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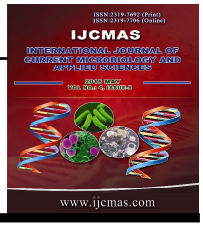

Original Research Article

An Industrial Dye Decolourisation by *Phlebia* sp.

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A B S T R A C T

In present study three ligninolytic white-rot fungi namely *Phanerochaete chrysosporium*, *Phlebia brevispora* and *Phlebia floridensis* were used for degradation of coracryl brilliant blue, an industrial dye. The biodegradation studies employing growing fungi for dye degradation suffer from problem of absorption by mycelia. This study employs cell free enzyme extracts obtained at different period of growth of the three fungi. The cell free enzyme extracts were used for production of ligninolytic enzyme and evaluation of their related dye degradation potential. In general, the two ligninolytic enzyme producing *Phlebia* sp. has been found to be good coracryl brilliant blue degraders.

Introduction

Dyes are coloured substances used for colouration of various substances including paper, leather, fur, hair, food, drugs, cosmetics and textiles materials etc. Azo dyes which form the largest & most important group are primary aromatic amines. White rot fungi are also capable of degrading a wide range of hazardous xenobiotic including various synthetic dyes (Coulibaly *et al.,* 2003; Gomi *et al.* 2011). These dyes are structurally related to triphenylmethane dyes. Acid azo dyes are characterized by the presence of a chromophoric azo group in addition to sulfonic acid group (Mautaoukkil *et al.,* 2004).

The chemical structure of azo linkage and aromatic sulpho group make them recalcitrant to biological degradation (Chander, 2014). The other chemicals which are used in industry as dyes are nitro, nitroso, diphenylamine dyes, heterocyclic dyes, vat dyes, anthraquinoid dyes, sulphur dyes, diphenyl and triphenyl methane dyes, xanthene dyes etc. These dyes are widely used in industries viz. textile, leather, paper, food and cosmetics etc. Such extensive use of colour often leads to problem of coloured waste waters, which need pre-treatment for colour removal prior to its disposal (Malaviya *et al.,* 2012). Some of the dyes are mutagenic and carcinogenic.

The waste water treatment systems used are unable to completely remove the recalcitrant dyes from the effluents. These dyes are released into the aquatic and terrestrial environment through the effluents emerging from the textile and dyestuff industries (Chander *et al.*, 2014).

Mutagenic and carcinogenic nature of these dyes and intense colouration which they exhibit are potent sources of soil and ground water pollution (Wesenberg *et al.*, 2002).

Environmental pollution is a worldwide problem and remediation of contaminated sites can be complex and expensive.

Dyes are mostly resistant to microbial attack and in certain cases, under anaerobic conditions bacteria reduce these dyes to carcinogenic aromatic amines (Chander and Arora, 2007). However ligninolytic enzymes of white rot fungi (WRF) can degrade these dyes under aerobic conditions.

These dyes must first be taken up by the bacterial cell for degradation (Coulibaly *et al.*, 2003), whereas in the fungal system dye degradation is carried out entirely by the extracellular enzymes. Therefore in recent years attention has been directed towards fungal decolourisation (Chander, 2014).

Most of the research work is centered on dye decolourisation using *Phanerochaete chrysosporium* and *Coriolus versicolor* which degrade many chemically diverse pollutants both in solid and liquid cultures.

In the present study three WRF including *Phanerochaete chrysosporium, Phlebia brevispora, Phlebia floridensis* have been used to study the decolourisation of a coracryl brilliant blue, a commercial textile dye (Disperse dye).

Materials and Methods

Microorganisms: Three white rot fungal cultures selected for the present study include

Phanerochaete chrysosporium (*BKM-F 1767), Phlebia brevispora* (*HHB-7030), Phlebia floridensis (HHB-9905)*. The WRF were procured from Dr. Rita Rentmeester, Forest Product Laboratory, USDA, Wisconsin, USA. The cultures were maintained on Glucose Yeast extract Agar plates and stocked at – 20°C.

Chemicals: Coracryl brilliant blue X, a disperse dye was procured from Colourtex Industries Limited, Vatva, Gujrat. Rest of the chemicals was of analytical grade and was purchased from Hi-Media Chemicals Mumbai.

Inoculum Preparation: Cultures were grown on Glucose Yeast extract Agar (GYA) plates. GYA contains, yeast extract - 5.0 g, peptone - 5.0 g, glucose - 10.0 g, agar - 15.0 and distilled water - 1l. The pH of medium was adjusted to 5.0 using 1N HCl. The GYA medium was autoclaved at 15psi for 15mins. After the medium solidified, each agar plate was inoculated at the centre with fungal mycelial disc (8 mm) taken from periphery of 6–8 days old grown fungal culture. *P. chrysosporium* was incubated at 37±0.5°C while *Phlebia* sp. were incubated at 25±0.5°C. These 7–8 days grown culture were used cut mycelia plugs with a 8mm cavity borer and used as inoculum.

Dye decolourisation studies in broth culture

The three white rot fungi were grown on mineral salt broth (MSB) containing as described by Gill *et al.* (2002) with scale up of medium. The Erlenmeyer flasks (250ml)

containing 120ml of MSB were autoclaved at 15psi for 15min and inoculated with 10 fungal discs (8mm) obtained from 7–8 days grown cultures on GYA plates and incubated on a shaker at 200rpm at their optimum growth temperature i.e. 37±0.5°C for *P. chrysosporium* and 25±0.5°C for *Phlebia* sp. On 6, 8 and 10th day of incubation, the extracellular medium containing dye-degrading enzymes was obtained by filtering through pre-weighed Whatmann filter paper no.1.

Hundred milliliters of cell free enzyme extract (CFEE) obtained as above was taken in sterilized 250ml conical flask and filter sterilized dye was added to obtained its final concentration of 30µg/ml. The dye containing extract was incubated on the shaker at 200 rpm at the optimum growth temperature of respective fungi, and decolourisation of dye was assayed as calculated as discussed previously (Chander and Arora, 2007). The enzyme extracts without dyes were used for estimation of three ligninolytic enzymes.

Enzyme assays

Lignin peroxidase (E.C.1.11.1.14)

LiP assay were done by monitoring the oxidation of dye Azure B is presence of H_2O_2 (Arora and Gill, 2001). The reaction mixture contained (final concentration) sodium tartarate buffer (50mm, pH 3.0), azure b (32µm), H_2O_2 (100 µm) and 0.5ml of enzyme extract. The reaction was initiated by adding 0.5ml of H_2O_2. One unit of enzymes activity is equivalent to an absorbance decrease of 0.1 units min^{-1} ml^{-1}.

Manganese peroxidase (E.C.1.11.1.13):

MnP was assayed by monitoring the oxidation of phenol red (Orth *et al.,* 1993). Five milliliters of reaction mixture contained 1.0ml sodium succinate buffer (50mM, pH 4.5), 1.0ml sodium lactate (50mM, pH 5.0), 0.4ml manganese sulphate (0.1mM), 0.7ml phenol red (0.1mM), 0.4ml H_2O_2 (50µm), gelatin 1mg ml^{-1} and 0.5ml of enzyme extract. The reaction was initiated by adding H_2O_2 and conducted at 30°C. One milliliter of reaction mixture was taken and 40µl of 5N NaOH was added to it. Absorbance was taken at 610nm. After every minute the same steps were repeated with 1ml of the reaction mixture up to 4 min. One unit of enzyme activity is equivalent to an absorbance increase of 0.1 units min^{-1} ml^{-1}.

Laccase (E.C.1.10.3.2)

Laccase activity was measured according to Arora and Sandhu (1985). The reaction mixture containing 3.8ml acetate buffer (10mM, pH 5.0), 1ml guaiacol (2mM) and 0.2ml of enzyme extract was incubated at 25°C for 2h. The absorbance was read at 450nm. Laccase activity has been expressed at colorimetric units ml^{-1} (CU ml^{-1}).

Results and Discussion

All the three fungi grew on Mineral Salt Broth cause decolourisation of dye to a variable extent. Though none of the fungi tested matched *P. floridensis* in causing decolourisation of coracryl brilliant blue *P. brevispora* gave a maximum dye decolourisation of 91% (Table 1). Dye decolourisation started at varied pace in different organism. On the 6th day of incubation, in *P. brevispora* 46%of the initial colour was removed in first 1h followed by *P. chrysosporium* and *P. floridensis* detected 37% and 36% respectively, while in 48h *P. floridensis* showed 49% (Figure 1). On 8th day of incubation *P. floridensis* showed 62% decolourisation in first 1h but the rate of decolourisation was lower in *P. brevispora*

and *P. chrysosporium* which causes dye decolourisation of 30% and 24% respectively in the same time (Figure 2). Maximum decolourisation was caused by *P. floridensis* i.e. 91% in 48h (Figure 3) followed by *P. brevispora* (64%) and *P. chrysosporium* (58%) in 48h. On 10^{th} day of incubation, decolourisation capacity of all the three fungi decreased *P. floridensis* caused 49% decolourisation in 1h while, 48% decolourisation was done by *P. brevispora* and rate was still slower in *P. chrysosporium* (26%). In 48h, 66% decolourisation was observed (Table 1-4).

P. floridensis while *P. brevispora* and *P. chrysosporium* caused 53% and 40% respectively. In *P. chrysosporium* dye decolourisation is less on 10^{th} day of incubation as compared to *P. floridensis* and *P. brevispora*. *P. floridensis* showed maximum decolourisation followed by *P. brevispora* and *P. chrysosporium*. Non-biological decolourisation was not evident from control studies and there was no pH change during entire incubation period in the culture fluids.

Ligninolytic enzyme activity

The percentage dye decolourisation was correlated with activities of LiP, MnP and Laccase. All the three white rot fungi produced three LE except, *P. chrysosporium* which do not produce laccase. LiP activity appeared on 6^{th} day of incubation in all the three fungi. Its activity was maximum in *P. floridensis* (Table 2) followed by *P. brevispora* and *P. chrysosporium*. As compared to 6^{th} day of incubation LiP activity was reduced on 8^{th} day of incubation but increased on further incubation i.e. 10^{th} day in all the fungi.

Laccase activity was higher in *P. brevispora* at all stages of incubation (0.24CU/ml) in comparison to *P. floridensis*. *Phlebia* sp. showed laccase activity on 6^{th} day of incubation which rose to a maximum on 8^{th} day of incubation, then decreased on 10^{th} day of incubation.

The MnP activity was maximum in the case of *P. floridensis* (0.018 units ml^{-1} min^{-1}) followed by *P. chrysosporium*. Only *P. brevispora* and *P. chrysosporium* showed MnP activity on 6^{th} day of incubation while in case of *P. floridensis* no activity was observed. MnP activity in *P. brevispora* decreased as the incubation period increased and no activity was observed on 10^{th} day of incubation (Table 2,3,4).

Various fungal strains have been known for their ability to degrade and decolourise various dyes. The important strains are *Phanerochaete chrysosporium, Geotrichum candidum, Trametes versicolor, Pleurotus* sp. etc. The decolourisation ability of fungi is ascribed to peroxidase and other ligninolytic enzymes, which are known to play a key role in the dye degradation. The important ones are lignin peroxidases and azo reductases ((Spadaro and Renganathan, 1994, Wesenberg *et al.*, 2002, Chander, 2014)).

Many commercially used dyes such as azo, triphenylmethane, heterocyclic and polymeric dyes were treated by crude enzyme preparation and it was found that enzyme lignin peroxidase plays a major role in decolourisation. The isozymes of lignin peroxidase (LiP4.65, LiP4.15 and LiP3.85) have been found with different specificities towards dyes as substrates (Ollikka *et al.*, 1993). The ligninolytic culture of *P. chrysosporium* decolourised several polyaromatic azo dyes. The oxidation rates of individual azo dyes depended on their structural configuration e.g. naphthalene ring was readily degraded by the powerful

degradation system of *P. chrysosporium*. Dye containing both methoxyl and hydroxyl groups, was degraded extensively than dye which lacked substitutions (Paszycynski *et al.,* 1992).

P. chrysosporium under N limiting and secondary metabolic ligninolytic conditions was able to degrade azo dyes, 4 phenyl azo phenol, 4 phenyl azo-2-methoxyphenol, disperse yellow 3, disperse orange 3 to much greater extent as compared to N_2 sufficient conditions. The degradation was monitored by the release of $^{14}CO_2$ from these labeled dyes (Spadaro and Renganathan, 1992; Paszycynski *et al.,* 1992).

Two strains of *P. chrysosporium* and another local isolate of white-rot fungus, when pre cultured in a high nitrogen medium with glucose, could decolourise two azo dyes (Amaranth and Orange G) and a heterocyclic dye (Azure B), when starch was used in the pre cultivation medium, decolourisation of Orange G occurred (Chao and Lee, 1994).

In the other study the potential of lignin degrading enzyme of *P. chrysosporium* have been shown to degrade 40–75% of the Recto fix Gold Yellow. It is based on decrease of colour. Both lignin degrading enzyme system as well as adsorption to its cell mass are involved in the degradation of the diazo dyes Recto fix Gold Yellow (Caplash and Sharma, 1992). The presence of lignin peroxidase and manganese peroxidase in addition to laccase in *Pleurotus ostreatus, Schizophyllum commune, Neurospora crassa* seemed to increase the degree of decolourisation of individual commercial dyes by 25% as in triarylmethane, anthraquinonic and indigoid textile dyes (Abdulla *et al.,* 2000). The main ligninolytic enzyme activities reported during dye decolourisation were laccase for *Phlebia tremellosa* (Kirby *et al.,* 2000, Robinson *et al.,* 2001) and lignin peroxidase *P. chrysosporium* and *Bjerkandera adusta* (Robinson *et al.,* 2001, Chander and Arora, 2014).

In a study conducted by Gill *et al.* (2002) decolourisation of all the dyes (final conc. 20μM) occurred when the cultures were grown under nitrogen-limited conditions. Almost complete decolourisation of dye Brilliant Green was achieved in 48h by *P. chrysosporium, P. floridensis* and *P. fascicularia.* The dyes Cresol Red and Crystal Violet are better decolourised by *Dichomitus squalens* and *Phlebia* sp. (almost complete) while *P. chrysosporium* decolourises only up to 20% after 5 days of incubation. These studies shows that *D. squalens* and *Phlebia* sp. Are much more efficient than *P. chrysosporium* as these caused total or near total decolourisation of all dyes (Gill *et al.,* 2002). Our work is in agreement with this study.

Disperse dyes are widely used for colouring polyester textiles. As disperse dyes are insoluble in water, they are dispersed with detergents, which may be a reason for their insusceptibility to biological degradation. A fungus *C. polymorpha* removed commonly used dispersed dyes that contain detergents and chelating agents. In synthetic medium *C. polymorpha* removed 59% of the Disperse Blue – 60 (DB-60; 0.1g/l) in 24h and 85% in 94h during cultivation. The fungal culture showed a delay of decolourisation until 16h and the dye removal was also incomplete. When the dye removal stopped, lysis of the fungal cells and drop in pH were observed. It is thought the pH drop may have caused the dye removal termination. But no such change in pH was observed during this study.

Table.1 Comparative percentage dye decolourisation by CFEE obtained from three white rot fungi

Time of incubation (hrs.)	% Dye decolourisation by CFEE obtained from *P. chrysosporium* on day			*% Dye decolourisation by CFEE obtained from* P. brevispora *on day*			% Dye decolourisation by *CFEE obtained from P. floridensis on day*		
	6	**8**	**10**	**6**	**8**	**10**	**6**	**8**	**10**
1	37	24	26	46	30	46	36	62	50
2	40	27	26	50	31	48	44	63	54
3	41	29	29	51	35	48	44	69	56
5	45	53	30	54	60	51	49	79	58
24	46	54	33	55	62	54	59	86	62
48	49	58	40	55	64	60	64	91	66

Table.2 Ligninolytic enzyme activity of *P. floridensis* on different period on incubation

Period of growth (Days)	LiP Activity Units ml^{-1} min^{-1})	MnP Activity (Units ml^{-1} min^{-1})	Laccase Activity (CU ml^{-1})
6	0.017	NA	0.0625
8	0.010	0.018	0.098
10	0.023	0.002	0.045

Table.3 Ligninolytic enzyme activity of *P. brevispora* on different period on incubation

Period of growth (Days)	LiP Activity (Units ml^{-1} min^{-1})	MnP Activity (Units ml^{-1} min^{-1})	Laccase Activity (CU ml^{-1})
6	0.02	0.003	0.125
8	0.008	0.0024	0.24
10	0.02	NA	0.08

Table.4 Ligninolytic enzyme activity of *P. chrysosporium* on different period on incubation

Period of growth (Days)	LiP Activity (Units ml^{-1} min^{-1})	MnP Activity (Units ml^{-1} min^{-1})	Laccase Activity (CU ml^{-1})
6	0.018	0.003	NA
8	0.004	0.015	NA
10	0.02	0.006	NA

Figure.1 Biodecolourisation of coracryl brilliant blue at different period of incubation by CFEE of *P. chrysosporium*

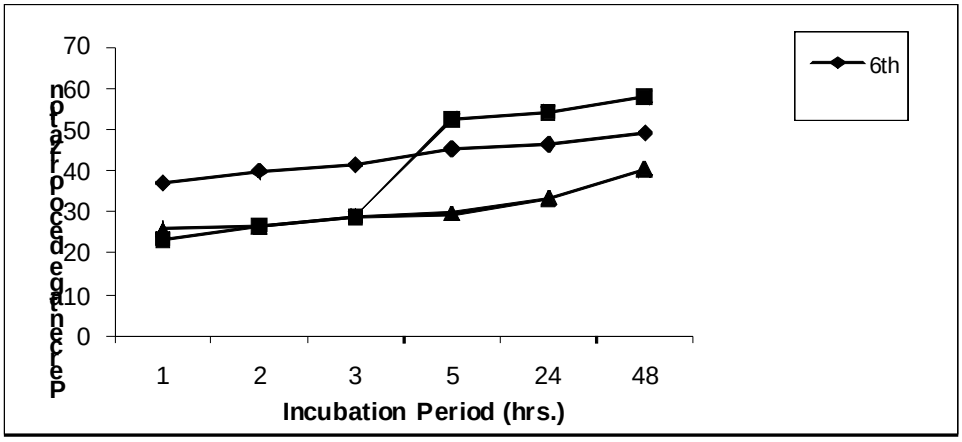


Figure.2 Biodecolourisation of coracryl brilliant blue at different period of incubation by CFEE of *P. brevispora*

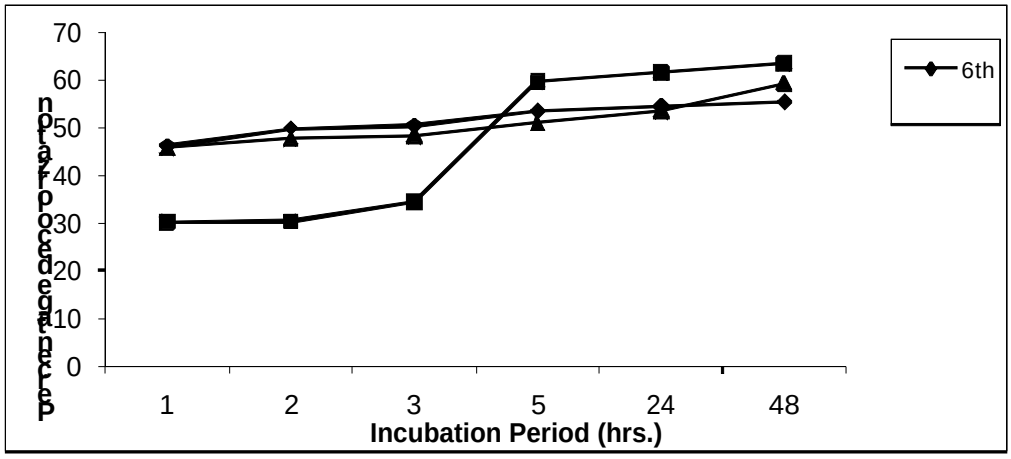
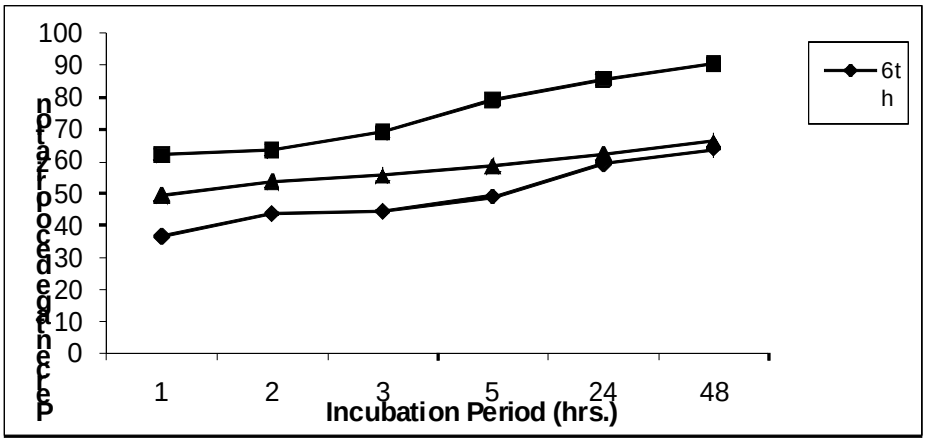


Figure.3 Biodecolourisation of coracryl brilliant blue at different period of incubation by CFEE of *P. floridensis*



In peptone glucose yeast agar (PGY) medium *C. polymorpha* removed 95% of the DB-60 in 36h and 100% of it in 120h without any decolourisation delay and fungal lysis. The pH of the medium decreases from 5.0 to 4.3. These results show that PGY medium is better than synthetic medium (Sugimori *et al.*, 1999).

Decolourisation of Poly R-478 and Poly S-119 occurred only when the fungal growth had decreased. In the absence of Mn (II) there was an 80% decrease in the levels of Poly R-478 and Poly S-119. Upon supplementation with Mn (II), fungal biomass was approximately 50% greater, with a concomitant increase in the degree of dye decolourisation with a marked decrease in the Poly R-478 and Poly S-119 level. The immobilized culture of the fungus causes 80% dye decolourisation both in the presence and absence of Mn (II) and 95% decolourisation Poly S-119 (Kevin *et al.*, 1998).

In the present study on decolourisation of coracryl brilliant blue *P. floridensis* caused its maximum decolourisation in Mineral Salt Broth, which was more efficient to cause its complete decolourisation in 120h in comparison to *P. chrysosporium*, which could cause colour loss of 64% in 120h. *P. brevispora* decolourise 64% dye in 48h and 83% in120h. 8th day of incubation showed maximum decolourisation as compared to 10th and 6th day of incubation. Rate of decolourisation by *P. chrysosporium* was slowest as compared to *P. brevispora* and *P. floridensis*. The similar observations have been made by Chander *et al.* (2014).

Three ligninolytic enzymes LiP, MnP and Laccase have been implicated in the decolourisation of dyes by white rot fungi. *Phlebia* sp. used in present study showed a better ligninolytic enzyme production potential as compared to *P. chrysosporium* (Arora and Gill, 2001). *P. floridensis*, which causes maximum decolourisation in Mineral Salt Broth, showed highest LiP activity and moderate laccase activity. It also showed MnP activity but to a lesser extent. *P. brevispora* also produced LiP, MnP and laccase enzyme. However LiP activity was relatively higher *P. chrysosporium* produced LiP and MnP enzyme but lacked laccase activity. Lack of laccase activity might be responsible for relatively lesser coracryl

brilliant blue dye decolourisation in comparison to *Phlebia* sp.

From the above discussion, it is apparent that either LiP or MnP production in association with laccase is causing dye decolourisation as in the case of *Phlebia* sp. and *P. chrysosporium*. However no single enzyme could be held responsible for dye decolourisation as the distribution of three ligninolytic enzymes varies a lot from fungus to fungus. In *P. chrysosporium* LiP-MnP combination dye decolourisation, while in others LiP-MnP-Laccase did the same job. *Phlebia* sp. capable of producing all the three enzymes gave the higher loss of dye colour in comparison to the much-studied *P. chrysosporium*. Among the three fungi studied *P. floridensis* produced maximum LiP and caused maximum dye decolourisation. The lower coracryl brilliant blue decolourisation by *P. chrysosporium* may be attributed to the comparatively lower LiP production and absence of laccase activity in it. MnP enzyme activity were found be relatively lower in all the three fungi. Thus it may be concluded that LiP in addition to MnP has a main role to play in coracryl brilliant blue decolourisation.

Dyes mainly used for colouring or dyeing purposes have many drawbacks viz. a potent source of pollution of soil and ground water, carcinogenic and mutagenic in nature. Because of these drawbacks many countries have banned the use of azo dyes and others and putting efforts to replace these with other chemicals, which are supposed to be less harmful.

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