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Sustainable Bioprospecting of Himalayan Lichens for the Production of Natural Dyes

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Abstract

Lichens are the combination of two organisms one is algae and another one is fungus, due to the combination of both the organisms, lichen as a whole organism contain various secondary metabolites, from these various secondary metabolites some chemicals act as colouring agents and are used from ancient times for the production of natural dye. There are various methods employed to extract lichen dyes from lichen thallus. In the beginning traditional cow urine method (CUM) was used for extraction of dyes from lichens which was replaced with ammonia fermentation method (AFM) and later boiling water method (BWM) was introduced. In addition to the traditional methods, DMSO extraction method (DEM) was also used for extraction of lichen dyes. Large amount of lichens are required in traditional methods for the extraction of dye which results overexploitation of lichens from the forests as lichens are extremely slow-growing organism, therefore cultured mycobiont (fungal part of lichen) can be used for extraction of dyes. Reports reveal that mycobiont can also produce lichen compounds which are responsible for production of coloured pigments. Lichen mycobiont culture in laboratory will not only useful for dye-yielding but also help to conserve these unique slow-growing organisms in nature. Lichens are also used for many purposes such as production of crude drugs for medicines, perfumery, agrochemicals etc. therefore lichens should be conserved. A brief explanation of traditional methods of natural dye extraction from lichens and how to overcome the overuse of lichens by extracting dyes from cultured mycobiont of lichens are explained in this review.

Keywords: Lichen Dye, Traditional Methods, Overexploitation, Mycobiont Culture, Conservation.

Introduction

Lichens are symbiotic organism consist of a photobiont (algae or blue-green algae) and a Mycobiont (fungus), together they formed a whole organism Known as lichen. Lichens are the organisms which grow in diverse condition and at all phytological regions of the world (Boustie and Grube, 2005). India is a mega diversity region having rich lichen diversity of 2,300 species belonging to 305 genera and 74 families, collected from different regions of the country (Shukla *et* al*.,* 2005). Lichens have been used for many purposes in India as well as in all over the world because lichens have tremendous properties in them due to the presence of various natural compounds known as primary and secondary metabolites they have varied chemistry and produced many polyketide derived phenolic compounds such as depsides and depsidones which are not known to other group of plants (unique with respect to higher plants), lichens are used for the preparation of natural dyes, crude drugs for medicines, perfumery, agrochemicals (Banerjee, 2002; Kumar and Upreti, 2008), also evaluate air quality, climate change, to detect accumulation of heavy metals. Lichens are the most significant indicators of air pollution and ecosystem health (Richardson, 1992; Upreti and Pandey, 1994; Wolseley *et* al., 1994; Upreti, 1995; Sloof, 1995; Mistry, 1998; Vokou *et al*., 1999). As lichens contain wide range of secondary metabolites which are believed to serve as antimicrobial, anti-herbivore and antigrowth agents (Hale, 1983; Manojlovic *et* al., 2005; Gupta *et* al., 2007). Due to various useful properties and uses lichens are considered as economically important herb. Secondary metabolites known 'lichen acids' are the main source in production of dyes which can colour fibers (Richardson, 1988). Lichen dyes

have affinity for natural fibers, wood, marble, leather, wine, and food materials. There are about 20,000 lichen species described all over the world and India represents 10% (2305) of the lichens known (Singh and Sinha, 2010). The Himalayan flora consists of large number of parmelioid lichen species that provide excellent source of dyes (Upreti *et* al., 2010) and 157 Indian lichen species belonging to 65 genera have potential dyeing properties. Parmelioid lichens contain characteristic compounds known as depsides and depsidones that are formed by joining two or sometimes three phenolic units, derived through acetate-polymelonate pathway (Asahina and Shibata, 1954), are the main source in production of dyes which can colour natural fibers ((Richardson, 1988). Few records of lichen dyes are recorded from India so far (Upreti *et* al., 2012). In India, the ethnic groups in Garhwal Himalayas are known to dye wool from dyes extracted from lichens species (Kala, 2002). The use of lichens as dyeing agent has a long history. The purple orchils have been the most important groups of lichen dyes in historical trade and Orchil (purple dye) was the first documented dye produced from *Roccella spp.* through ammonia fermentation method. The purple dye from *Roccella* was historically very important as 'Royal-purple' during the classical Greek and Roman period. The process generally consisted of obtaining the desired lichen, adding it to stale urine and slaked lime. During the seventeenth century, a growth in usage of orchils developed with the discovery of ammonia treatment process. Later, shortage of supplies developed and new sources of lichens were developed. In twentieth century, usage of lichen dyes of all types has declined due to competition from lower costs and progressively improving synthetic dyestuffs. With the discovery of first synthetic dye in 1856 (Margareta, 1981), the use of natural dyes was replaced completely by synthetic compounds due to their easy extraction methods and cost-effectiveness. Methods of lichen dyeing were kept as secrets in early times. The earliest known description of the preparation of orcein (purple dye) was given by Roseto in the year 1540. Along with dyeing textiles, orecin was also used as food colouring agent, microscopical stain and preparation of litmus (acid/base indicator). Cow urine method (CUM), ammonia fermentation method (AFM), boiling water method (BWM) and dimethyl sulphoxide extraction method (DEM) are some popular lichen dye extraction methods which yield beautiful purple, pink, yellow, brown, orange, and green colours. Together with the colour, lichen dyes also give characteristic odour to the dyed fibers. Traditional dyeing methods such as fermentation in urine and ammonia were followed in ancient times to dye natural fibers. Sometimes, ammonium salts were added to the fermenting solution that functions as mordant. BWM and DMSO extraction methods were later developed. The colours obtained through ammonia/urine fermentation methods were best when compared with the colours obtained with BWM and DEM. (Casselman, 2001) detailed a list of over 100 common species of lichen worldwide that produce reliable colour dyes. As the synthetic dyes have tremendous environmental impact due to their toxic, carcinogenic and non-biodegradable nature, the demand of dyes and colours for textile, food and cosmetics from natural sources has increased in the recent years. Several attempts are now being made for the development of environment friendly dyes from the natural sources. Lichens are the best option for natural dye Production but the main problem is the availability of the lichens in large amount because the traditional methods of extraction dye from lichen required large amount of raw material and we know lichens are extremely slow-growing organism and not available in greater amount for their commercial exploitation. To avoid overexploitations and to conserve lichens recently researchers found a new alternative method of dye production from lichens by culturing its fungal part, this method is known as mycobiont culture method. In this method fungal part is isolated and cultured in artificial media which is able to produce some of the secondary metabolites that can produce coloured compounds through chemical reactions. Because majority of organic compounds found in lichens are secondary metabolites of fungal origin, the mycobiont culture is the only way to exploit the secondary metabolites produced by lichens. In recent years, methods for cultivating lichen mycobiont and lichen tissues have been developed, and these have given rise to hopes for the production of metabolites. Research reports on extraction methods, other than traditional ones, are available, and extraction of dyes from natural thalli and cultured mycobiont is possible. In this review some approaches of extraction of lichen dyes from natural thallus as well as from the mycobiont (cultured fungal part) is discussed.

Methods Used for Extraction of Dyes from Lichen Thallus

Processing of collected lichen samples for dye production

Lichens are grow on various substrates such as tree bark, soil, leaves, boulders etc therefore while removing them from their substrates many impurities are intact with them so it is necessary to remove these impurities to overcome mixing of other substances which cause contamination in dye production. To remove impurities lichen samples are washed thoroughly and dried the clean samples at room temperature till the samples dried. It is emphasized that whole thallus must not be harvested in order to conserve them in their natural habitat. Commonly there are four methods which are used to extract natural dyes from Lichen thallus which are as follows:-

Cow Urine Method (CUM)

Cow Urine Method (CUM) is the oldest method of dye extraction from lichens, but now days it is not in use. In this method, the cleaned dried lichen thalluses are merged in stale urine for 3 to 4 weeks. After that urine thallus mixture is filtered and the fibers are added to the filtrate and again left for few days to get colour. In countries such as Sweden and Scotland, the mixture of cow urine and lichens is kept at moderate heat for long time and substance having a thick and strong texture is taken out and small

cakes are made from it. These pieces are wrapped in dock leaves and hung up to dry in peat smoke. The dried dye can be stored for years, and when needed for dyeing, it can be dissolved in warm water to dye fibers. Later, cow urine was replaced with Ammonia Fermentation Method (AFM).

Ammonia Fermentation Method (AFM)

During eighteenth and nineteenth centuries Ammonia Fermentation Method (AFM) is the most widely used method. In this method dye is extracted with the help of diluted ammonia. Lichen thallus is crushed into powder and this powder is added and mixed thoroughly in diluted ammonium hydroxide solution (add NH4OH and distilled water in1:10), and left for 3 to 4 weeks at room temperature. The extract is then filtered and fibers are added in it and again left for 3 to 4 weeks. After this, fibers are removed, rinsed and dried. In some modified versions of the AFM, the lichens are first boiled in the solution of ammonium carbonate. The mixture is then cooled and ammonia is again added to make the mixture damp and is kept for 3 to 4 weeks. In another processes, the extracted lichens in ammonia are acidified so that the dissolved dye precipitates and is washed. Ammonia is again added, and the solution is heated in air until some colour is developed. The colour is again precipitated with calcium chloride and this resulting in insoluble coloured solid that does not fade in light.

Boiling Water Method (BWM)

The powdered lichen samples are added to distilled water and heated till boiling. The mixture is maintained at low heat for 1 hour. The content is filtered into a clean flask, and the filtrate is again maintained at simmer for at least 2 hour until some colour is obtained. Pre-soaked fibers in distilled water are then immersed in the filtrate and are slowly heated at maximum 90 °C for 2 hour. After dyeing the threads are rinsed in cold water and dried.

DMSO Extraction Method (DEM)

The DMSO Extraction Method (DEM) also known as solvent extraction method was developed just before the dawn of twentieth century. In this method, the powdered lichen samples are added to 50 ml crude dimethyl sulphoxide solution. The content is stirred vigorously and left for 1 month at room temperature. After 1 month, the content is filtered into another clean flask and pre-soaked threads are added for dyeing. The threads are removed from the flask after 1 month, washed with distilled water and were left for drying. After dyeing, the fibers can be stored at room temperature.

Naming and Quantification of prepared lichen dyes

The colours can be named with those matching Ridgway colours (Ridgway, 1912). The solvents used for extraction of dyes are evaporated using rotary evaporator to get dry weight of the dyes this helps in quantification of dye production from different species of lichens. According to (Casselman, 2001), there is no need of mordant as lichens are substantive dyes and do not require a mordant; however, the use of mordant increases uptake of colour, improves fastness and varies the colour.

Table 1. List of Himalayan lichens used for producing colour dyes

S.No.	Species name	colour produced after dyeing	References
1.	Bulbothrix setschwanensis	Buffy brown, Mikado brown	(Shukla and Upreti, 2015)
2.	Cetrelia braunsiana	Ivory yellow, Light yellowis	(Shukla and Upreti, 2015)
3.	Cladonia pyxidata	Purple	(Kok, 1966).
4.	Dermatocarpon miniatum	Brown to grey	(Brough, 1988, Upreti *et* al., 2010)
5.	Dermatocarpon vellereum	Green to yellow	(Shukla and Upreti, 2014b)
6.	Evernia mesomorpha	Pink to yellow	(Shukla and Upreti, 2014a)
7.	Everniastrum cirrhatum	Sayal brown, Light yellowish	(Shukla and Upreti, 2015)
8.	Everniastrum nepalense	Chamois, Mikado Brown , Reed yellow	(Shukla and Upreti, 2015)
9.	Flavoparmelia caperata	Light brownish olive, Cartridge buff	(Shukla and Upreti, 2015)
10.	Flavopunctelia soredica	Buffy brown, Marguerite Yellow	(Shukla and Upreti, 2015)
11.	Heterodermia diademata	White, Vinaceous russet, Cartridge buff	(Shukla and Upreti, 2015)
12.	Lobaria retigera	Cartridge buff Vinaceous Buff Clear fluorite	(Shukla and Upreti, 2015)
13.	Nephromopsis nephromoides	Green, brown to Yellow,	(Shukla and Upreti, 2014a)
14.	Parmelaria subthomsonii	acid Ivory yellow Isabella color Marguerite yellow	(Shukla and Upreti, 2015)
15.	Parmelaria thomsonii	Ivory yellow Isabella color Marguerite	(Shukla and Upreti, 2015)
16.	Parmelia marmariza	Brown to yellow	(Kok, 1966; Brough, 1988; Casselman, 1994; Hodge, 2006)
17.	Parmelia meiopbora	Brown to yellow	(Kok, 1966; Brough,1988; Casselman, 1994; Hodge, 2006)
18.	Parmelia saxatilis	Brown to yellow	(Kok, 1966; Brough, 1988; Casselman, 1994; Hodge, 2006)

19.	Parmelia sulcata	Brown to yellow	(Kok, 1966; Brough, 1988; Casselman,1994; Hodge, 2006)
20.	Parmelinella wallichiana	Clay color ,Deep olive, Wood brown	(Shukla and Upreti, 2015)
21.	Parmotrema nilgherrense	Ivory yellow, Avellaneous	(Shukla and Upreti, 2015)
22.	Parmotrema reticulatum	Brown to yellow	(Shukla and Upreti, 2014a)
23.	Parmotrema tinctorum	Purple to golden brown	(Upreti *et* al., 2010; Shukla and Upreti, 2014a)
24.	Peltigera rufescens	Green to yellow	(Upreti *et* al., 2010; Shukla and Upreti 2014a, b)
25.	Punctelia rudecta	Pink to yellow	(Shukla and Upreti, 2014a)
26.	Ramalina baltica	Yellow	(Kok, 1966; Upreti *et* al., 2010)
27.	Ramalina farinacea	Yellow	(Kok, 1966; Upreti *et* al., 2010)
28.	Ramalina himalayensis	Yellow	(Kok, 1966; Upreti *et* al., 2010)
29.	Ramalina hossei	Yellow	(Kok, 1966; Upreti *et* al., 2010)
30.	Ramalina.intermedia	Yellow	(Kok, 1966; Upreti *et* al., 2010)
31.	Ramalina pollinaria	Yellow	(Kok, 1966; Upreti *et* al., 2010)
32.	Ramalina roesleri	Yellow	(Kok, 1966; Upreti *et* al., 2010)
33.	Ramalina sinensis	Yellow	(Kok, 1966; Upreti *et* al., 2010)
34.	Stereocaulon foliolosum	Atranorin and lobaric acid, Chamois Isabella color Colonial buff	(Shukla and Upreti, 2015)
35.	Sticta nylanderiana	Chamois Dark vinaceous Dark olive	(Shukla and Upreti, 2015)
36.	Sticta platyphylloides	White Isabella color Olive buff	(Shukla and Upreti, 2015)
37.	Usnea longissima	brown to yellow	(Shukla and Upreti, 2014b)
38.	Usnea stigmatoides	Pinkish buff Light brownish olive White	(Shukla and Upreti, 2015)
39.	Usnea undulata	Orange to brown and yellow	(Kok, 1966; Dean *et* al., 2012; Shukla and Upreti, 2014a, b)
40.	Xanthoparmelia stenophylla	Mikado Brown Buffy olive Deep colonial	(Shukla and Upreti, 2015)
41.	Xanthoria elegans	Red to yellow	(Shukla and Upreti, 2014b)

Extraction of dyes from lichens by mycobiont culture

Mycobiont Culture

In most of the lichens, 90 percent part is fungus and it is reported that secondary metabolites which are responsible for dye production are derived from lichen fungal part therefore these desired fungus are cultured (Mycobiont Culture) in laboratory conditions and produced in large amount for the production of lichen dyes. Freshly collected lichens gives best result for culturing and production of dyes as compared to stored and old samples. The mycobiont culture can be cultured by following two different methods which are 'spore discharge method' and 'thallus fragment-derived culture method' (Yoshimura *et* al., 2001).

Spore-Derived Culture

It is the standard method of lichen culture in which culture is initiated from lichen spores. The method is established by (Ahmadjian, 1973), and later, many modifications have been made by practitioners (Ahmadjian, 1993; Yoshimura *et* al., 2001). In this method, the thalli collected are screened under dissecting zoom microscope, and healthy thalli bearing fruiting bodies (apothecia) are selected for sterilization. The mature apothecia are cut off from the clean part of thalli. The apothecia are re-hydrated by placing them in a sterile water-saturated atmosphere for 24 hour at 18 to 20 °C. The apothecia are washed in running tap water for 30 min to 1 hour and then treated with Tween 80 (2 %) for 5 min. The surfactants are removed by washing the apothecia in double distilled water. The sterilized apothecia are dried in autoclaved petri plates lined with dry filter paper and used for mycobiont culture. The sterilized apothecia are attached to the inside of the petri plate lids with the help of petroleum jelly. Petri plates containing solidified media were then inverted over the lids, and ascospores are allowed to discharge onto the agar medium. Plates are incubated in BOD incubator and are observed periodically over 3 to 5 months. The suitable media for culturing fungus is prepared by mixing the ready-made powder media in distilled water, heating to boiling, to dissolve the media completely. The media are sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Thallus Fragment-Derived Culture

The thallus fragment-derived culture can be used to obtain mycobiont of lichen species that do not produce apothecia. The method was described by (Yamamoto, 1985) and popularly known as 'lichen tissue culture method'. Generally, the Thallus fragment-derived cultures are liable to contamination with micro-organisms present in the thallus surface; therefore, small thallus fragment (in µm range) is recommended to use (Yoshimura *et* al., 2001). A segment of about 1 cm length from the thallus is separated by scissor or knife and washed under tap water for 30 min to 1 hour. The thallus fragment is then homogenized with mortar pestle in distilled water. The homogenate is filtered through nylon sieve mesh 500 µm. The solution is again filtered through nylon sieve mesh 150 µm, and the desired thallus fragments thus obtained on the mesh are picked up

with the sterile needle under the dissecting microscope and inoculated onto the surface of slant malt– yeast medium. The slants are maintained at 15 °C in dark. Two weeks after the inoculation, the mycobiont hyphae and algal cells can be observed on slants. The symbionts can be isolated and further sub-cultured in desired culture medium that suits the growth and metabolite production in the cultured mycobiont. Although the production of lichen compounds by a mycobiont sometimes stops or is quite meager in amount suggested the mycobiont needs algal stimulation for the production of secondary metabolites. Enough quantities of desired molecules can be achieved by batch cultures and fermentation.

Culture Conditions and Mycobiont Extraction

Petri plates containing the cultured mycobiont should be incubated in the dark at 22 °C and 70– 80 % relative humidity in BOD incubator and observed periodically over 3 to 5 months. When the mycobiont is fully grown, the cultured mycelia (150 days old or more) can be used for dye preparation. Mycobiont is removed from petri plate by keeping plates in the water bath at 80 °C and dried with lyophilizer for 12 hours. Lichens and the cultured mycobiont samples selected for extraction of dyes should be thoroughly washed under tap water. The samples are then shadow-dried in air or sun-dried within a temperature range of 37–40 °C so as to reduce the moisture content of the samples. The dried samples are crushed to form powder and are used for dye preparation by the same methods as applied to extraction through natural thallus (as explained above).

Conclusion

Lichens are the organisms, found in different habitats of the world. Lichens are used for many purposes natural dye production is one of them, there are different types of traditional methods available for dye production, but in these methods large amount of lichens are required for the production of dyes, as they are very slow growing organisms it is very harmful to collect lichens in large amount because this results in disappearing of lichens which are very useful and are not available in large amount, so there is need of safety measures and sustainable use of these type of organisms therefore while collecting lichens for bio prospecting it is necessary to collect fully developed thallus and a small part having fruiting body (apothecia) on it is left behind to conserve useful species of lichens having economical importance but these safety measures are not enough for the conservation of lichens. Lichens are effectively conserved if the use of large amount of lichens is overcome by modifying the extraction methods therefore extraction of dyes from mycobionts of lichens by culturing them in labs reduce the use of lichens in large amount because in this method only a small piece or a fruiting body is enough to produce large amount of dyes by this method overexploitation of lichens is stopped. There is need of establishment of labs which are capable of culturing desired parts which are responsible for bio prospecting of natural products.

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