.13	91.29 ± 9.75
NegC 24 h		18.09 ± 3.52	47.61 ± 7.09	7.30 ± 1.89
HQ 24 h	0	12.90 ± 1.88	60.97 ± 5.97	4.73 ± 1.25
	9.08	22.05 ± 3.42	75.76 ± 8.63	10.79 ± 2.70
	45.4	17.74 ± 3.16	87.37 ± 6.55	8.16 ± 1.97
	90.8	17.13 ± 4.28	95.93 ± 11.11	12.25 ± 5.44
	227.0	85.45 ± 4.60	298.40 ± 31.50	150.22 ± 16.42
	454.1	89.15 ± 1.44	320.78 ± 26.82	163.35 ± 10.95
Fibroblasts				
Etop 15 min	50.0*	46.12 ± 3.24	55.97 ± 2.23	19.78 ± 1.76
NegC 24 h		10.22 ± 1.05	35.09 ± 1.82	9.99 ± 2.46
HQ 24 h	454.1	82.82 ± 6.31	244.30 ± 34.40	123.50 ± 21.90
	908.0	87.42 ± 2.31	215.00 ± 14.05	107.20 ± 6.60

HQ, hydroquinone; Etop, etoposide; NegC, no drug.
[a] mg/l.

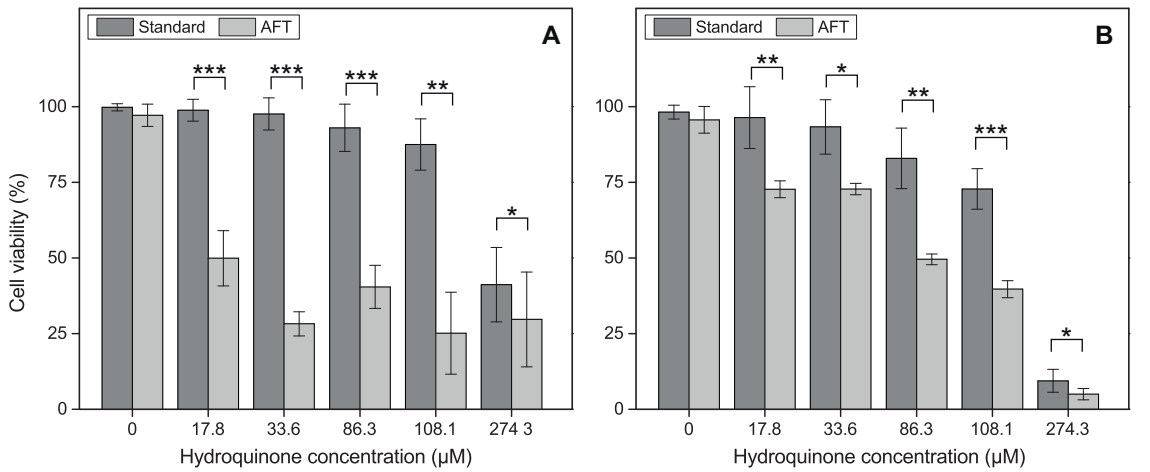

Fig. 2. Effects of the remaining hydroquinone concentrations after fungal treatment (AFT) on cell viability of fibroblasts (A) and HCT116 (B) cells after 24 h of exposure. Data are expressed as the mean with SD of three independent experiments. The probability in the ANOVA one-way test for the difference between controls and each treatment is given in parentheses: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

different degradation yields. Fungal mycelium was then separated by centrifugation and the supernatants buffered to pH 7.4 and isotonic conditions. Those samples obtained after fungal treatment (AFT) were then added to the fibroblast and HCT116 cells growing in McCoy's medium (Fig. 2). Cell survival was evaluated by a well-established method based on the fluorescent conversion of a redox indicator (Alamar Blue®) after 24 h of culture on AFT samples. Controls were provided by fibroblasts and HCT116 cells cultivated exactly for the same periods of time in plain MMFe medium i.e. in which the fraction of saline medium was freshly prepared without hydroquinone. The data show a

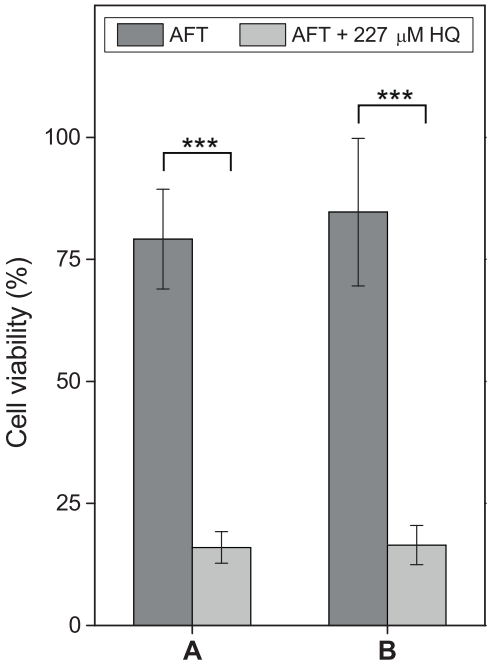

Fig. 3. Cytotoxicity effects of fungal treated samples (AFT) in HCT 116 cells observed in the presence or absence of hydroquinone addition (227 μM). (A) Sample from a batch with an initial concentration of hydroquinone of 4541 μM; (B) sample from a batch with an initial concentration of hydroquinone of 7265 μM. Data are expressed as the mean with SD of three independent experiments. The probability in the ANOVA one-way test for the difference between controls and each treatment is given in parentheses: ***, $P < 0.001$.

strong correlation between higher remaining concentrations of hydroquinone and reduced survival of HCT116 cells (Fig. 2). A different survival pattern was observed on fibroblasts; data depicted in Fig. 2 shows that concentrations of 33.6 μM of hydroquinone obtained after fungal treatment can reduce approximately 70% of the survival of fibroblasts cells. These data suggests that *P. chrysogenum* var. *halophenolicum* produces one or more metabolites during hydroquinone degradation that increase its toxicity, in particularly to fibroblasts cells. On the other hand, the salt medium composition (controls) did not affect cell viability.

To further address whether hydroquinone itself did play the key role in reduced survival of human cells, we cultivated HCT116 cells in medium in which hydroquinone had been reduced to undetectable levels by *P. chrysogenum* from initial concentrations of 4541 or 7265 μM (Fig. 3). The results show that, irrespectively of the initial concentration of hydroquinone, survival of HCT116 cells is only minimally affected when compared to controls cultured in freshly prepared salt medium (Figs. 2 and 3). Importantly, when purified hydroquinone was added back to a final concentration of 227 μM, survival of HCT116 cells were reduced to levels comparable to those observed when hydroquinone reached similar concentrations via *P. chrysogenum*-dependent degradation (Figs. 2 and 3). Together, these data demonstrate that *P. chrysogenum var. halophenolicum* is able to reduce the toxicity exerted by hydroquinone on cultured human cells.

3.4. *P. chrysogenum var. halophenolicum eliminate toxicity via degradation of hydroquinone*

We subsequently tested whether the capacity *P. chrysogenum* to eliminate the negative effect of hydroquinone on fibroblasts and HCT116 cells observed previously, was due to the hydroquinone degradation to undetectable levels in culture. To do so, batch cultures with *P. chrysogenum* var. *halophenolicum* and hydroquinone at different initial concentrations of 4541 and 7265 μM in saline liquid media (MMFe) were performed. The results are shown in Fig. 4. Since no abiotic loss of hydroquinone was detected in controls and less than 3% of hydroquinone becomes adsorbed to fungal cell surface, the decrease of hydroquinone concentration in the presence of fungus can be mostly attributed to cell metabolism. Hydroquinone at initial concentration of 4541 μM was completely removed within 56 h of treatment; while 75% of hydroquinone was removed in fungal cultures when the initial concentration was 7265 μM after the same time of treatment. These results demonstrate that *Penicillium* var. *halophenolicum* can remove hydroquinone to undetectable concentrations by HPLC method.

Additional studies were done to assess the complete biological conversion of hydroquinone to CO_2 and H_2O by the *P. chrysogenum* strain, using the OxiTop® respirometric system. The OxiTop® respirometric system is a simple, batch device, which is appropriate and sensitive for determination and analysis of wastewater biological oxygen demand (BOD). Fig. 5 shows hydroquinone BOD data from the respirometric study. Each BOD value was corrected for endogenous respiration (i.e., BOD obtained from the fungal blank). Since the biodegradation test was carried out within a brown dark bottle container and in the absence of light, the possible existence of photodegradation was withdrawn. The 5-day BOD for the initial concentrations of 4541 and 7265 μM of hydroquinone was 440 mg/l and 720 mg/l, respectively. The initial mineralization of the biodegraded hydroquinone is slightly lower at the initial concentration of 7265 μM than that at 4541 μM up to the first day. This fact suggests that hydroquinone at high concentrations induces smaller rates of respiration than low initial concentrations and agrees with the observation that hydroquinone can reduce enzyme activity of microbial biomass [8].

3.5. *Effect of P. chrysogenum var. halophenolicum on hydroquinone genotoxic activity*

Finally, we tested whether *P. chrysogenum* could degrade hydroquinone to levels that were non-genotoxic to cultured human cells. HCT116 and fibroblasts cells were thus exposed for 24 h to fungal treated samples containing different concentrations of hydroquinone as the result of progressive degradation of this compound by *P. chrysogenum* and then subjected to the alkaline comet assay protocol; controls were provided by cells exposed to plain medium without hydroquinone for the same duration (Table 2 and Fig. 6). As expected for a genotoxic agent, metabolites coming from an incomplete degradation of hydroquinone still might led to significant DNA damage in

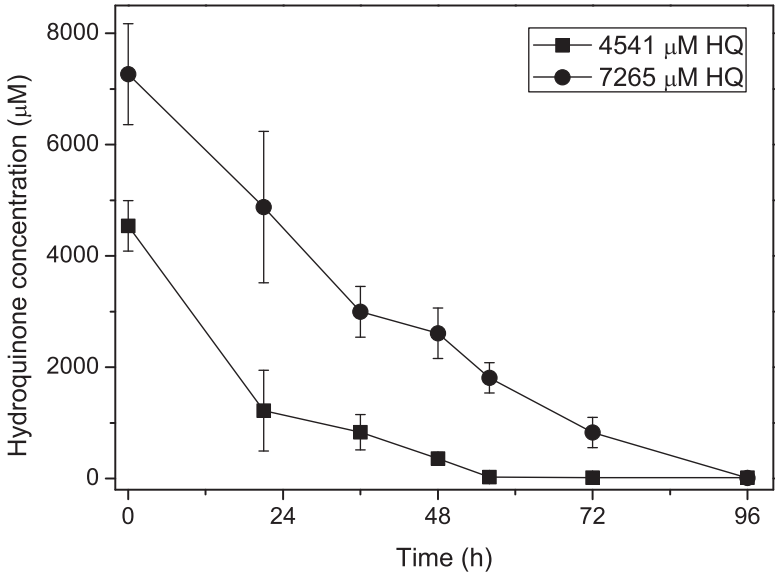

Fig. 4. Hydroquinone removal by *P. chrysogenum* var. *halophenolicum* at different initial concentrations as indicated in the legend. Data shown represents average of triplicates ± standard deviations.

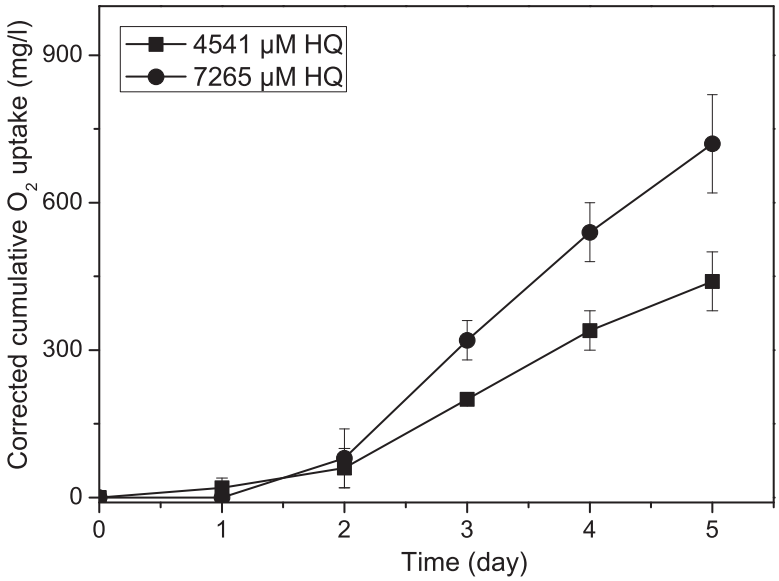

Fig. 5. Biochemical oxygen demand of hydroquinone. Data are corrected against the fungal blank BOD from respirometric analysis. Error bars are based on samples from duplicate reactors.

HCT116 or fibroblasts cells. HCT116 cells exposed to 86.3, 108.1 and 274.3 μM of remaining hydroquinone after fungal treatment showed in the range between 40% and 80% of total DNA fractured enough to leave the cell nucleus and form the comet tail (Fig. 6 and Table 2). In the case of fibroblasts, a remaining hydroquinone concentration of 86.3 μM did not induce a noticeable increase in DNA damage, while with 274.3 μM more than 80% of DNA in the comet tail was observed (Table 2). However, when hydroquinone was either fully degraded (0 μM) or degraded almost to completion (33.6 μM final concentration) by *P. chrysogenum*, the amount of DNA damage induced in HCT116 and fibroblasts cells was similar to that observed in the control cells (NegC) (Table 2).

Overall, these data show that *P. chrysogenum* var. *halophenolicum* is capable of degrading hydroquinone from highly cytotoxic initial concentrations to levels that are non-genotoxic and are well tolerated by fibroblasts and HCT116 cell (Fig. 7).

4. Discussion

The toxicity of hydroquinone may have been underestimated, given the small number of studies performed

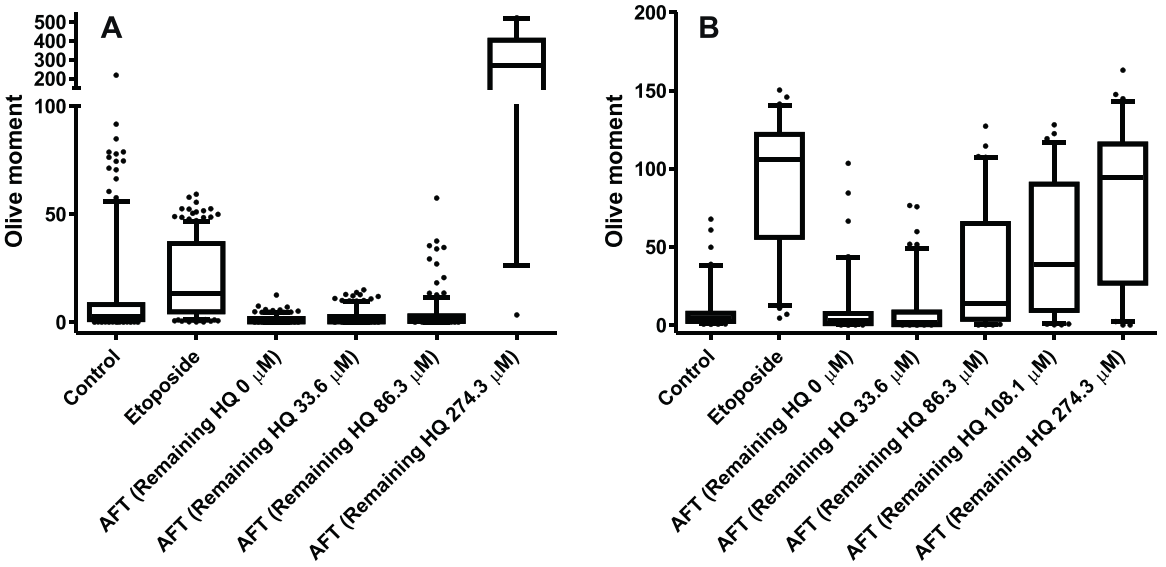

Fig. 6. Depicted are the results of alkaline comet assays performed using fibroblasts (a) and HCT116 cells (b) cultured for 24 h in medium containing either drug solvent alone (controls), etoposide (50 μM for 15 min), AFT medium containing varying final concentrations of hydroquinone. The different concentrations of hydroquinone were obtained by progressive treatment of hydroquinone by *P. chrysogenum* var. *halophenolicum* (initial concentration 4541 and 7265 μM). The 0 μM concentration corresponds to full (maximal) degradation. Controls correspond to HCT116 cells and fibroblasts grown in the absence of hydroquinone. In the graphs, boxes correspond to the 75th percentile, whiskers to the 95th percentile and lines identify the median obtained from triplicates.

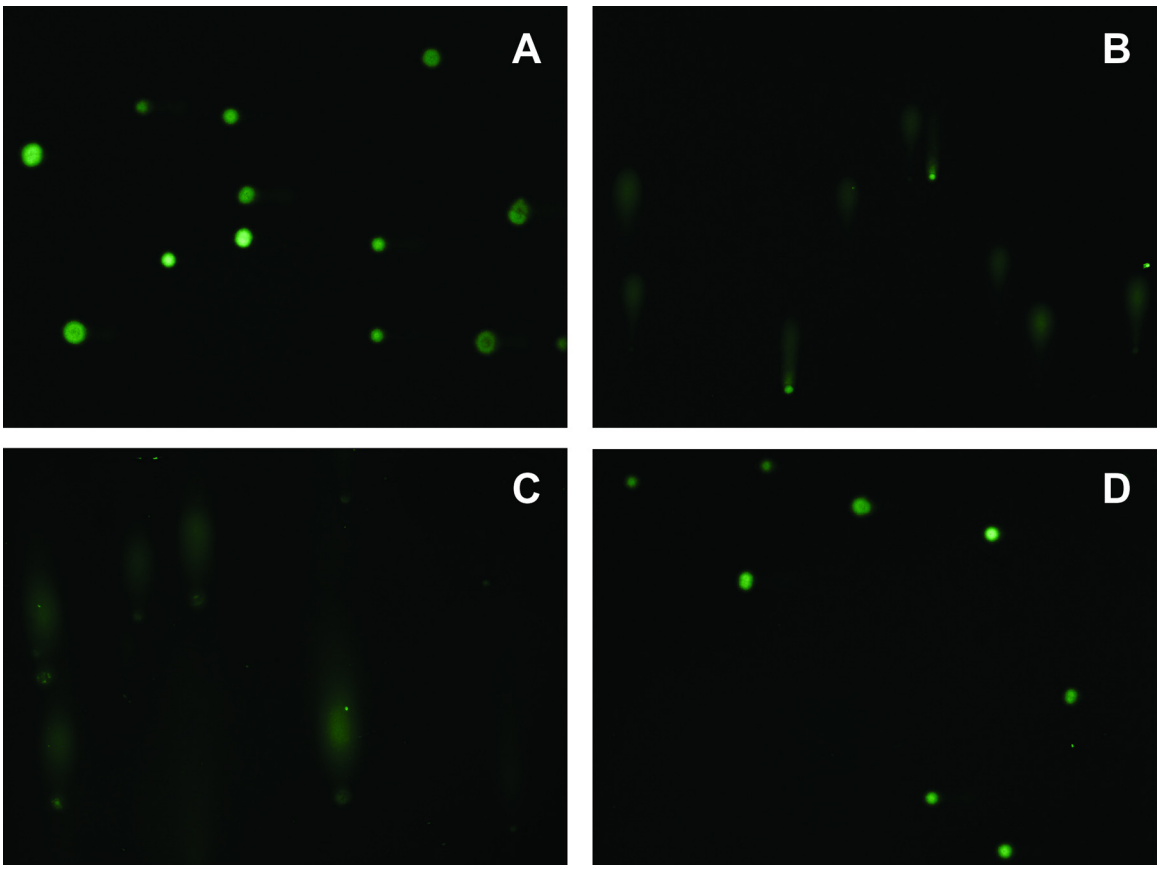

Fig. 7. Hydroquinone induces DNA double-strand breaks in HCT116 cancer cells. Representative photos are shown for (A) negative control (medium), (B) positive control (etoposide), (C) standard hydroquinone (454 μM) and (D) AFT sample where the remaining concentration of hydroquinone is zero.

Table 2
Measurement of DNA damage-related parameters obtained by alkaline comet assay in HCT 116 and fibroblast cells following exposure to AFT medium. Data are expressed as the mean with SD of three independent experiments. The probability in the ANOVA one-way test for the difference between controls and each treatment is given in parentheses: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$.

Cell treatment	Concentration (μM)	% DNA in tail	Tail length (pixels)
HCT116			
Control		18.09 ± 3.52	47.61 ± 3.56
HQ 24 h	0	17.77 ± 4.50 (ns)	38.01 ± 3.88 (ns)
	33.6	16.06 ± 4.30 (ns)	40.10 ± 3.24 (ns)
	86.3	43.61 ± 7.86 (***)	93.26 ± 5.81 (***)
	108.1	57.77 ± 7.43 (***)	110.00 ± 5.51 (***)
	274.3	73.97 ± 7.63 (***)	153.70 ± 8.64 (***)
Fibroblasts			
Control		10.22 ± 1.05	35.09 ± 0.93
HQ 24 h	0	5.58 ± 0.84 (***)	21.89 ± 0.94 (**)
	33.6	6.47 ± 1.06 (***)	29.10 ± 1.30 (ns)
	86.3	11.03 ± 1.90 (ns)	27.40 ± 1.11 (ns)
	274.3	87.26 ± 5.50 (***)	506.00 ± 39.28 (***)

HQ, hydroquinone.

in animal models, the difficulty to extrapolate to humans most of the data obtained in models, and the limited statistical power of cohort studies already performed in human subjects [30]. There is growing evidence that hydroquinone and some of its metabolites have genotoxic activity to mammalian cells, namely human cells, either primary or transformed [11].

In initial work on the cytotoxicity of hydroquinone on mammalian cells a requirement for copper was described [25]. Indeed, Cu(II) through a copper-redox cycling mechanism promotes the oxidation of hydroquinone with generation of benzoquinone and reactive oxygen species (ROS) [26], and several reports have subsequently implicated oxidative damage to DNA as a major mechanism for the cytotoxic effects of hydroquinone (reviewed in [11]). Later, Luo and coworkers showed that hydroquinone induced genotoxicity and oxidative DNA damage in human hepatoma HepG2 cells independently of the presence of transition metals, and afterwards several articles were published supporting these researchers [16,29,33]. In this study, *P. chrysogenum* var. *halophenolicum* ability to degrade hydroquinone was investigated using saline medium (MMFe) with iron in its composition. The presence of iron did not affect the toxicity of hydroquinone over fibroblasts and HCT116 cells. These findings in fibroblasts and HCT116 cells, are in agreement with previously published data obtained using other cell types [24], not excluding a role for endogenous copper in mediating the cellular effects of hydroquinone. The median effective concentration (EC_{50}) of hydroquinone in several cancer lines was reported to be 8.5 μM, 10.0 μM, 88 μM for HL-60, HL-60/MX2 and Huh7, respectively, and >100 μM for Hep3B and HepG2 [16]. Our data showed that hydroquinone decreased cell viability of HCT116 cells (EC_{50} = 132.3 μM) and, to a lesser extent, primary human fibroblasts (EC_{50} = 329.2 μM). These data are in agreement with the data published by other researcher who has found that primary human fibroblasts were relatively more resistant to hydroquinone compared to lymphocytes [24]. As it was previously reported, differences between a cancer cell line and primary fibroblasts can be attributed to differences in cell sensitivity to the compound that was assayed and would be mainly related with the cell division rate [36].

Reactive species generated by hydroquinone have been implicated in the formation of modified bases (e.g., 8-oxo-deoxyguanine) in the DNA molecule, which appear to be removed with fast kinetics [33], but also single and double-stranded DNA strand breaks [17,29,33]. Moreover, both hydroquinone and its degradation product benzoquinone are topoisomerase II poisons which inhibit the final ligation step of the catalytic cycle of the enzyme, thus stabilizing topoisomerase-mediated DNA scissions [27]. Although the relative contributions of reactive oxygen species and topoisomerases in hydroquinone-mediated genotoxicity remain to be elucidated, it is clear that that DNA breaks generated by hydroquinone pose a serious challenge to genome integrity [5,11]. Herein, we have analyzed the capacity of hydroquinone to generate both single and double-strand DNA breaks using the well characterized comet assay under alkaline conditions (cf. Table 1). We showed that the hydroquinone-induced increment in DNA strand breaks in HCT116 cells was dose-related. In HCT116 cells, hydroquinone at concentrations of 227.0 and 454.1 μM caused a marked increase of the olive tail moment (the product of % tail DNA and tail length) compared to lower concentrations. Hydroquinone concentrations up to 90.8 μM induced a gradual but slow increment of the olive tail moments and this was due more to the increase in the tail length of comets than to the amount of DNA in the tail. The relative amount of DNA in the comet tail (the % tail DNA or tail intensity) has been related to DNA break frequency over a wide genome range, while tail length has been related to the frequency of the smallest detectable DNA fragments and, since it quickly reaches a maximum, its useful only for low levels of damage [2]. Taking this into account, we can say that hydroquinone concentrations higher than 90.8 μM are required in order to induce a high frequency of DNA breaks throughout the whole genome of HCT116 cells, resulting in overall cell death, as evidenced by the survivability assay (Fig. 2). Hydroquinone alone induced greater loss of viability in HTC116 cells than in fibroblasts cells (cf. Fig. 1) but surprisingly, when cells were exposed to medium

previously incubated with *P. chrysogenum* var. *halophenolicum*, fibroblast survivability seemed to be dependent on more than just the remaining hydroquinone concentration in the medium. This suggests that fibroblasts are more sensitive than HCT116 cells to the metabolites resulting from hydroquinone degradation. Interestingly, the comet assay data also indicates that, except for very high remaining hydroquinone concentrations, DNA strand breaks are not the major cause of the viability loss in fibroblasts after fungal treatment (compare Figs. 2 and 6). This data suggest that the toxic effect of the hydroquinone metabolites originated by fungal treatment on primary fibroblasts may be due to a mechanism which does not involve DNA damage.

This increase of DNA damage on fibroblasts and HCT116 cells may be due to fungal metabolites originated during hydroquinone degradation. Nevertheless, this fungal strain showed the capacity to reduce hydroquinone to concentrations at which DNA strand breaks become basal level in human fibroblasts and HCT116 cells (Table 2).

Given that hydroquinone is a relevant environment pollutant, and that bioremediation has obvious advantages over chemical degradation, efforts have been made to identify microorganisms capable of hydroquinone degradation under harsh conditions [6,11,23,35]. However, studies monitoring the efficiency of hydroquinone removal have remained scarce. The present study shows that *P. chrysogenum* var. *halophenolicum* exhibits high tolerance and degradation capacity to hydroquinone, as it was able to remove up to 7265 μM of the aromatic compound under 1 M NaCl. Furthermore, a cumulative O_2 uptake of 440 and 720 mg/l was obtained in respirometric assays for initial hydroquinone concentrations of 4541 μM and 7265 μM, respectively. Since the theoretical carbonaceous oxygen demand (ThOD) for 4541 and 7265 μM of hydroquinone was calculated to be 872 mg/l and 1395 mg/l, respectively, our results indicate that at least 50% of carbon from hydroquinone is converted to CO_2, supporting the hypothesis that hydroquinone is a substrate readily and efficiently used by fungus.

In conclusion, *in vitro* tests showed that hydroquinone is cytotoxic for human fibroblasts and HCT116 cells. Moreover, hydroquinone induces DNA damage to fibroblast and HCT116 cells in the form of DNA single and double strand breaks as it was demonstrated by alkaline comet assay. Our data provides also the first evidence that, without prior acclimation, *P. chrysogenum* var. *halophenolicum* has the capacity to degrade hydroquinone present at high initial concentrations in hypersaline media to levels that are non-genotoxic to human cells. Overall, the present study supports the potential of *P. chrysogenum* var. *halophenolicum* for the treatment of salty phenolic-contaminated wastewaters.

Conflict of interest

None declared.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

[1] T.J. Atkinson, A review of the role of benzene metabolites and mechanisms in malignant transformation: summative evidence for a lack of research in nonmyelogenous cancer types, Int. J. Hyg. Environ. Health 212 (2009) 1–10.
[2] A. Azqueta, A.R. Collins, The essential comet assay: a comprehensive guide to measuring DNA damage and repair, Arch. Toxicol. 87 (2013) 949–968.
[3] H.S. Bae, J.M. Lee, S.T. Lee, Biodegradation of 4-chlorophenol via a hydroquinone pathway by *Arthrobacter ureafaciens* CPR706, FEMS Microbiol. Lett. 145 (1996) 125–129.
[4] H. Bahrs, A. Putschew, C.E. Steinberg, Toxicity of hydroquinone to different freshwater phototrophs is influenced by time of exposure and pH, Environ. Sci. Pollut. Res. Int. 20 (2013) 146–154.
[5] G. Barreto, D. Madureira, F. Capani, L. Aon-Bertolino, E. Saraceno, L.D. Alvarez-Giraldez, The role of catechols and free radicals in benzene toxicity: an oxidative DNA damage pathway, Environ. Mol. Mutagen. 50 (2009) 771–780.
[6] P. Bergauer, P.A. Fonteyne, N. Nolard, F. Schinner, R. Margesin, Biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts, Chemosphere 59 (2005) 909–918.
[7] J.J. Black, P.C. Baumann, Carcinogens and cancers in freshwater fishes, Environ. Health Perspect. 90 (1991) 27–33.
[8] H. Chen, J. Yao, F. Wang, M.M. Choi, E. Bramanti, G. Zaray, Study on the toxic effects of diphenol compounds on soil microbial activity by a combination of methods, J. Hazard. Mater. 167 (2009) 846–851.
[9] G.M. DeGraeve, D.L. Greiger, J.S. Meyer, H.L. Bergman, Acute and embryo-larval toxicity of phenolic compounds to aquatic biota, Arch. Environ. Contam. Toxicol. 9 (2008) 557–568.
[10] J.E. Deweese, N. Osheroff, The DNA cleavage reaction of topoisomerase II: wolf in sheep's clothing, Nucleic Acids Res. 37 (2009) 738–748.
[11] F.J. Enguita, A.L. Leitão, Hydroquinone: environmental pollution, toxicity, and microbial answers, BioMed Res. Int. (2013), http://dx.doi.org/10.1155/2013/542168.
[12] J. Griffith, R.C. Duncan, W.B. Riggan, A.C. Pellom, Cancer mortality in US. counties with hazardous waste sites and ground water pollution, Arch. Environ. Health 44 (1989) 69–74.
[13] S.F. Guedes, B. Mendes, A.L. Leitão, Resorcinol degradation by a *Penicillium chrysogenum* strain under osmotic stress: mono and binary substrate matrices with phenol, Biodegradation 22 (2011) 409–419.
[14] R. Guerra, Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents, Chemosphere 44 (2001) 1737–1747.
[15] M. Hendryx, J. Conley, E. Fedorko, J. Luo, M. Armistead, Permitted water pollution discharges and population cancer and non-cancer mortality: toxicity weights and upstream discharge effects in US rural–urban areas, Int. J. Health Geogr. 11 (2012) 9.
[16] C.P. Huang, W.H. Fang, L.I. Lin, R.Y. Chiou, L.S. Kan, N.H. Chi, Y.R. Chen, T.Y. Lin, S.B. Lin, Anticancer activity of botanical alkyl hydroquinones attributed to topoisomerase II poisoning, Toxicol. Appl. Pharmacol. 227 (2008) 331–338.
[17] M. Ishihama, T. Toyooka, Y. Ibuki, Generation of phosphorylated histone H2AX by benzene metabolites, Toxicol. In Vitro 22 (2008) 1861–1868.
[18] K.H. Jones, P.W. Trudgill, D.J. Hopper, 4-Ethylphenol metabolism by *Aspergillus fumigatus*, Appl. Environ. Microbiol. 60 (1994) 1978–1983.
[19] K.L.E. Kaiser, V.S. Palabrica, *Photobacterium phosphoreum* toxicity data index, Water Poll. Res. J. Can. 26 (1991) 361–431.
[20] F. Kamada, S. Abe, N. Hiratsuka, H. Wariishi, H. Tanaka, Mineralization of aromatic compounds by brown-rot basidiomycetes – mechanisms involved in initial attack on the aromatic ring, Microbiology 148 (2002) 1939–1946.
[21] M. Kiffe, P. Christen, P. Arni, Characterization of cytotoxic and genotoxic effects of different compounds in CHO K5 cells with the comet assay (single-cell gel electrophoresis assay), Mutat. Res. 537 (2003) 151–168.

[22] A.L. Leitão, M.P. Duarte, J. Santos Oliveira, Degradation of phenol by a halotolerant strain of *Penicillium chrysogenum*, Int. Biodeterior. Biodegrad. 59 (2007) 220–225.
[23] A.L. Leitão, C. Garcia-Estrada, R.V. Ullan, S.F. Guedes, P. Martin-Jimenez, B. Mendes, J.F. Martin, *Penicillium chrysogenum* var. *halophenolicum* a new halotolerant strain with potential in the remediation of aromatic compounds in high salt environments, Microbiol. Res. 167 (2012) 79–89.
[24] Q. Li, M.T. Aubrey, T. Christian, B.M. Freed, Differential inhibition of DNA synthesis in human T cells by the cigarette tar components hydroquinone and catechol, Fundam. Appl. Toxicol. 38 (1997) 158–165.
[25] Y. Li, M.A. Trush, DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation, Carcinogenesis 14 (1993) 1303–1311.
[26] Y. Li, M.A. Trush, Oxidation of hydroquinone by copper: chemical mechanism and biological effects, Arch. Biochem. Biophys. 300 (1993) 346–355.
[27] R.H. Lindsey Jr., R.P. Bender, N. Osheroff, Effects of benzene metabolites on DNA cleavage mediated by human topoisomerase II alpha: 1,4-hydroquinone is a topoisomerase II poison, Chem. Res. Toxicol. 18 (2005) 761–770.
[28] P.D. Lovell, T. Omori, Statistical issues in the use of the comet assay, Mutagenesis 23 (2008) 171–182.
[29] L. Luo, L. Jiang, C. Geng, J. Cao, L. Zhong, Hydroquinone-induced genotoxicity and oxidative DNA damage in HepG2 cells, Chem. Biol. Interact. 173 (2008) 1–8.
[30] D. McGregor, Hydroquinone: an evaluation of the human risks from its carcinogenic and mutagenic properties, Crit. Rev. Toxicol. 37 (2007) 887–914.
[31] M.J. Moonen, S.A. Synowsky, W.A. van den Berg, A.H. Westphal, A.J. Heck, R.H. van den Heuvel, M.W. Fraaije, W.J. van Berkel, Hydroquinone dioxygenase from *Pseudomonas fluorescens* ACB: a novel member of the family of nonheme-iron(II)-dependent dioxygenases, J. Bacteriol. 190 (2008) 5199–5209.
[32] M. North, V.J. Tandon, R. Thomas, A. Loguinov, I. Gerlovina, A.E. Hubbard, L. Zhang, M.T. Smith, C.D. Vulpe, Genome-wide functional profiling reveals genes required for tolerance to benzene metabolites in yeast, PLoS ONE 6 (2011) e24205.
[33] C. Peng, D. Arthur, F. Liu, J. Lee, Q. Xia, M.F. Lavin, J.C. Ng, Genotoxicity of hydroquinone in A549 cells, Cell Biol. Toxicol. 29 (2013) 213–227.
[34] M.T. Smith, Advances in understanding benzene health effects and susceptibility, Annu. Rev. Public Health 31 (2010) 133–148.
[35] U. Szewzyk, B. Schink, Degradation of hydroquinone, gentisate, and benzoate by a fermenting bacterium in pure or defined mixed culture, Arch. Microbiol. 151 (1989) 541–545.
[36] V. Ugartondo, M. Mitjans, M.P. Vinardell, Comparative antioxidant and cytotoxic effects of lignins from different sources, Biores. Technol. 99 (2008) 6683–6687.