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Original article

Extraction and characterization of biocolors from bacterial isolates of *Pseudomonas* sp. M1 and MS2

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Abstract

In the current study, two pigments, *i.e.*, yellow (M1) and orange (MS2) were extracted from *Pseudomonas* sp, isolated from hot water spring of Himachal Pradesh. For the extraction, different solvents were used and maximum extraction was obtained in methanol followed by acetone. Extracted pigments showed λ_{max} at 508 nm (M1) and 459 nm (MS2), indicating that both the pigments are carotenoid. Thin Layer Chromatographic (TLC) analysis showed R_f values of 0.90 and 0.91 for M1 and 0.75 and 0.18 for MS2. Spectral data of TLC fractions revealed that there are three fractions present such as β-carotene, torulene and torularhodin, respectively. The M1 and MS2 yielded stable colors and showed 28.04 (M1) and 25 per cent (MS2) antioxidant activities confirmed by DPPH reduction method. Pigments were evaluated for their potential as dye by dyeing cotton fabric material. The findings revealed less color loss when dye was used with mordant thiourea to dye cotton material. Thus, potential of biocolor from bacteria inhabiting hot water springs of Himachal Pradesh can be exploited in dyeing industries.

Key words: Extraction, identification, DPPH, stability, carotenoids, cloth dyeing

1. Introduction

Dyeing is an ancient art which predates written records. Dyeing of cloths with the commercially available synthetic dyes imparts very strong colors but causes carcinogenicity (Adeel *et al*., 2009). Thus, interest in use of natural colorants is increasing worldwide due to public awareness. Natural dyes are important alternative to harmful synthetic dyes (Sivakumar *et al*., 2009). The utilization of natural pigments in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing processes has increased in the recent years due to their non-toxic nature (Unagal *et al*., 2005). Moreover, their eco-friendly, antioxidant, anticancer and antimicrobial activities further add to their positive effects. The significant growth in the naturally derived colors has been attributed to their stability and consumer perception. Further, the annual growth rate of naturally derived colors has been predicted to be 5-10 per cent in comparison to synthetic colors with a low growth rate of 3-5 per cent (Parmar and Phutela, 2015). Natural colors are generally extracted from fruits, vegetables, roots and microorganisms which are often called as bio-colors due to their biological origin. In spite of the availability of variety of pigments from fruits and vegetables, there is an ever growing interest in microbial pigments due to several reasons like their natural character and safety to use, production being independent of seasons and geographical conditions, controllable and predictable yield (Kim *et al*., 1998; Gunasekaran and Poornima, 2008). The rapid growth of microbes reduces the production time to a matter of days compared to plant and animal sources, the production is flexible and can easily be controlled (Joshi *et al*., 2003). Microorganisms produce a variety of pigments like carotenoids, melanin, flavins, *etc*. Carotenoids are a group of pigments responsible for yellow, red and orange pigments of various plants, animals and microorganisms (Wilhelm and Helmut, 1996). These pigments have an important function to act as protective agents against oxidative damage. Recently carotenoids have attracted greater attention due to the beneficial role on human health. Carotenoids can inhibit various types of cancer and it enhanced the immune response (Krinsky and Johnson, 2005).

Hot water springs have been a natural source from years for isolation of pigment producing bacteria. Hot springs have high sulphur content (Debnath *et al*., 2009) and, hence they perceived to warrant a therapeutic value to the human body. Also, biocolors possess antioxidant activities. A limited research studies have been conducted for exploration of such pigment producing microorganisms. The hot springs of Himachal Pradesh are untapped sources for pigments that can have wide range of applications in industries. Hence, the present study was focused on pigment production by bacteria and their extraction for industrial applications.

2. Materials and Methods

2.1 Extraction of pigment

Solvent extraction is the conventional method which is used for extraction of pigments. After the growth of pigment producing

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bacterial isolates the fresh biomass was harvested on solid medium and dried at temperature 60°C for 24 h. The dried biomass was dissolved in different solvent *viz*., acetone, petroleum either, ethanol, and hexane. Optical density was measured in each solvent for maximum absorbance. The color was measured in tintometer where ever specified (Joshi *et al*., 2003).

2.2 Identification and characterization of pigment

2.2.1 Identification of the pigment by spectral analysis

1g of biocolor from pigmented bacteria dissolved in 30 ml of the solvent. The clear supernatant was used for the identification of extracted pigment by scanning at different wavelength region from 200-1000 nm at 50 nm interval under UV-Visible spectrophotometer. Methanol was set as blank (Sharma, 2014).

2.2.2 Tintometer color determination

Pigment produced was evaluated with tintometer (Ranganna, 1986). The color measured in the form of red, yellow and blue units.

2.2.3 Identification by thin layer chromatography

The pigment was extracted and then the spots were developed on the TLC plate using a micropipette as described by Ranganna (1986). The chromatogram was run using chloroform : methanol : water as a solvent and R_f (resolution front) value of spots were calculated by using the following formula

$$\text{Resolution front} = \frac{\text{Distance traveled by the solvent}}{\text{Distance traveled by solvent front}}$$

2.2.4 Estimation of antioxidant activity

DPPH (1, 1-Dipheny-2- picryl-hydazil) free radical scavenging activity of the biocolor was measured by DPPH method described by Sasidharan *et al*. (2013). The absorbance was taken at 517 nm by using UV-Visible spectrophotometer. The percentage of DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A0 - A1}{A0} \times 100$$

where, A0 will be absorbance of the control and A1 will be the absorbance in presence of sample.

2.2.5 Estimation of total carotenoids

Carotenoids content was determined as per the method given by Ranganna (1986). One gm of pigment was dissolved in methanol and grounded till the whole color was not extracted. Then, the liquid was transferred to a separating funnel, adding 3 per cent sodium sulphate and 10-15 ml petroleum ether and separated colored portion was collected and final volume was made to 25 ml. The optical density was taken at 449 nm and the reading was compared with standard curve of β-carotene.The quantity was calculated and expressed as:

$$\mu g \text{ of carotenoids} = \frac{\text{Concentration} \times \text{final volume} \times \text{dilution} \times 100}{\text{Weight of sample}}$$

2.3 Applications of the extracted biocolor

2.3.1 Dying of textile material

White cotton material was dyed using bacterial pigment in solvent as stock solution and white cloth material as control. 50 ml of stock was applied to the cloth in a warm surface and was allowed to dry at room temperature for 1h. One set of experiment was done with the application of thiourea as a mordant. The dyed cloth material was dipped in thiourea solution (1%) for 30 min at 70°C (Shirata *et al*., 2000).

2.3.2 Wash performance of textile material

Dyed textile material was washed with soap solution (sunlight 0.7 per cent w/v) and in plane water for 30 min. at room temperature and at 40°C. After 30 min, clothes were washed under tap water and allowed to dry at room temperature. Absorbance of soap solution and water was measured at 535 nm in UV-Visible spectrophotometer. The procedure was repeated for dyed textile material treated with thiourea (Shirata *et al*., 2000).

3. Results

3.1 Extraction of biocolor

Two *Pseudomonas* sp. (M1 and MS2) isolated from Manikaran hot water spring of Himachal Pradesh, were found to produce yellow (M1) and orange (MS2) pigmented colonies. Different solvents were used for extraction of biocolor from pigment producing bacterial isolates (M1 and MS2) and the results are depicted in Figure 1. Results showed that maximum absorption was observed in methanol followed by acetone for pigment producing strains namely; M1 and MS2.

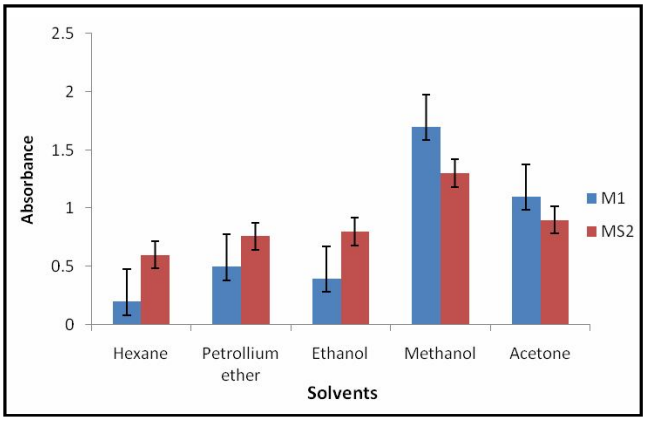


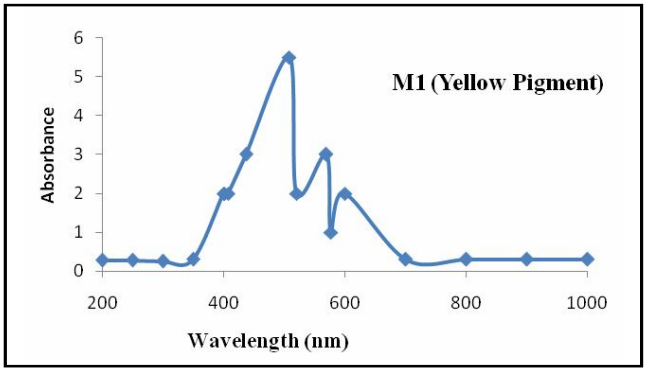
Figure 1: Effect of different solvents on pigment extraction.

3.2 Identification and characterization of extracted biocolor

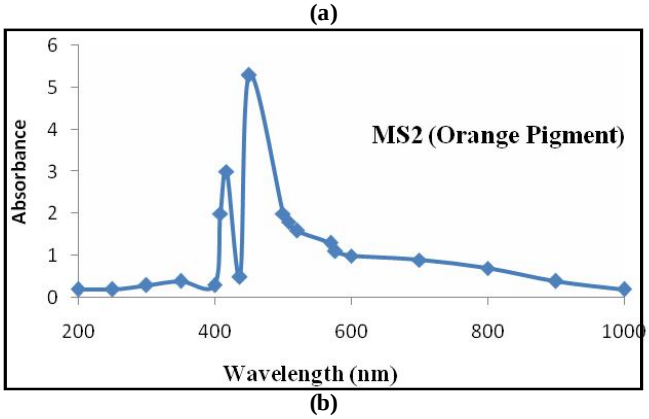
3.2.1 Spectrophotometric analysis of pigments

The UV-Visible spectrum of the extracted pigments were generated and spectrophotometric analysis was carried out at a wavelength region of 200-1000 nm and results are depicted in Figure 2(a) and 2(b). The absorption maximum for the extracted biocolor was found

to be between 400-600 nm which was a typical pattern of absorption spectrum of carotenoids. The absorption maxima were observed at 508 and 450 nm, for pigments of isolates M1 and MS2. Results of UV-VIS spectral analysis of pigment in the present study showed that all the pigments gave the absorption maxima between 400-600 nm which is absorption spectrum of carotenoids.



(a)



(b)

Figure 2: Identification of the extracted pigment by spectral analysis

3.2.2 Chromatographic analysis of pigment

Pigments produced by *Pseudomonas* sp. (M1 and MS2) were identified by thin layer chromatography. Thin layer chromatographic separation of the pigments with chloroform, methanol, and water in the ratio of 90:25:4 revealed the presence of three major pigments from these two isolates (Figure 3) and their R_f values are presented in the Table 1. The R_f value of yellow color was noted as 0.90 which was similar to that of standard β-carotene (0.91). The orange color pigment exhibited two spots showing R_f values of 0.75 similar to torulene (0.78) and 0.18 which was close to torularhodin (0.20). The chromatographic analysis showed that the biocolors produced by these two bacterial isolates M1 and MS2 belonged to carotenoid family.

Table 1: Identification of pigments from selected bacterial isolates by thin layer chromatography (TLC)

Bacterial Isolates	Spots	R_f value	R_f as per literature	Compound	Reference
M1	1	0.9	0.92	β-carotene	Latha and Jeevarthnam, 2010
	2	0.91	0.92	β-carotene	
MS2	1	0.75	0.78	Torulene	
	2	0.18	0.2	Torularhodin	

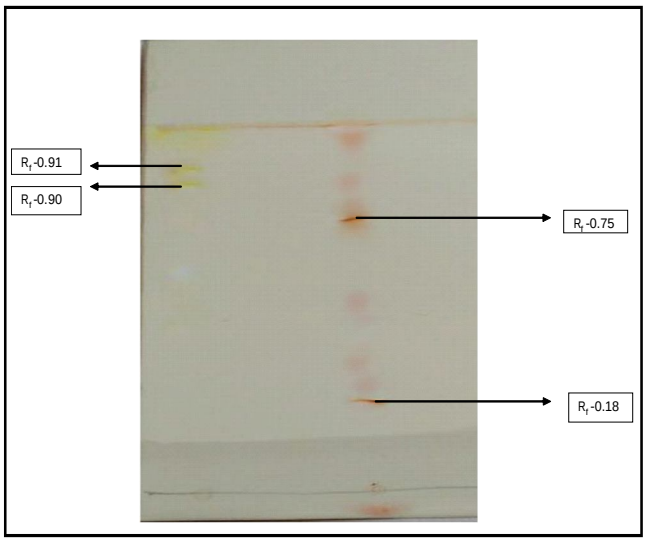


Figure 3: Thin layer chromatographic analysis of pigments.

3.2.3 Estimation of carotenoid content

Extraction of total carotenoid content from both pigment producing bacterial isolates was carried out using methanol as a solvent. The results are presented in Figure 4, indicating that M1 recorded the maximum (11.64 mg/100 g) amount of carotenoid followed by MS2 (10.96 mg/100 g).

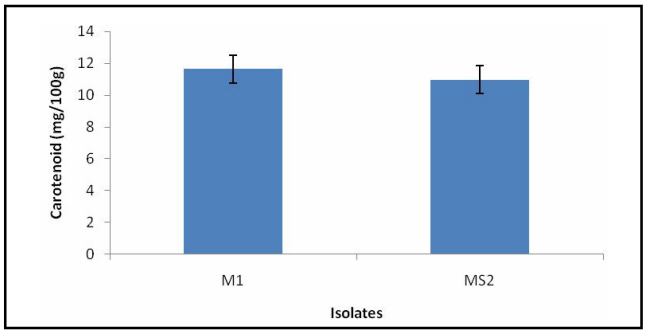


Figure 4: Carotenoid content of pigments.

3.2.4 Estimation of antioxidant activity

Pigments extracted from the bacterial isolates M1 and MS2 were analyzed for antioxidant activity by reducing the DPPH (1, 1-Dipheny-2-picryl-hydazil) radical. The per cent value of inhibition of DPPH for methanolic extract for the selected pigments were found to be 28.04 per cent for M1 and 25 per cent for MS2 (Figure 5).

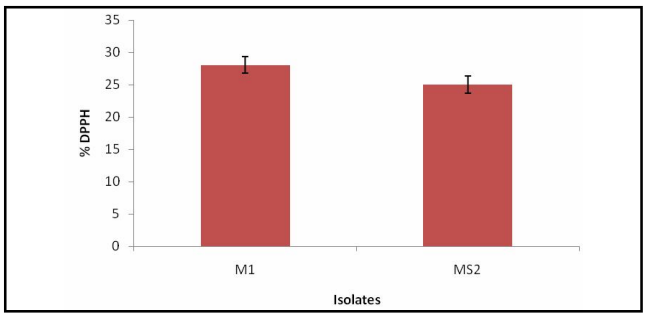


Figure 5: DPPH (1, 1-Diphenyl-2-picryl-hydrazil) free radical scavenging activity of isolates.

3.2.5 Stability of pigment

The observation on the stability of pigments using tintometer revealed that red and yellow units were recorded in the 3 colors of the selected strains, however, blue units were not found. It was observed that the pigments from bacterial isolates M1 and MS2 were stable for 6 days (Figures 6(a) and 6(b)). The highest red units were recorded for MS2 (30) and 20 for M1. The highest yellow units were recorded in the color of M1 (25) and 22 for MS2. Hence, the two stable bacterial isolates, namely; M1 and MS2 were studied further for commercial applications.

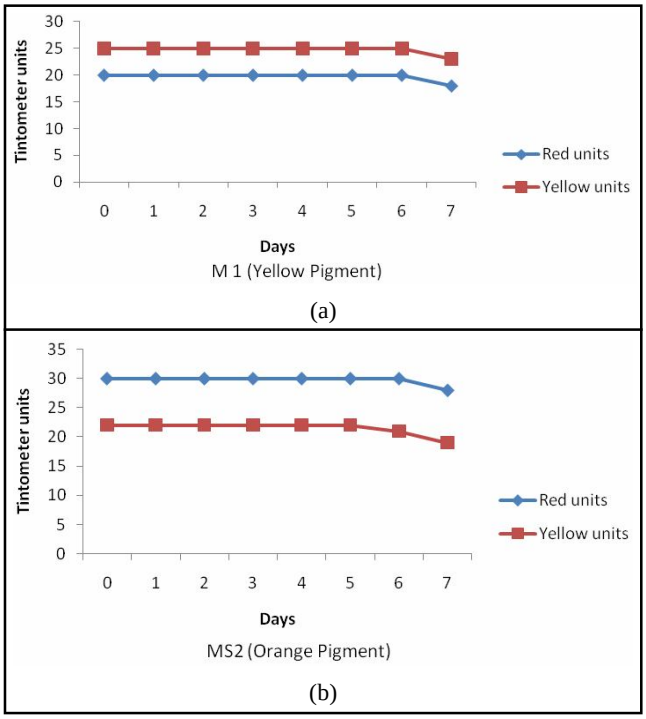


Figure 6: Stability of biocolor from bacterial isolates.

3.3 Application of extracted pigment from selected bacterial isolates

The pigment produced from M1 and MS2 pigmented bacterial isolates from hot springs of Himachal Pradesh were subject to application in cotton fabrics. It is evident that the pigment extracted (Figure7) from the pigment producing bacterial isolates M1 and MS2, can be effectively used to dye the cotton textile material. In order to check the stability of color, wash performance studies were also carried out (Figures 8(a) and 8(b)). It was found that the maximum dye is lost from the cotton textile material after washing in soap solution as indicated by increase in optical density of soap solution at 535 nm at room temperature (Figures 9(a) and 9(b)) and minimum loss, *i.e.*, decrease in optical density was observed in cold water.

To prevent the loss of pigment from cotton material after washing the cotton material was treated with mordant like thiourea and dyed with the pigments. The results are illustrated in Figures10(a) and 10(b) which revealed that the fading of pigment was reduced when the dyed cotton textile material was subjected to treatment with thiourea solution. This was indicated by the decrease in optical density of soap solution after washing of colored fabrics (Figures 11(a) and 11(b)). It was concluded that thiourea is an effective mordant for dyeing of textile material. The pigment from two bacterial isolates, namely; M1 and MS2 were also found to withstand at hot wash conditions with soap solution.

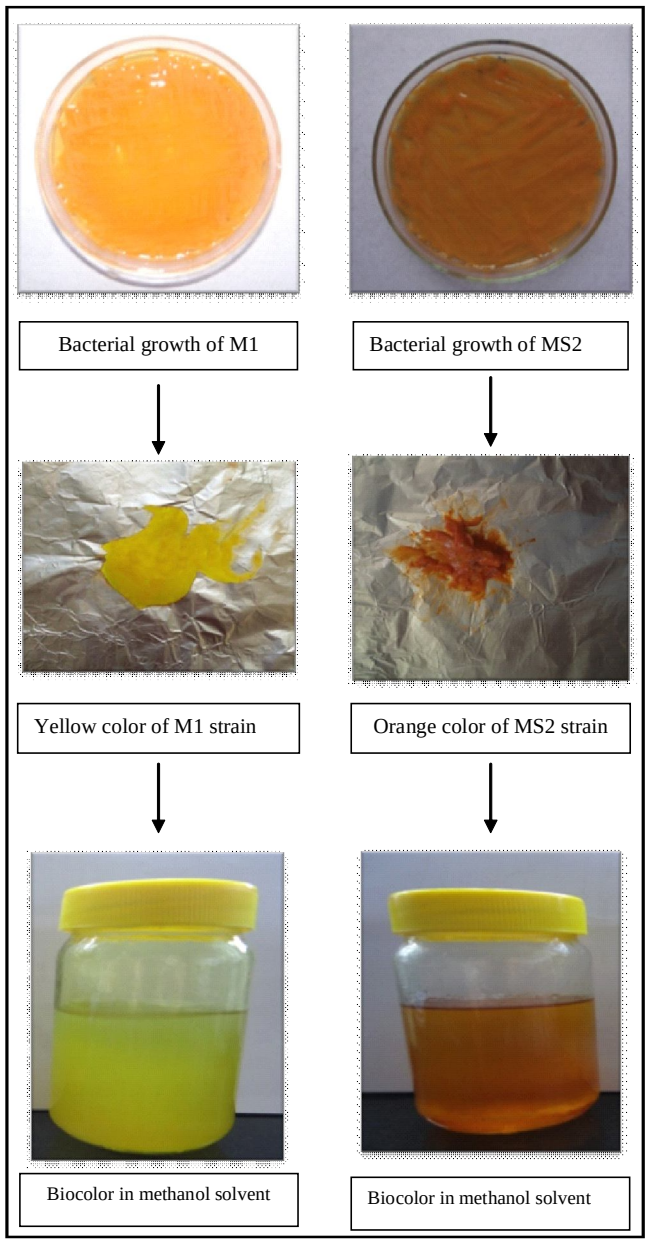
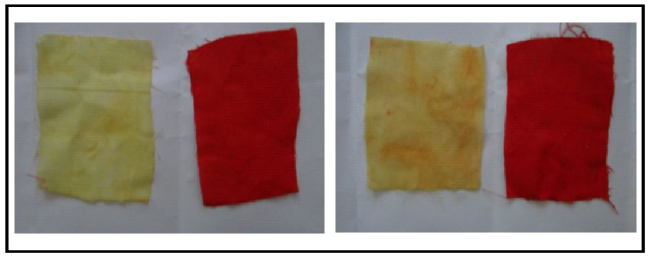
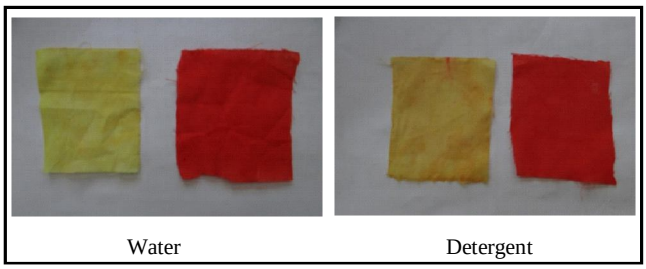


Figure 7: Growth and pigment extraction from bacterial isolates.



(a) Textile material dyed with extracted pigments from bacterial isolates M1 (Yellow) and MS2 (Orange)



(b) Wash performance of dyed textile material

Figure 8: Dyeing and washing performance of cotton fabric material with extracted biocolor.

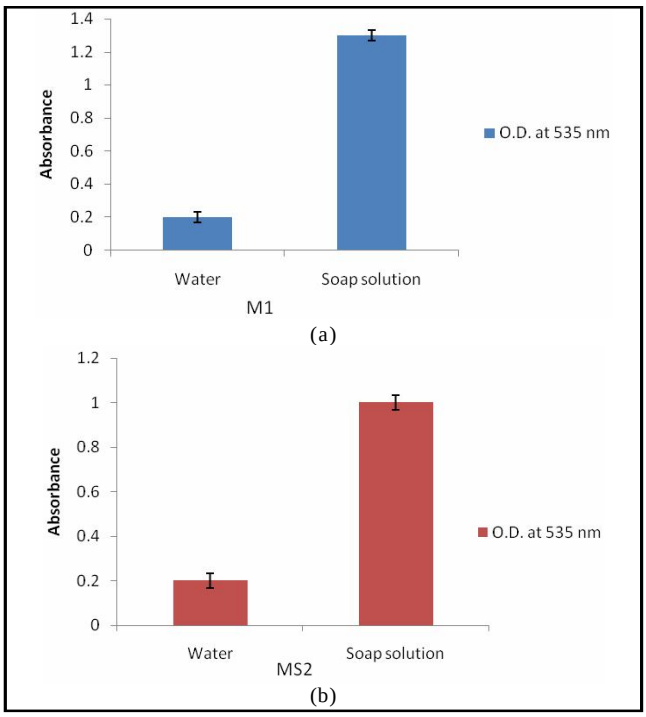
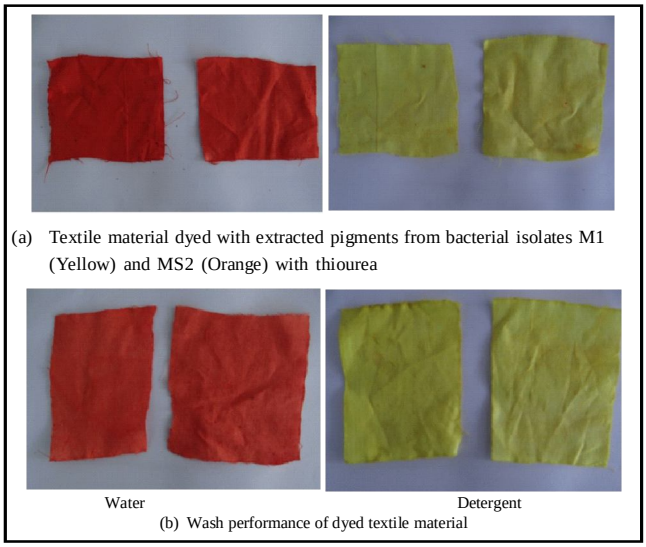


Figure 9: Wash performance of dyed textile material.



(a) Textile material dyed with extracted pigments from bacterial isolates M1 (Yellow) and MS2 (Orange) with thiourea

(b) Wash performance of dyed textile material

Figure 10: Dyeing and washing performance of cotton fabric material with extracted biocolor by using mordant (Thiourea).

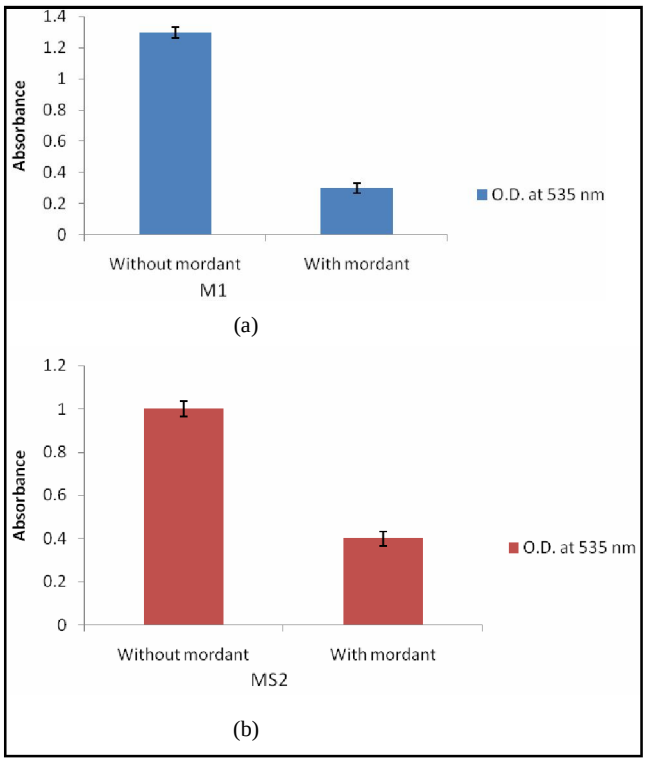


Figure 11: Wash performance of dyed textile material in soap solution with mordant (thiourea).

4. Discussion

In the present investigation, yellow and orange pigments were extracted from *Pseudomonas* sp. and pigment extracted was evaluated for cloth dying. The extraction was carried out in different solvents to obtain maximum pigment. Extraction of pigment in methanol was maximum and beneficial because it is environment friendly, relatively safe to human health (Chaudhari, 2013). Moreover, it is more effective as it interacts with the pigments through non-covalent interactions and promotes a rapid diffusion of the pigments into the solution. Velmurugn *et al.* (2010), Sasihdaran *et al.* (2013), Arulselvi *et al.* (2014) and Bhat and Marar (2015) used a mixture of methanol and acetone as the best extraction solvents. The peak and spot values obtained from the spectrophotometric and chromatographic analysis showed that the extracted pigments were carotenoids (Arulselvi *et al.*, 2014) belonging to β-carotene, torulene and torularhodin. Pigment from *Pseudomonas aeruginosa* showed maximum absorbance at 690 nm and 682 nm (Masi *et al.* 2014). Latha and Jeevarthnam (2010) and Antony *et al.* (2011) have also reported similar results for identification of pigments, using thin layer chromatography for 3 carotenoid compounds, *viz.*, β-carotene (R_f value 0.92), torulene (R_f value 0.78) and torularhodin (R_f value 0.20).

Microorganisms are known to produce a variety of biologically and pharmacologically active compounds. A number of studies have been carried out to find antioxidant, anticancer, antimicrobial activities using microbial pigments. It can be an alternative for synthetic compounds in food and pharmaceutical technology in order to treat various pathological disorders. The DPPH is a stable radical which reacts with an antioxidant compound and can donate

hydrogen or electron. It is reduced to yellow colored diphenylpicryl-hyfrazine. Sasidharan *et al.* (2013) reported that yellow pigment from the four isolates namely, RS7, RSS3, RS13 and RS14 exhibited higher antioxidant acivity. The isolate RS7 showed highest amount of DPPH free radical scavenging activity (70%), Arulselvi *et al.* (2014) reported that among the 8 isolates the methanolic extract of isolate YCD3b resulted in higher (78%) amount of DPPH free radical scavenging effect. Masi *et al.* (2014) also reported anti-oxidant activity using DPPH assay as 54.7 per cent free radical scavenging effect.

The present study indicated that the two biocolrs, *viz.*, yellow from M1 strain and orange from MS2 can be utilized for dyeing of fabrics. These results are also supported by the findings of Shirata *et al.* (2000). They reported that dying and wash performance of textile material with mordant like thiourea can prevent the loss of color in dyed textile material. Krishna *et al.* (2008) have evaluated the prodigiosin like pigments, from marine *Serratia* sp. for application as dye in the textile industry. The study indicated that pigment could be used as natural dye for imparting red color of various grades to the textile materials. The color was observed to be stable after wash performance studies. Similar results have also been demonstrated by Shahitha and Poornima (2012) for dying of cotton material with the pigment prodigiosin extracted from *Serratia marcescens*. The cotton fabrics exhibited good color tone and did not change after washing.

5. Conclusion

From the present investigations, it was concluded that isolates M1 and MS2 collected from Manikaran hot springs of Himachal Pradesh are potential source for pigment production. The two colors, *i.e.*, yellow from M1 isolate and orange from MS2 isolate was stable and can be used for dyeing of cotton fabrics with thiourea as mordant. The color was also stable with soap solution and withstands at hot water conditions. The biocolors was identified as carotenoid with high antioxidant activity. Hence, microbial pigments have potential in industries as natural and attractive sources of color.

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Conflict of interest

We declare that we have no conflict of interest.

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