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Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather

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ABSTRACT

The present study aimed to evaluate and optimize the dyeing potential of fungal pigments for pre tanned leather samples. Five different water-soluble pigments were extracted from *Monascus purpureus*, *Isaria* spp., *Emericella* spp., *Fusarium* spp. and *Penicillium* spp., purified and used for dyeing processes. The effect of process parameters of dyeing such as pH, temperature, time duration, exhaustion of colour, shade brightness, colour intensity and fastness properties have been studied and the conditions were optimized. The results showed that the optimum concentration of the pigments were 6% on weight of leather. However, the optimum condition for dyeing was 70 °C, at a pH 5, and the time duration 120 min. The maximum uptake of pigments in the leather samples varied from 40% to 70 ± 0.2%. The changes in shades of the samples were high in *M. purpureus* – red pigment and it compared with visual assessment data. The fungal pigments did not significantly alter the organoleptic properties of the leather sample.

1. Introduction

Highly coloured substances, known as dyes or colourants, are widely used to impart colour to a boundless variety of materials described technically as substrates (McLaren, 1983; Sivakumar, Lakshmi, Vijayeeswaree, & Swaminathan, 2009). The increased application of these dyes or colourants in dyeing industries and inefficiencies in dyeing result in a large amount of dyestuff being directly lost in the wastewater, which ultimately finds way into the environment. It is estimated that 10–35% of the dye is lost in the effluent during the dyeing process, while in the case of reactive dyes, as much as 50% of the initial dye load is present in the dye bath effluent (Rai et al., 2005). Moreover, some of these dyes contain potential colon carcinogens, which is a possible hazard to humans when chronically exposed (Osman, Sharaf, Osman, El-Khouly, & Ahmed, 2004). Hence, there is a growing demand for eco-friendly/non-toxic colourants, specifically for health sensitive applications such as colouration of food and dyeing of child textile and leather garments.

Natural dyes and pigments are emerged as an important alternative to potentially harmful synthetic dyes (Sivakumar et al., 2009). The application of these natural dyes and pigments in dyeing of cotton, silk and wool samples has reported in several studies (Kamel, El-Zawahrym, Ahmed, & Abdelghaffar, 2009; Rekaby, Salem, & Nassar, 2008). However, the main disadvantage of these natural dyes or pigments lies in the order of magnitude of their extraction yield factors (a few grams of pigment per kg of dried raw material). This makes their current market price about USD 1/g, thus limiting their application to high-value-added natural-coloured garments only. To defeat this constraint, it is suggested to exploit the potentiality of other biological sources such as fungi, bacteria and cell cultures, since appropriate selection, mutation or genetic engineering techniques are likely to improve significantly the pigment production yields with respect to wild organisms (Mapari et al., 2005).

Fungi are reported as potent pigment producing microorganisms (Babitha, Soccol, & Pandey, 2007). Hamlyn (1995) reported the importance of pigments such as anthraquinone, anthraquinone carboxylic acids, pre-anthraquinones extracted from filamentous fungi. The application of these fungal pigments in dyeing of cotton, silk and wool has reported in several studies (De Santis, Gallo, & Petruccioli, 2005; Nagia & EL-Mohamedy, 2007). However, the application of these fungal pigments for leather sample has not been determined. Hence, the main objective of the present study is to develop simple methodology for both extractions of pigment from filamentous fungi as well as for application in the leather dyeing. Moreover, the dyeing conditions were optimized and characteristics of dyed leather samples were assessed by standard methods.

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2. Materials and methods

2.1. Origin and identification of fungi

Soil samples were collected from The Nilgris, a high altitude and biodiversity hotspot in the Western Ghats of state Tamil Nadu, India (Manoharachary et al., 2005). Fifty-one soil samples were collected from diversified locations and transported on ice to the laboratory and processed within 24 h. Pigment producing fungi were isolated by serially diluting 1 g of the soil sample in sterile distilled water and 1 ml of the appropriate dilution were plated by pour plate technique on Potato Dextrose Agar (PDA). The isolates were purified and stored in a PDA slants at 4 °C for further studies.

The selected four isolates were identified by Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, G.G. Agharkar Road, Pune, India. The reference culture *Monascus purpureus* MTCC 410 was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

2.2. Cultivation, extraction and purification of fungal pigments

The isolated fungi was cultivated individually on (3 L) of defined mineral salts-glucose medium contained (per liter of deionized water): glucose 30 g; 1.0 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4{\cdot}7H_2O$; 1.4 g K_2HPO_4; 0.6 g KH_2PO_4; 0.8 mg $ZnSO_4{\cdot}H_2O$; 0.8 mg $FeC1_3{\cdot}6H_2O$; 0.8 mg $NaMoO_4{\cdot}2H_2O$; 0.4 mg $MnSO_4{\cdot}2H_2O$; 0.08 mg $CuSO_4{\cdot}5H_2O$; and pH 5.6 in Haffins flask (Judd & Wyeszchi, 1975). All flasks were inoculated by a mycelial disk by using cork borer (12 mm diameter) from PDA culture of *M. purpureus*, *Isaria* spp., *Emericella* spp., *Fusarium* spp. and *Penicillium* spp. which were grown at 27 °C in the dark as stationary cultures for 4–6 wk (Nagia & EL-Mohamedy, 2007). After an incubation period of 6 wk, the mycelium was harvested, and the supernatant was filtered in a sterilized muslin cloth. Later, two volumes of 95% (v/v) ethanol was added to exhausted culture broth according to the following procedure: (i) after dilution with about 60% of the solvent volume needed, the resulting mixture was kept on the rotary shaker at 180 rpm at 30 °C for 30 min; (ii) the ethanolic mixture was centrifuged at 3780 rpm for 15 min; (iii) once the supernatant had been recovered, the residue was dispersed in the remaining volume of ethanol and centrifuged again at 3780 rpm for 5 min; and (iv) the supernatants were then collected and filtered through a preweighed Whatman GF/C filter paper (47 mm) and further diluted with 95% (v/v) ethanol to a final volumetric dilution factor of 20. Next, the absorption spectrum was observed at 300–600 nm using Hitachi U-2000 spectrometer (Hitachi Ltd., Tokyo, Japan) (De Santis et al., 2005). The purified pigments were concentrated in a buchi rotary evaporator and lyophilized to obtain powder.

The optical density (OD) was measured at 400, 550, 520 and 530 nm (a wavelength which represents the absorption maxima for yellow, pink, reddish brown and red pigments, respectively) and multiplied by the above dilution factor, thus yielding the so-called yellow, pink, reddish brown and red pigment production (Fig. 1), expressed in units of absorbance (λUA) at a given wavelength (λ). Finally, the mycelial biomass recovered by centrifugation was dispersed in deionised water, filtered through a preweighed Whatman GF/C filter paper (47 mm), dried at 105 °C for 12–15 h and weighed to yield the biomass (X) concentration.

2.3. Leather dyeing

The conventional chromed wet blue goat tanned leathers sample was purchased from the Sri Lakshmi Tannery Industry, Vaniampadi, India. Chrome tanned wet blue goat leather was cut into 9-cm diameter circular pieces as per SLTC 1996 official method of sampling and neutralized to pH 6.0–6.5 using sodium formate (1%) and sodium bicarbonate (1%) solution. Dyeing was carried out by shaking leather tad with a optimized concentration of 6% on weight of leather (owl) of each pigment in 100 ml deionized water at 70 °C for 120 min in a thermostatted shaker bath operated at 100 strokes/min with 1.5 g sodium sulphate (Sivakumar et al., 2009) at pH 5. The dyed samples were rinsed with cold water to remove the unfixed pigment, in a bath of liquor ratio (L:R) 40:1 using 3 g/L nonionic detergent (Hostapal CV, Clariant) at 50 °C for 30 min, and then air dried. The dye bath pH was monitored with cyberscan pH meter and adjusted with dilute solutions of 1 M sodium carbonate.

2.4. Optimization of leather dyeing

The leather samples were dyed with different pigment concentrations (2–14% owl) at different temperatures (30–80 °C), pH (2–9), time (30–140 min) and at a fixed salt concentrations (1.5 g/L). After optimization, the samples were dyed with the optimum conditions (pigment concentrations 6% owl, pH 5, 70 °C for 120 min).

2.5. Pigment exhaustion in the process liquor

The unspent pigment in the exhausted process liquor (before and after dyeing) was analyzed using Hitachi U-2000 spectrometer. The percentage of pigment exhaustion was calculated using the following equation:

$$\% \text{ Pigment exhaustion} = [(C_g - C_t)/C_g] \times 100 \quad (1)$$

where C_g is the concentration of pigment used and C_t is the concentration of pigment in the spent liquor.

2.6. Colour measurement analysis

Quantification of pigment dyed leather colour was made according to the Commission Internationale de l'Eclairage (CIE) system of colour measurement with 100 standard observer data (McLaren, 1983). L^*, a^*, b^*, c^* and h values for both the grain shade of the dyed leathers were obtained using Data colour SF 600 spectrophotometer (Data colour, Dietikon, Switzerland). The values L^*, a^*, b^*, c^* and h are the variables in the CIELAB colour space and explained as follows. More negative value of L^* denotes darker shade and more positive value of L^* for more light shade of the colour. More negative value of a^* implies more green colour and more positive value of a^* for more red colour. More negative value of b^* means more blue colour and more positive value of b^* for more yellow colour. c^* represents chroma or purity of colour. h represent hue (shade) of colour.

2.7. Evaluation of visual colour

Dyed leather samples were subjected to visual assessment for uniformity of colour, depth of shade, colour shift from control and general appearance by standard tactile evaluation technique. Four experienced tanners rated the leathers on a scale of 0–10 points for each functional property with 0 as the lowest and 10 as the best. The average rating was calculated for each parameter and used for comparison studies.

2.8. Determination of fastness properties on dyed leather

Dyed leather samples were tested for light fastness after conditioning according to IS 6191–1971 (LF: 4), (Indian Standards IS 6191, 1971). The samples were exposed to xenon arc light under

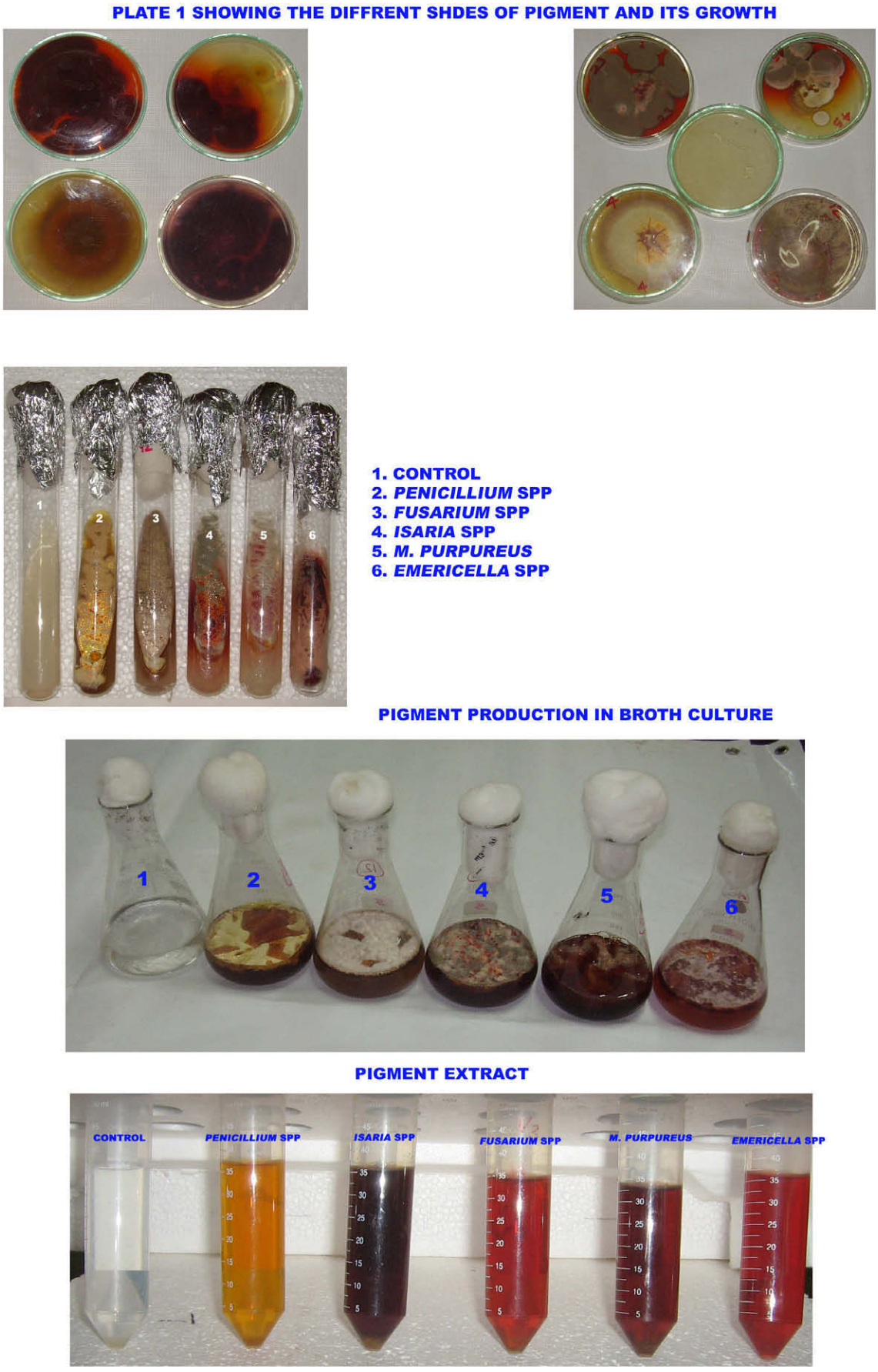

Fig. 1. Different shades of water-soluble pigment produced by five different genus of fungi. (For interpretation of colour mentioned in this figure, the reader is referred to the web version of this article.)

prescribed conditions for 20 h along with the dyed blue wool standards.

3. Results and discussions

In the present study, an attempt has made to evaluate and optimize the dyeing potential of filamentous fungal pigments for processed leather samples. The dyeing approach was based on the concept that the fungal pigment may contain some biocatalysts (Ferreira-Leitao, Andrade de Carvalho, & Bon, 2007), which improves the swelling of the fibrous leather network and thereby increasing the diffusion of the pigment containing molecules into the leather samples. Improved exhaustion of fungal pigment will not only reduce the pollution but also result in safety leather products for humans.

One hundred and fifty-three morphologically different fungi were isolated from the collected soil samples. Among the 153 isolates, four fungi were selected based on its ability to produce different shades (red, reddish brown, pink and yellow) of pigments. The four potent isolates were identified as *Isaria* spp., *Emericella* spp., *Fusarium* spp. and *Penicillium* spp. based on the morphological and cultural characteristics. Table 1 shows the shades of the pigment, biomass yield and pigment concentration extracted from the isolated fungus. Each isolate exhibited a marked difference in the pigment shade, pigment concentration and biomass yield. Maximum production ($\geqslant$20 UA) of pigment was observed in the *M. purpureus* and *Penicillium* spp. However, minimum production ($\leqslant$20 UA) was observed in *Isaria* spp., *Emericella* spp. and *Fusarium* spp. Similarly, maximum biomass was observed in *M. purpureus* (6.2 ± 0.3 g dm^{-3}) followed by *Penicillium* spp. (5.2 ± 1.6 g dm^{-3}), *Isaria* spp. (5.1 ± 0.9 g dm^{-3}), *Fusarium* spp. (4.1 ± 1.2 g dm^{-3}) and *Emericella* spp. (3.6 ± 0.6 g dm^{-3}), respectively.

3.1. Optimization of pH, time duration and temperature

In order to attain improved exhaustion and distribution of pigments into the leather, conditions on the usage of pigments have to be optimized. The optimized concentration of all the five pigments at 6% owl at a salt concentration of 1.5 g/L in (*L*:*R*) liquor to goods ratio 40:1, were used for further studies. The red pigment, produced by *M. purpureus* showed 70% of pigment exhaustion, followed by 60% exhaustion of *Penicillium* spp. (yellow) pigment, respectively. However, the fungus *Isaria* spp. (reddish brown pigment), *Emericella* spp. (pink pigment), *Fusarium* spp. (red pigment), produced maximum pigment exhaustion 40% and 35% at 6% owl of pigment, respectively. The results obtained with respect to the effect of pH, temperature and time duration on the exhaustion of pigment in the process liquor is shown in Fig. 2a, b and c. From the Fig. 2a, it can be observed that the exhaustion of pigment increases gradually up to pH 5.0, and further increase in pH resulted in decrease in exhaustion of pigment. The increased exhaustion of pigment in pH 5 could be due several factors; the acidic pH may increase the fiber swelling of leather samples, alternatively the stability and nature of pigments could not altered at this pH. The fixation of pigments to the leather samples at different time duration is shown in Fig. 2b. The results exhibited that the uptake of colour increases gradually according to the time. The sample requires maximum of 120 min to bring about significant exhaustion in dye bath. Hence, 120 min time interval has taken as optimum duration.

The results obtained for the exhaustion of pigment at different temperatures is shown in Fig. 2c. The results showed that there was an increase in exhaustion of pigments according to the temperature from 30 to 70 °C, whereas the pigment exhaustion was decreased afterwards (80–120 °C). The maximum pigment exhaustion at 70 °C indicates that the functional molecules present in the pigments were highly active at this temperature, whereas the decreased exhaustion from 80 °C could be due to instability of the functional molecules and biocatalysts at higher temperature. The optimized pigment concentration of 6% owl, pH 5, salt concentration 1.5 g/L, 120 min, temperature 70 °C, resulted in maximum pigment uptake of 94.70% (*M. purpureus* – red), 90% (*Penicillium* spp. – yellow), 79.8% (*Fusarium* spp. – reddish brown), 77% (*Emericella* spp. – red) and 81.6% (*Isaria* spp. – pink).

3.2. Colour analysis by reflectance measurement

Quantification of colour value of the leathers dyed by conventional dyeing method with different shades of pigment obtained from five filamentous fungi was analyzed by reflectance measurement. The colour values of the samples L^*, a^*, b^*, c^* and h are shown in Table 2. Marked improvement in the colour intensity was observed in red and yellow pigment produced by *M. purpureus* and *Penicillium* spp. dyed with 6% owl. However, remaining pigment produced by *Isaria* spp. (reddish brown), *Emericella* spp. (pink), *Fusarium* spp. (red) did not give a significant shades in leather dyed with same percentage of pigment.

3.3. Visual assessment of leathers

Visual assessment for shift in colour from control, uniformity of colour, depth of shade and general appearance (optimized dyeing conditions) was carried out by standard tactile evaluation technique, and the values are detailed in Table 2. The depth of shade was moderate uniformity for the reddish brown, pink, red pigments produced by *Isaria* spp., *Emericella* spp. and *Fusarium* spp., and further better uniformity in pigments of *M. purpureus* – red and *Penicillium* spp. – yellow at 6% owl. The intensity of the pigment dyed leather was comparatively lower than the control. There was no appreciable change or shift in colour for the experimental leathers compared to control. These results were in agreement with the reflectance measurement values. The uniformity of colour, pigment penetration and shade was moderate for the pigment dyed leather samples. In general, a moderate improvement

Table 1
Pigment produced by five filamentous fungi and the production concentration of pigment, final pH and biomass.

Fungi	pH	X (g dm^{-3})	UA_{400}	UA_{550}	UA_{600}	Extract colour[a]
M. purpureus	5.2	6.02 ± 0.3	25.0 ± 1.6	20.1 ± 1.4	16.6 ± 1.8	R
Isaria spp.	4.6	5.10 ± 0.9	10.1 ± 1.2	7.2 ± 1.2	3.1 ± 0.3	P
Emericella spp.	5.9	3.68 ± 0.6	16.0 ± 0.6	10.1 ± 1.4	6.2 ± 0.2	R
Fusarium spp.	5.6	4.16 ± 1.2	12.2 ± 0.2	8.2 ± 1.4	3.3 ± 0.4	RB
Penicillium spp.	4.6	5.20 ± 1.6	23.0 ± 0.3	19.0 ± 1.2	14.4 ± 0.2	Y

[a] R, red; P, pink; RB, reddish brown; Y, yellow.

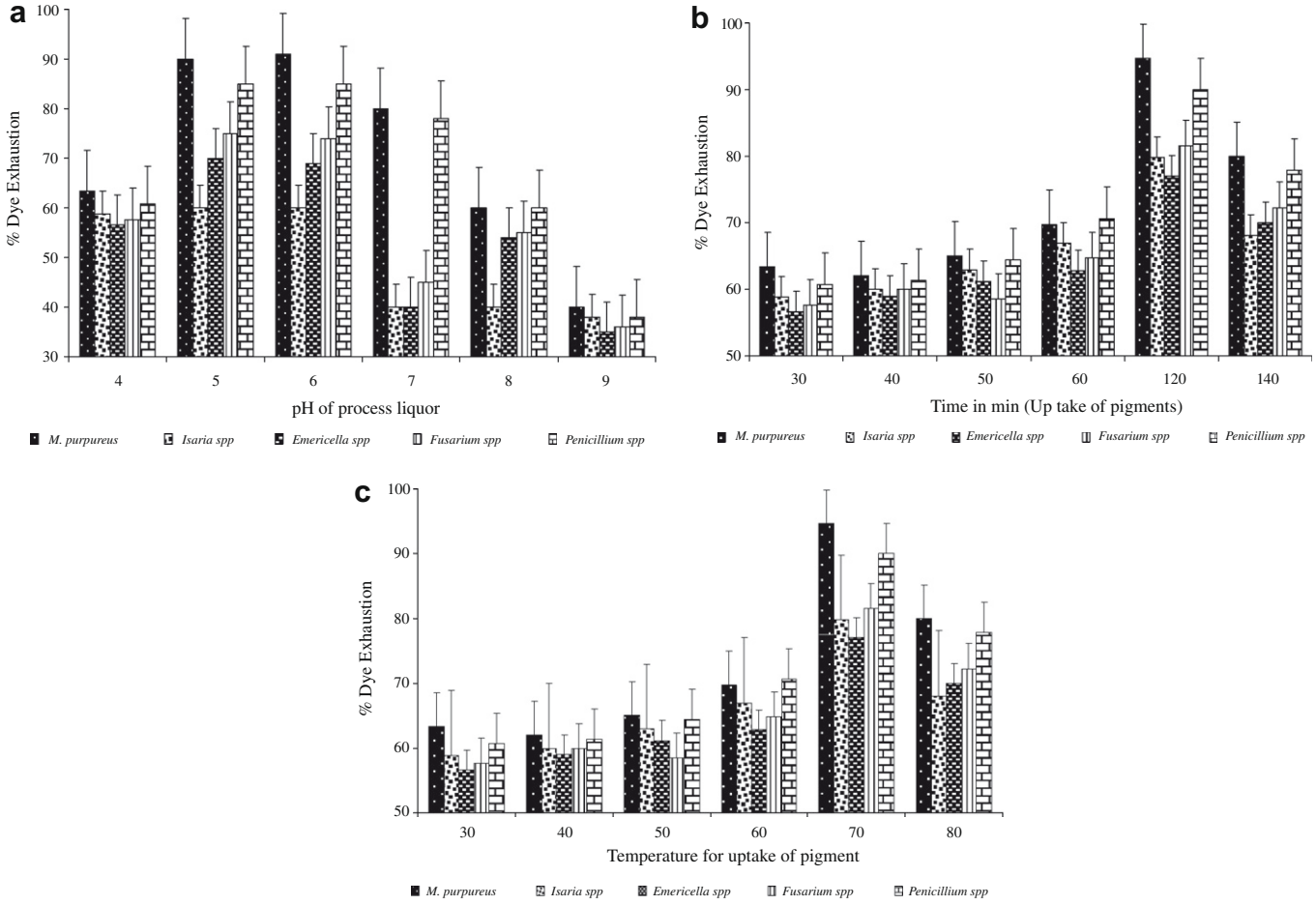

Fig. 2. (a) Effect of pH on pigment uptake (6% owl) in room temperature for 30 min. (b) Effect of time on pigment uptake (6% owl) in room temperature at pH 5.0. (c) Effect of temperature (°C) on the pigment uptake at (6% owl) for 120 min in room temperature at pH 5.0.

in the appearance of the leathers dyed with fungal pigments (optimized conditions) was observed.

3.4. Assessment of bulk properties of dyed leather

The various strength characteristics of the experimental crust leathers dyed with fungal pigments at optimized conditions are given in Fig. 3. The results clearly indicate that the fungal pigments did not affect the strength characteristics of the leathers. The values of various strength properties of experimental leather were compared with the controls (Indian standards IS 576, 1975). The ratings of the bulk properties such as softness, fullness, grain smoothness, general tightness and general appearance of the pigment dyed leathers are presented in Fig. 3. The results showed that all the organoleptic properties are comparable with of chemically dyed leather samples. Especially the grain smoothness and softness of the pigment dyed leathers have been found to be moderate than chemically dyed leather.

3.5. Fastness properties of dyed leathers

The rubbing and light fastness character of the optimized dyed leather sample is given in Table 3. The results showed that the fastness to wet and dry rubbing of the pigment dyed leather was lower than control leathers. Pigment dyed leathers in general exhibited moderate light fastness (rating 3 on grey scale), equivalent to the blue wool standards. The effect of ageing (3 months) on the fastness of leather has also been studied. However, the value for wet

Table 2
L^*, a^*, b^*, c^*, h, dye exhaustion and visual assessment data of dyed leather in a optimized condition.

Fungi and pigments[a]	Colour co-ordinates					Dye exhaustion and visual colour				
	L^*	a^*	b^*	c^*	h	% Pigment exhaustion[b]	Colour shift	Uniformity	Depth of shade	Intensity of dyeing
Control (white)	66.8	5.7	17.0	27.6	78.0	75 ± 1.8	8	7.5	7	7.5
M. purpureus^{R}	67.0	24.1	18.0	17.7	25.2	70 ± 1.2	8	7.0	6	7
Isaria spp.^{P}	54.5	22.8	18.9	19.6	25.3	58 ± 1.4	7	6.5	5.5	6
Emericella spp.^{R}	55.7	23.2	17.6	16.4	30.2	40 ± 1.4	6.5	6.5	5.5	6.5
Fusarium spp.^{RB}	55.6	22.0	19.3	25.4	42.4	38 ± 1.6	6.5	6.5	6	7
Penicillium spp.^{Y}	66.2	24.7	16.8	18.2	41.0	60 ± 1.8	7	7.5	6.5	7.0

[a] R, red; P, pink; RB, reddish brown; Y, yellow.
[b] Values represents here is average of four samples ± standard deviation. Pigment concentration 6% owl.

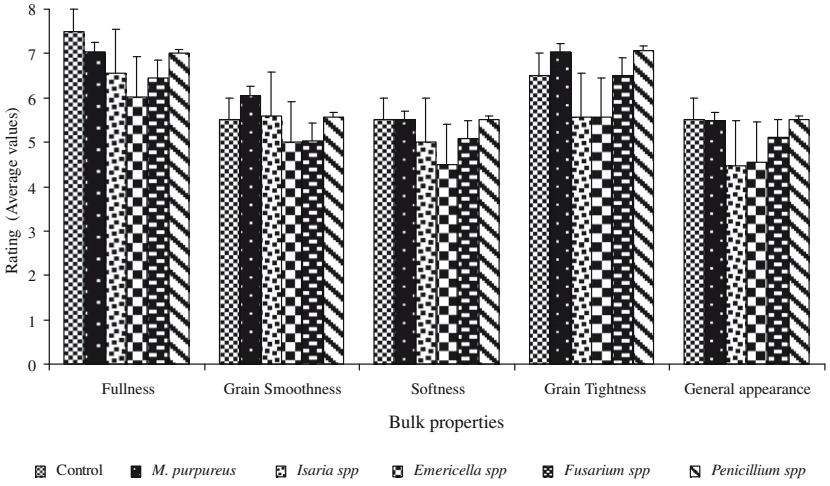

Fig. 3. Graphical representation of organoleptic properties of dyed leather.

rub and perspiration resistance is poor, which is not unexpected because mordant was not employed during the natural leather dyeing process. It is a general practice to use conventional metal mordents in dyeing with natural materials. Since the application here involves health sensitivity, metal mordents may pose toxicity, which is undesirable. Hence, research on suitable eco-friendly alternative mordents is being pursued in our laboratory for improving the wet rub and perspiration resistance as well.

3.6. Realistic mechanism of the process

Numerous studies reported that the proteinase, metalloproteinase and some other enzymes produced by the microorganisms were effectively alter the bulk properties and matrix of the leather materials (McHenry, Christeller, Slade, & Laing, 1996; Murao, Oda, Tsuji, & Terashita, 1986; Terashita, Oda, Kono, & Murao, 1985). Gomez, Rojas, Rojas, and Cruz (2004) reported that the fungi could produce a variety of proteases enzymes that will be active over a wide pH (4–11) and exhibit broad substrate specificity. Aguilar, Contreras-Esquivel, and Favela-Torres (2002) reported that the enzymes produced by the filamentous fungi are utilized in leather dyeing process for making the leather softer. Hence, presence of these kinds of enzymes in pigments results in opening-up of the fibrous leather network, which enhances the penetration of pigments. Moreover, the enzymes present in pigments can exhibit some binding, such binding can catalyze in exposing more number of functional sites for the binding of pigments. However, more studies are required to confirm the mechanism of pigment binding on leather materials.

4. Environmental and economic benefits

Since the process involves natural material with non-toxic, highly suitable for dyeing of fibrous substances such as leather for health sensitive applications. Earlier studies confirm that *M. purpureus*, *Emericella* spp. and *Penicillium* spp. pose no toxic effects (Martinkova, Jzlova, & Vesely, 1995; Youssef, El-Maghraby, & Ibrahimm, 2008) as well as the pigment produced by these fungus is biodegradable (Daniel, Silvana, Plinho, & Adriano, 2007) and contain negligible amount of phenolics component (Alvarez et al., 2002; Cheng et al., 2004). Therefore, there is no environmental risk involved in spent dye liquor unlike in the case of azo dyes. Even the spent dye liquor can be recycled for the next operation and could not develop any unpleasant odours. In addition, the use of fungal pigment for dyeing process offers significant improvements in exhaustion as an in-plant control measure to achieve near zero discharge concepts. Fungal pigment obtained can be spray dried or lyophilize to get various colourants in powder form and solvent ethanol can be recovered by suitable solvent recovery system.

Table 3
Fastness properties of the dyed leather sample at optimized condition.

Sample	Before ageing			After ageing		
Optimized pigment conc. [a] (%)	Wet rubbing	Dry rubbing	Light fastness	Wet rubbing	Dry rubbing	Light fastness
6	4–5	4.0–4.5	3.5–4.0	4.0–4.5	4.0–4.5	3.0–3.5
pH 4–8	4–5	pH[b] 4.0–4.5	3.5–4.0	4.0–4.5	4.0–4.5	3.0–3.5
30–140 (min)	4–5	Time[c] 4.0–4.5	3.5–4.0	4.0–4.5	4.0–4.5	3.0–3.5
30–80 (°C)	4–5	Temp[d] 4.0–4.5	3.5–4.0	4.0–4.5	4.0–4.5	3.0–3.5
Dyeing at optimized state pH 5, temp 70 °C and 120 min	4–5	4.0–4.5	3.5–4.0	4.0–4.5	4.0–4.5	3.0–3.5

[a] Dye concentration 6% owl.
[b] pH 5.
[c] 70 °C.
[d] 120 min.

There is a significant improvement in the yields of extract obtained due to the use of perfect cultivation process, even for the two liter of cultivation broth used. This indicates scale-up viability of the process. The environmental factor and health concern involved in the dyeing application overweigh the cost factor as compared to toxic synthetic dyes.

5. Conclusions

To conclude, the pigment concentrations 6% owl, at 70 °C, in pH 5.0 and 120 min duration were found to be optimum with respect to the uptake of pigment, pigment penetration and intensity of the colour. The pigment dyed leather at the optimized conditions resulted with uniform dyeing, intense and bright shade. The results of reflectance measurements and visual assessment data indicate that the samples have moderate chroma or purity of colour with fungal pigments. The overall fastness of the pigment dyed leather samples was moderate. Ageing of the samples does not alter the fastness significantly. The strength characteristics and organoleptic properties of the dyed leathers were not significantly altered. The results of the present study clearly indicate that the fungal pigments could be an alternate for leather dyeing as a natural dye. The present approach of reducing pollution load in dyeing process with the use of fungal pigment at optimized conditions provides a new possibility for eco-friendly dyeing process. However, the naturally occurring components present in filamentous fungi as in the nature of pigment contribute to the dyeing of leather require further fractionation and elucidation of chemical structure are currently under investigation. Such an operation is regarded as too expensive for the specific novel application tested. Here, we used an economical method of fungal pigment extraction. Briefly, the fermentation broth was exhausted and the pigments were diluted in ethanol, and the suspension was clarified by centrifugation to remove microbial biomass before using the product for dyeing.

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