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Densitometric quantification for the validation of decolorization of Disperse Orange ERL by lichen *Parmelia* sp.

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Densitometric high performance thin layer chromatography (HPTLC) quantification method was developed to validate the decolorization/biotransformation of Disperse Orange ERL and dye mixture by lichen *Parmelia* sp. which release several colored compounds during decolorization process, hence unable to use colorimetric estimation. Percent decolorization of Disperse Orange ERL and dye mixture by lichen *Parmelia* sp. was observed when estimated using developed HPTLC method. Limit of detection and limit of quantification for both dyes in mixture were obtained as 0.3 and 1 μg/μl, respectively. Area of peak of control Disperse Orange ERL was reduced by 43% after 12 h, 71% after 48 h and upto 82% after 72 h of incubation. Precision and repeatability of data elucidated the % relative standard deviation less than 3 for all the values thus indicating statistically acceptable. Biodegradation of dye and mixture was confirmed with Fourier transform infrared spectroscopy analysis, i.e., altered fingerprinting spectral pattern.

[**Key words:** Quantification; Densitometry; High performance thin layer chromatography; Silica gel; Biodegradation]

Chromatographic methods possess a broad spectrum of application in fields such as pharmacological, biochemical, botanical, downstream processing and purification protocols. Thin layer chromatography (TLC) is based on the principle of separation; specifically, the affinity of test compounds towards stationary phase, solubility into mobile phase and their densities. TLC is referred as key identity test in pharmacopeial guidelines for product analysis due to its robustness, simplicity and efficiency in parallel analysis of samples. High performance thin layer chromatography (HPTLC), an advanced and more developed form is intended for resolution of a compound. HPTLC stands as unique combination of contemporary instrumentation, standardized protocol, concrete theoretical foundation and ability to produce photographic image data which leads to a robust quantification technique (1).

Densitometric quantification using chromatographic separation method is the leading chromatographic protocol widely used for estimation of various active principle molecules from plant extracts (2). Estimation of desired compound from mixture can be achieved with HPTLC. Qualitative as well as quantitative account of detection can be carried out using this technique and therefore it has become a leading trustworthy technology within a short time (3). Interspecies distribution of reserpine within *Rauvolfia* genus has been competently demonstrated with qualitative HPTLC (4). Eight bioactive secondary metabolites namely β-sitosterol-3-*O*-glycoside, luteolin, apigenin, gentiacaulein, swertiaperenine, β-sitosterol, taraxeryl-3-acetate and 3β-acetoxyoleanane-12-one from *Codonopsis ovata* were simultaneously quantified with validated HPTLC protocol (5). Antioxidant activities of phenolic compound from sweet potatoes such as chlorogenic (CGA) acid and dicaffeoylquinic acids (diCQA) have been successfully demonstrated along with quantitative estimation (6). Naringin, a flavonone glycoside was successfully quantified from partially purified *Labisia pumila* dichloromethane extracts with validated densitometric HPTLC protocol (2). Simultaneous detection of indole 3-acetic acid (IAA) and indole 3-butyric acid by *Rhizobacteria* was performed with qualitative HPTLC (7). Quantitative detection of food dyes Tertrazine, Azorubin and Sunset Yellow from commercially available dried concentrated juices has also been performed using HPTLC (8). Scanning densitometry is most accepted and considered indubitably robust method for quantitative estimation of chromatographic plates based on measurement of absorbance or fluorescence of different lanes with a monochromatic beam of light. Secoisolariciresinol diglucoside determination from flaxseeds was successfully performed with validated densitometric scanning at 282 nm with TLC Scanner 3 (CAMAG, Muttenz, Switzerland) (9). Rapid densitometric TLC method was developed for simultaneous quantification of costunolide and dehydrocostus lactone from medicinal plant *Saussurea costus* (10).

Textile dyes are virtual recalcitrants imparting detrimentally hazardous effects upto chromosomal level (11). Generally, dye removal is evaluated with well-developed and widely applied

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spectrophotometric protocols (12,13). HPTLC has been applied previously in qualitative analysis of dye removal. Preferential degradation of structurally different dyes from mixture containing Golden Yellow HER, Rubin GFL, Brown 3REL, Methyl Red, Brilliant Blue by *Brevibacillus laterosporus* was demonstrated with qualitative HPTLC protocol (14). The lichen *Parmelia* sp. (pale colored dorsally and greenish brown colored ventrally) biomass when exposed to Disperse Orange ERL (OERL, Chemilene Orange ERL, Chemiequip Ltd., Mumbai, India) and a simulated dye mixture gave colored metabolites interfering with the spectrophotometric results. In the present study, densitometric quantification method is developed for sophisticated evaluation of color removal using HPTLC (CAMAG). Biodegradation/biotransformation is further confirmed by comparing Fourier transform infrared spectroscopy (FTIR) spectra of OERL, dye mixture and their respective extracted metabolites.

MATERIALS AND METHODS

Procuring chemicals, lichen biomass and identification Tartaric acid, n-Propanol, potassium iodide was purchased from Sisco Research Laboratory, Mumbai, India. Dyestuff was purchased from Mahesh Dying Industry, Ichalkaranji, India. HPLC grade methanol, isopropanol, toluene and acetic acid were purchased from SD Fine Chemicals Ltd. (Mumbai, Maharashtra, India). Collection location was situated at 16°49′12″ north and 74°7′12″ east at elevation 2473 feet above mean sea level in the area near Yamai temple, Jyotiba hill situated at 18 km northwest of Kolhapur, Maharashtra, India. The lichen sample was procured from tree trunk. The collected biomass was brought to laboratory, washed to remove attached tree-trunk particles with soft brush, air dried and stored at 4 °C temperature. Aliquots of stored biomass were used for experiments. Herbarium of the sample was identified by National Botanical Research Institute, Lucknow up to genus level as *Parmelia* sp.

Preparation of standard solutions and samples by solvent extraction Stock concentration of OERL was taken as 20 mg per 10 ml methanol in the volumetric flask. Dilution of sample was done as 1:1 to obtain working concentration 2 mg per ml MeOH. For dye mixture, 10 mg per 10 ml of each dye (10 mg OERL +10 mg Brown 3REL) were referred as working standard. After incubation, decolorized solutions were centrifuged at 4000 rpm for 10 min. The metabolites in supernatant were extracted with 1:1 (v/v) ethyl acetate and dried into sterile Petri plate. Two milligrams of dried metabolites obtained by biodegradation of OERL and 1 mg of that of dye mixture was dissolved in 1 ml MeOH to obtain the test sample.

Instrumentation and validation of method HPTLC instrument (CAMAG) was used for experiments of densitometric quantification. Linomat automatic spray-on sample applicator was used to apply samples on the HPTLC plate precoated with silica gel K60. Nitrogen was applicator gas. The band width was kept 6 mm. Plates were applied with OERL and dye mixture separately; and developed in pre-saturated twin trough chamber. Solvent system for mobile phase was standardized as toluene: methanol: ethyl acetate, in a ratio of 7: 2.5: 0.5. Ascendingly developed plate was dried and scanned in TLC scanner 3 with slit dimensions 5 × 0.3 cm. Derivatization was not required due to colored nature of the test components.

Validation was performed as per guidelines provided by International Committee on Hormonization (ICH) for limit of detection and quantification, linearity, precision and repeatability (15). Precision was determined in terms of percent relative standard deviation (%RSD) by performing the HPTLC experiments intraday and interday at different times with same instrument and laboratory. Repeatability of method was expressed in terms of %RSD by analyzing area of peaks of dyes after separate application cycles. %RSD was calculated as per equation (Eq. 1):

$$\%\text{RSD} = \frac{\text{Averag of peak area}}{\text{Standard deviation of peak area}} \times 100 \quad (1)$$

Experimental procedure and quantification using HPTLC Standard graph of OERL was plotted by applying the samples with range of 8 standards. Concentration of dye was ranging from 2 to 16 μg/spot. Eight standards of 2, 4, 6, 8, 10, 12, 14 and 16 μg/spot were applied on silica gel K60 pre-coated plate (10 cm × 20 cm) from single vial. Eight standards were corresponding to the 20, 40, 60, 80, 100, 120, 140 and 160 mg L^{-1} OERL solution. Mixture of OERL and Brown 3REL (B3REL) in MeOH was applied in the plate with same size mentioned as above in same manner to obtain the coefficient of regression for mixture. The concentration of each dye in the mixture per spot was 1, 2, 3, 4, 5, 6, 7 and 8 μg corresponding to the equivalent concentration in 100 ml dye mixture solution (i.e., 10, 20, 30, 40, 50, 60, 70 and 80 mg L^{-1} each dye). The volume of test samples applied was 6 μl falling within the range of the standard concentration. Six μg/spot was referred as control value for all experiments of optimization of pH, temperature and time dependant decolorization. Standard graph was plotted with area of spot against concentration to get linearity and coefficient of regression. Equation for line was obtained with Microsoft Excel intended for statistical analysis. Values of unknown samples or test samples were determined with equation.

Screening and decolorization experiments Lichen *Parmelia* sp. was found to produce lot of colored metabolites interfering with the UV–Vis spectrophotometric readings. Hence control and test samples were extracted into (1:1, v/v) ethyl acetate and then analyzed by spectrophotometer to get an idea about maximum decolorization. Cuvettes of 3 ml quantity and 10 mm path length with stoppers (Japan Cell Co., Ltd., Machida, Tokyo, Japan) were used during the experiment. Different dyes such as Red M5B, Golden Yellow HE4B, Solo Blue GL, OERL, Orange 3R were screened. OERL was selected as model dye. One gram of partially air-dried biomass of lichen *Parmelia* sp. in 10 ml dye solution was applied for decolorization experiments. Lichen biomass was treated with mild surface sterilizing agent (0.5% hypochlorite solution) to get rid of surface micro-flora. OERL (6 mg/100 ml) solution was inoculated with 1 g of partially air-dried lichen biomass in Erlenmeyer's flask for 72 h. Different combinations of dyes were studied. The mixture containing OERL and B3REL (6 mg/100 ml each dye) was selected for further experiments. Same experimental procedure was followed for dye mixture.

Optimization of conditions for decolorization of OERL by *Parmelia* sp. with HPTLC Optimum pH and temperature for decolorization of OERL was determined by subjecting the biomass of *Parmelia* sp. to dye solution (6 g/100 ml OERL concentration) with different pH (pH 2, 4, 6, 8 and 10) and by incubating at different temperatures (10, 20, 30, 40 and 50 °C) for 72 h. After incubation, decolorized solutions were extracted with procedure given in earlier section.

Study of time dependent decolorization of OERL and dye mixture by *Parmelia* sp. with HPTLC Precise study of decolorization of OERL and dye mixture was performed by tracking the decolorization with time intervals. OERL solution (6 mg/100 ml) was added with 1 g of lichen biomass and incubated at 40 °C until complete color removal. Samples were intermittently procured after 12, 24, 48 and 72 h. Obtained samples were extracted with ethyl acetate using the procedure mentioned earlier. Obtained samples were dissolved in MeOH and applied on a plate (8 × 10 cm) along with control sample and developed ascendingly. Time dependent decolorization study of dye mixture was performed by procuring samples after 24, 48 and 72 h with similar extraction protocol. MeOH dissolved samples were applied in silica gel K60 plate (20 × 10 cm) along with standards and developed ascendingly in trough chamber. Both developed plates were analyzed with TLC Scanner 3 at 530 nm with tungsten lamp. Results were observed in terms of area of peaks obtained. Percent reduction in peak area due to decolorization was calculated as per equation (Eq. 2):

$$\%\text{reduction in peak area} = \frac{\text{Control peak area} - \text{Test peak area}}{\text{Control peak area}} \times 100 \quad (2)$$

FTIR analysis before and after biodegradation of OERL and dye mixture by *Parmelia* sp. FTIR analysis was performed for the confirmation of biodegradation. Samples obtained before and after the biodegradation were extracted and prepared for FTIR with the method reported for lichens (16). Sample was analyzed with 16 scan speed for development of spectrum of mid IR region (400–4000 cm^{-1}).

Statistical analysis The statistical analysis of data was performed with one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test using the software Graph Pad InStat version 3.06. Average of peak area and standard deviation was calculated with Microsoft Excel.

RESULTS

Screening for selection of model dye *Parmelia* sp. was explored for ability of biodecolorization of various dyes which showed decolorization percentage Red M5B (20%), Green HE4B (16%), Solo Blue GL (36%), Orange 3R (31%). OERL and B3REL showed maximum decolorization 85% and 80%, respectively, after 72 h incubation, hence selected for further studies.

Standard graph for OERL and dye mixture Linear graphs were obtained after mounting standard solutions. Graph of standard OERL based on area of spot showed linear tendency with standard deviation 6.25% and R^2 value of 0.981. Line equation obtained for OERL was Y = 739.9x + 5453. Two linear graphs for mixture were obtained based on area of spot. The mixture contained two dyes namely OERL and B3REL were run with provided conditions, showed R^2 0.975 for OERL and 0.997 for B3REL. Line equations obtained were Y= 1474x + 801.7 for OERL and Y= 1782x + 7200 for B3REL composing mixture (Figs. 1 and 2, Table S1).

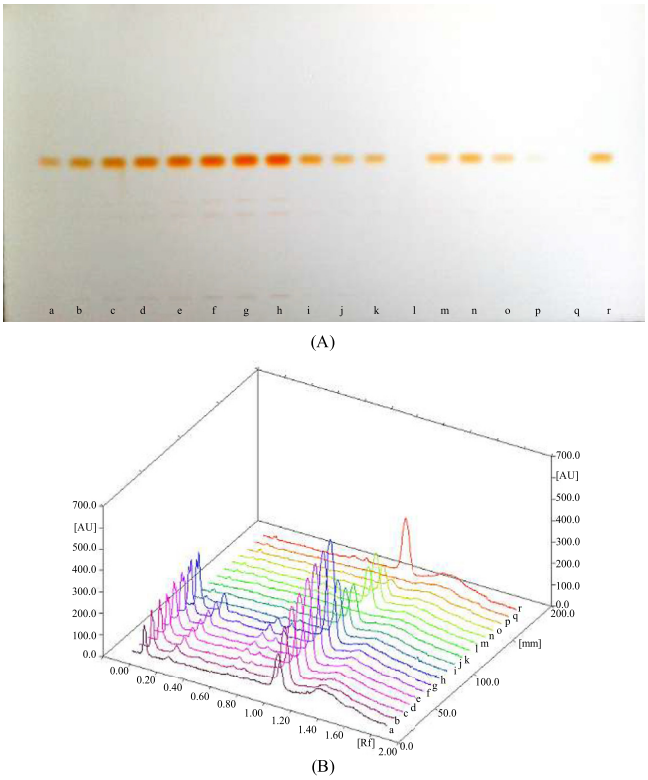

FIG. 1. Biodecolorization analysis of OERL by lichen *Parmelia* sp. (A) Ascendingly developed TLC plate with standard concentrations (lanes a–h), samples exposed to different pH (lanes i–m), samples exposed to different temperatures (lanes n–r), (B) scanning spectra of the same samples showing increasing peaks with increasing concentrations of standards and reduced peaks at optimum experimental conditions.

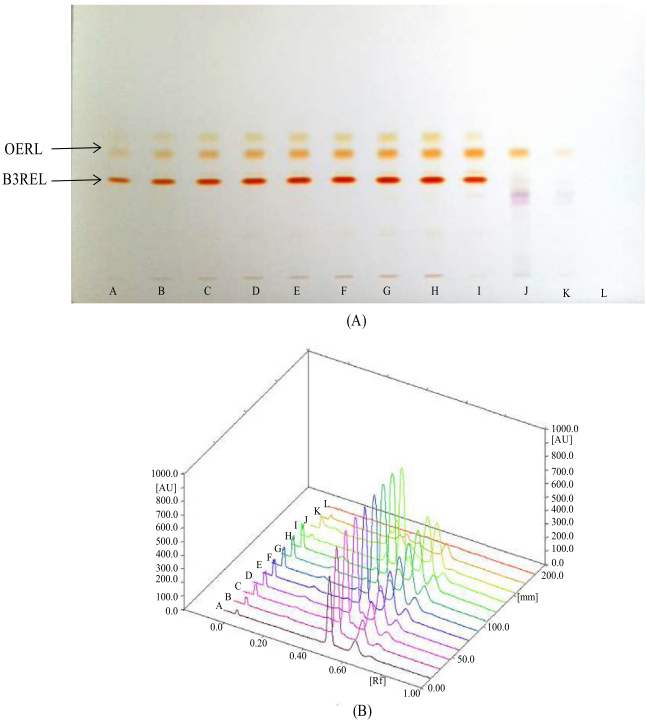

FIG. 2. Biodecolorization of dye mixture containing two different dyes OERL and B3REL. (A) Developed TLC plate with standard concentrations of both dyes (lanes A–I) and decolorized samples after incubation of 24 h (lane J), 48 h (lane K) and 72 h (lane L). (B) Scanning spectra of the same plate showing increasing area of peak as per increasing concentrations of standards and reduced peaks after biodecolorization.

Area of peaks for 2, 4, 6, 8 10, 12, 14 and 16 μg/spot concentration of standard OERL were 4799.91, 6576.29, 9198.25, 12,949.20, 15,569.48, 18,675.86, 21,468.37 and 25,315.05 AU, respectively (Table 1). Dye mixture was composed of equal amounts of both dyes. Values of area of peak for 1, 2, 3, 4, 5, 6, 7 and 8 μg/spot of OERL were 2806.54, 3741.85, 4766.21, 6943.35, 7080.67, 9746.65, 11,856.57 and 12,548.15, respectively, and those for the equal amount of B3REL were 8772.68, 10,882.79, 12,621.61, 14,703.48, 15,940.05, 17,598.13, 19,695.77 and 21,557.58, respectively (Table 2).

Precision and reproducibility Precision and reproducibility are the measures of robustness of chromatographic protocols (1). These two parameters were studied by considering the area of peaks of OERL, dye mixture samples and their respective extracted metabolites obtained after biodecolorization. Interday precision for OERL and its extracted metabolites were obtained as mean area of peak 17,613.33 AU for AM, and 16,903.0 AU for PM with respective %RSD values 1.73 and 0.92. Mean area of peaks (with %RSD) obtained for intraday precision for OERL were 17,613.33, 16,528.33 and 14,418.0 AU for 1, 2 and 3 d, respectively. Their respective %RSD was 1.73, 0.77 and 1.79 for respective days. However, interday and intraday mean peak areas in AU for extracted metabolite of OERL (Rf– 0.14) were 835.66 (2.15) for AM, 841.33 (1.73) for PM, 835.66 (2.15) for day 1, 930.33 (1.45) for day 2 and 936.33 (2.18) for day 3 (with %RSD mentioned in the bracket). For dye mixture, mean peak areas for OERL and B3REL were obtained separately due to differences in Rf values. For intraday precision mean peak area values (%RSD) for OERL, B3REL and extracted metabolite of mixture (with Rf– 0.89) were 9283.33 (1.40), 14,713.67 (1.32) and 835.33 (1.92) AU for AM, respectively; whereas 9340.66 (1.39), 14,836.0 (1.54) and 834.33 (0.60) AU for PM, respectively. For interday precision, OERL, B3REL and extracted metabolite showed mean peak area 9283.33 (1.40), 14,713.67 (1.32) and 835.33 (1.92) AU for day 1, 9318.33 (1.40), 14,694.33 (1.77) and 954.0 (1.61) AU for day 2 and 8766.66 (2.55), 13,630.67 (2.18) and 917.0 (2.07) AU for day 3 (Table 3).

Repeatability was studied by obtaining samples after three batches of decolorization experiments. The mean peak areas (with % RSD) obtained after three respective batches for OERL were 16,444.0 (1.10), 16,577.67 (1.19) and 16,106.67 (2.73) AU; however, those for its extracted metabolite were 846.66 (2.85), 872.33 (2.80) and 892.33 (1.08) AU, respectively. The mean peak areas for OERL and B3REL in dye mixture and its extracted metabolite were 9160.66 (0.87), 14,790.0 (1.78) and 996.33 (1.21) for batch 1, 9683.66 (1.80), 14,574.33 (1.93) and 973.33 (0.93) for batch 2 and 9574.33 (2.70), 13,589.67 (2.02) and 944.33 (1.38) for batch 3 (Table 3).

Optimization of effective parameters for maximum decolorization of OERL Samples applied from i to m lanes of dye removal at different pH values show area of peak (with %RSD) as 8505.41 (2.39), 7783.58 (2.93), 6865.90 (1.72), 0 and 7607.38 (2.69) for pH 2, 4, 6, 8 and 10, respectively (Fig. 1). Values of amount of dye per spot for respective pH value were 4.12, 3.14, 1.90, 0 and 2.91 μg calculated with line equation of standard graph. Lane l shows per spot dye concentration below limit of detection (i.e., 0) thus suggesting complete removal of dye which denoted optimum pH 8 for decolorization of OERL. Lanes n to r show samples exposed to different temperature values as peak areas 8748.21 (2.03), 7318.32 (2.63), 2985.88 (2.89), 0 and 8764.58 (2.26) for 10, 20, 30, 40 and 50 °C, respectively. Calculated values of μg of OERL/band for respective temperature values were 4.45, 2.52, 0, 0 and 4.47 μg using obtained line equation. Percent OERL removal for pH 2, 4, 6 and 10 was 31.33, 47.66, 68.33 and 51.5, respectively, wherein complete decolorization was observed at pH 8. However, percent decolorization for 10, 20 and 50 °C was 25.83, 58 and 25.5, respectively. Complete decolorization was observed at 30 and 40 °C (Fig. 1, Table S2).

TABLE 1. Values of area of peak for standard curve for OERL concentrations [standard concentration: 2 mg/ml (2 μg/spot)].

Sr. No.	Lane	Application volume (μl)	Area of peak (%RSD)	Concentration of OERL/spot (μg)	Dye concentration (mg/L)
1	a	1	4799.91 (1.38)	2	20
2	b	2	6576.29 (2.39)	4	40
3	c	3	9198.25 (2.49)	6	60
4	d	4	12949.20 (2.36)	8	80
5	e	5	15569.48 (2.52)	10	100
6	f	6	18675.86 (1.55)	12	120
7	g	7	21468.37 (2.04)	14	140
8	h	8	25315.05 (1.58)	16	160

%RSD, repeatability standard deviation in percentage.

TABLE 2. Values of area of peak for standard curves of each dye in mixture.

Sr. No.	Lane	Concentration of each dye/spot (μg)	Dye 1, OERL with %RSD (lane 1, Rf-0.96)	Dye 2, B3REL with %RSD (lane 2, Rf-0.36)	x-calculated (concentration, mg/L)		% dye removal	
					OERL	B3REL	OERL	B3REL
1	A	1	2806.54 (2.21)	8772.68 (2.17)	10	10	–	–
2	B	2	3741.85 (2.04)	10882.79 (1.74)	20	20	–	–
3	C	3	4766.21 (2.49)	12621.61 (1.72)	30	30	–	–
4	D	4	6943.35 (2.21)	14703.48 (1.43)	40	40	–	–
5	E	5	7080.67 (2.57)	15940.05 (1.22)	50	50	–	–
6	F	6	9746.65 (1.49)	17598.13 (1.16)	60	60	–	–
7	G	7	11856.57 (2.03)	19695.77 (0.92)	70	70	–	–
8	H	8	12548.15 (2.11)	21557.58 (1.36)	80	80	–	–
9	I	–	6890.56 (1.84)	15359.22 (2.29)	4.13	4.57	17.40	8.60
10	J	–	4867.03 (1.75)	5830.14 (2.32)	2.75	(−0.76)	45.00	Below LOQ
11	K	–	2598.83 (1.20)	1066.70 (2.08)	1.21	(−3.44)	75.80	Below LOQ
12	L	–	0	0	0	0	CD	CD

%RSD, repeatability standard deviation in percentage; LOQ, limit of quantitation; CD, complete decolorization.

Time dependent biodecolorization of OERL by *Parmelia* sp. Study of decolorization by time factor is necessary from optimization as well as acclimatization point of view under certain conditions. Lichen *Parmelia* sp. showed satisfactory removal of OERL after 12 h of incubation. Peak of control OERL at Rf value 0.98 was with peak area 9848.2 AU reduced to 5588.9 AU after 12 h, upto 2868.2 after 24 h and upto 1802.7 AU after 48 h of incubation. Additional peak at Rf value 0.42 was observed after 24 h incubation with peak area 2868.0 AU, that further reduced to 1066.6 after 48 h and to 472.6 AU after 72 h of incubation. Similarly, another set of peaks was observed at Rf 0.29 which showed peak area 281.6 AU after 24 h and reduced to 255.4 AU after 48 h. This peak was removed completely after 72 h (Fig. 3, Table S2).

Time dependent decolorization of dye mixture by *Parmelia* sp. Biodecolorization of mixture was studied by mounting the control and test samples on the same TLC plate. Lanes I to L are mounted with samples procured after 0, 24, 48 and 72 h, respectively. At 0 h, area of peak for OERL and B3REL were 6390.56 (1.84) and 15,559.22 (2.29) AU, respectively. After 24 h incubation, area of peak for OERL was lowered by 1523.53 AU, however, it was exponentially lowered by 9729.08 AU in case of B3REL. After 48 h, area of peak was lowered by 3791.73 AU for OERL, whereas, it was reduced by 14,490.52 AU for B3REL. Calculated values of concentration of OERL in μg within dye mixture after 6, 24, 48, and 72 h were 4.13, 2.75, 1.21 and 0, respectively; however, in case of B3REL, after 6 h incubation, dye concentration was 4.57 μg and after that the concentration was below the limit of quantification indicating satisfactory dye removal. Percent decolorization of OERL in the mixture was recorded as 17.40, 45.00, 75.80 at 6, 24, 48 h, respectively. B3REL was removed by 8.60% within 6 h of incubation and after that the concentration was below limit of quantitation (Fig. 2, Table S2).

Confirmation of biodegradation with FTIR analysis FTIR spectra of control and test samples were compared for their differential pattern. Spectrum of OERL revealed its structural constitution. C—H stretching was observed through peak at position 2920 cm^{-1}. Presence of nitro compound was detected at 1638 cm^{-1}. Phenolics and H-bonded compounds were found present (1430 and 1166 cm^{-1}). Peak at 1039 cm^{-1} indicated presence of C—OH stretching. Peak at 897 cm^{-1} indicated presence of disubstituted carbon. Presence of halogen group was detected as a peak at 612 cm^{-1}. However, spectrum after biodegradation analysis was found altered. Disubstituted carbon might have been deformed into monosubstituted compound. Deformation of phenolic group might be carried out to form substituted six carbon membered benzene ring. Peak nature and pattern were found altered after biodegradation protocol thus confirming the activity of lichen. Some peaks in control spectra of OERL and mixture were found similar as mixture also constituted the OERL dye. Presence of N═O stretching, and thus nitro compound was confirmed by peaks at 3422 and 3855 cm^{-1}, respectively. Peaks at positions 2921 and 1639 cm^{-1} indicated presence of C—H and C—N stretching. Skeletal carbon was found present in mixture spectrum (1249 cm^{-1}). Peak at 1042 cm^{-1} indicated presence of cyclic carbon where as presence of disubstituted C—H bond containing compound detected at peak position 896 cm^{-1} (Fig. S1). Absence of peaks of complex functional groups in test samples suggested the mineralization of dye into simpler compounds which confirmed the biodegradation by lichen *Parmelia* sp.

DISCUSSION

HPTLC technique possesses several advantages hence has a wide application spectrum. It requires lesser amount of solvents making it cost effective as well as able to analyze several samples simultaneously on the same plate thus promoting comparative approach of study (6). Densitometric chromatographic procedures focus on concentration of desired compound on TLC plate with reference to its peak obtained by scanning of plate (9). Textile dyes are heterocyclic nitrogen-containing phenolic compounds attached with aliphatic or aromatic groups composing chromophore structure which is evidently responsible to impart color

TABLE 3. Precision and repeatability of HPTLC quantification method for analysis of biodegradation of OERL and dye mixture.

S.N	Name of substance	Rf value	Precision (with %RSD)					Repeatability (with %RSD)		
			Intraday		Interday			Batch 1	Batch 2	Batch 3
			AM	PM	Day 1	Day 2	Day 3			
1	OERL	0.96	17613.33 (1.73)	16903.0 (0.92)	17613.33 (1.73)	16528.33 (0.77)	14418.0 (1.79)	16444.0 (1.10)	16577.67 (1.19)	16106.67 (2.73)
2	EM of OERL	0.14	835.66 (2.15)	841.33 (1.73)	835.66 (2.15)	930.33 (1.45)	936.33 (2.18)	846.66 (2.85)	872.33 (2.80)	892.33 (1.08)
3	OERL in dye mixture	0.96	9283.33 (1.40)	9340.66 (1.39)	9283.33 (1.40)	9318.33 (1.40)	8766.66 (2.55)	9160.66 (0.87)	9683.66 (1.80)	9574.33 (2.70)
4	B3REL in dye mixture	0.36	14713.67 (1.32)	14836.0 (1.54)	14713.67 (1.32)	14694.33 (1.77)	13630.67 (2.18)	14790.0 (1.78)	14574.33 (1.93)	13589.67 (2.02)
5	EM of dye mixture	0.89	835.33 (1.92)	834.33 (0.60)	835.33 (1.92)	954.0 (1.61)	917.0 (2.07)	996.33 (1.21)	973.33 (0.93)	944.33 (1.38)

%RSD, repeatability standard deviation in percentage.

(17). OERL and B3REL both belong to disperse dyes having poor solubility in universal polar solvent, i.e., water but an exceptional solubility in ethyl acetate and MeOH, thereby suggesting their non-polar nature (18). Solubility of a dye into any solvent is assuredly dependent upon its polarity (8). Standard curve of OERL was with $R^2 > 0.99$ showing competent linearity with about 6% SDV. However, two graphs obtained after chromatographic separation of dye mixture were also having the similar R^2 values. Lanes p and q in Fig. 1 show dye concentration below detection limits thus showing a temperature range of 30—40 °C for decolorization of OERL. Similar optimum pH and temperature values have been observed for lichen *Parmelia perlata* in biodegradation of solvent Red 24 dye (16).

Different linearities were observed for OERL after application in mixture than that when applied individually. There might be a role of presence of other dye in the mixture in altering the solubility of test dye. Polar dyes are soluble into polar solvents and non-polar in non-polar or less-polar solvents. The polarity of any dye is consequence of charge distribution or charged groups present on surface of dye molecule (19). Rf value of a dye can be explained based on the affinity towards stationary phase. OERL showed higher Rf value than that of B3REL thus suggesting its lesser affinity towards silica gel K60 than B3REL (Fig. 2). In the present study, Time dependant biodecolorization of OERL was studied with densitometric quantification for calculating percent reduction in peak area suggesting color removal. Area of peak of control OERL was reduced by 43% after 12 h, 71% after 48 h and upto 82% after 72 h of incubation. Peak of intermediate metabolite produced during biodegradation at Rf value 0.42 was appeared after 24 h incubation which was reduced by 63% after 48 h and 84% after 72 h of incubation. Peak observed at 0.29 Rf value which was appeared after 24 h. However, after 72 h incubation all peaks were found significantly reduced suggesting satisfactory color removal. Appearance of additional peaks after 12 h incubation suggested possible formation of colored intermediate metabolites by degradation of OERL (Fig. 3). These peaks were found with reduced peak area after incubation of 72 h showing their further processing by lichen enzymes.

Mobile phase was composed of toluene, ethyl acetate and MeOH which belong to group of less-polar solvents. This suggested the less-polar nature of both disperse dyes based on "like dissolves like" rule. Time dependant decolorization of dye mixture elucidated preferential removal of B3REL over OERL by *Parmelia* sp. After incubation duration of 24 h, peak area of B3REL was reduced exponentially by 63%; however, the same for OERL was 24%. After 48 h, area of peak for B3REL was reduced significantly by 93%, OERL was reduced by 59%, whereas after 72 h both dye was removed completely from the solution by lichen *Parmelia* sp. (Fig. 2).

Precision and repeatability of the data elucidated the % relative standard deviation (%RSD) less than 3 for all the values thus indicating statistically acceptable values. Peaks of metabolites of OERL and dye mixture were selected based on their frequent appearance on the same Rf value in all spectra. Altered Rf values during biodecolorization elucidated mineralization of dyes into different intermediates with different Rf values and their remineralization into relatively smaller products with higher Rf values. The produced intermediate metabolite with lesser Rf value probably had acceptable affinity towards stationary phase. They traveled along with solvent front on TLC plate due to their lighter weights than that of parent dyes.

In conclusion, densitometric validated HPTLC protocol was successfully implemented for assessment of biodecolorization of disperse OERL and dye mixture by lichen *Parmelia* sp. Precision and repeatability studies elucidated the validation of protocol with % RSD values of less than 3 thus suggesting effective implementation of protocol. Biotransformation was also confirmed with FTIR. This

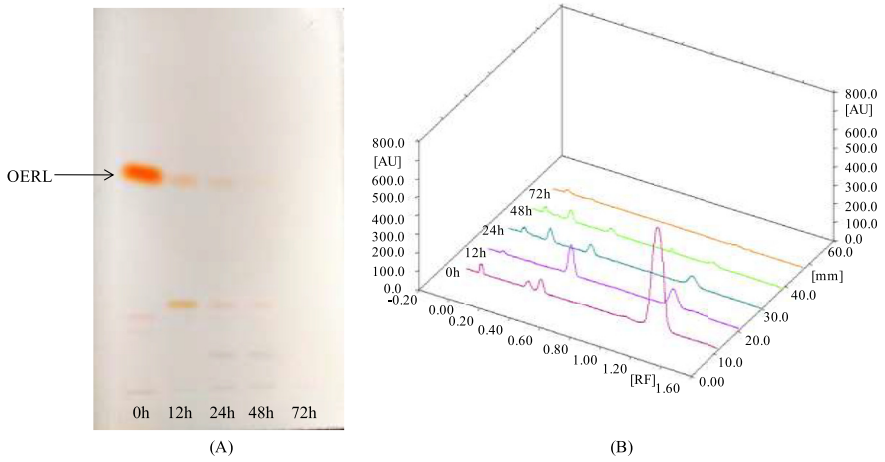

FIG. 3. Time dependant decolorization of OERL. (A) Developed plate with OERL at 0, 12, 24, 48 and 72 h. (B) Scanning spectra of same plate showing gradual reduction in peaks of OERL after biodecolorization by lichen *Parmelia* sp.

methodology will be highly useful where colored metabolites are produced during metabolism of textile dyes.

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