ORIGINAL ARTICLE

Optimization of extraction methods for natural pigment from *Lawsonia inermis*

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Abstract

Background: Natural extract obtained from the Henna leaves has antimicrobial and antifungal and also acts as a fluorescent compound in the gel electrophoresis. It can be coated in silk, cotton, woolen fabric, and combination. **Introduction:** Dye from the Henna leaves can be obtained by various methods such as alkaline extraction, ethanolic extraction, hot water extraction, Soxhlet extraction, and enzymatic extraction. It paves the alternative way for the use of synthetic dyes and also reduces the environmental hazards and health hazards caused by synthetic dyes. **Materials and Methods:** The three methods (hot water, ethanolic, and enzymatic extraction) were used to extract with the optimized parameters. Hot water extraction of the dye carried with the distilled water; ethanolic extraction is carried out by 1:1 ratio of water and ethanol and the enzymatic extraction is done with the help of cellulase enzyme produced by *Brevibacillus borstelensis* obtained from the guts of termite, collected from the residential area. Based on the concentration the pigment enzymatic extraction efficiency is approximately equal to ethanolic. **Results and Discussion:** While considering the cost and eco-friendly nature, enzymatic extraction is more efficient for the extraction of dye. Extracted pigment is analyzed by the results from UV-Vis spectrophotometer and Fourier-transform infrared results.

Key words: Extraction, Fourier-transform infrared, Henna leaves, natural dye, synthetic dye, UV-Vis

INTRODUCTION

Globally, the major pollutants of water bodies in major cities are the effluent from the dyeing industries.[1-3] In South India, Tiruppur is the industrial town where the dyeing is an occupation for the most of the people. The effluent from those industries is directed toward the path of water bodies which leads to water pollution. The main consequence of water pollution is damaging or unbalancing the ecosystem by killing the living things in it. The water ecosystem is majorly polluted due to the release of dye industry effluent from various regions. The effluent consists of organic, inorganic substance, and synthetic colored pigments. Due to this discharge, the living environment gets polluted and causes several diseases to the human beings. Removal of synthetic colors or dyes from the wastewater will be more expensive than the other treatment.[4-6] However, the natural dyestuffs are colors which are obtained from the plant or any living organism which is used as a substitute for the synthetic colors. It enhances the eco-friendly nature of the living ecosystem. Most commonly the pigments are isolated from the plant which also contains many other substances such as alkaloids, flavonoids, phenolics, pigments, and essential and non-essential oils. The natural pigment can be obtained from various sources such as marigold, beetroot, peel of sweet potato, Henna leaves[7], and opuntia, varieties of *Nerium oleander* and carminic acid from *Dactylopius coccus* which is a cochineals which is mainly for red dye carmine. Henna leaves are the commonly used for the pigment extraction. Most commonly dyeing process in industry utilizes the synthetic dyes. Enzyme-assisted extraction involves the use of enzymes such as pectinase, cellulase, pancreatin, and pepsin. Based on the source and nature of the material the enzyme can be chosen for the process. If the extraction is from the plant source cellulase can be used, from the fruit or other plant body pectinase or cellulase can be used. The advantages of EAE are decreased solvent usage and retain the

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eco-friendly nature. However, the limitation is the preparation of enzyme, cost of enzyme related to raw material, and difficult to scale-up. The extraction of natural pigment can be extracted mainly from plant source such as marigold[7], Henna leaves, cochineal, and algae (red algae, brown algae, and blue-green algae). It can be various methods of extraction such as conventional and non-conventional methods.

Traditional methods[9] include the maceration, infusion, percolation, decoction, and Soxhlet extraction.[10] Maceration is the process of saturating the samples in the solvent for the particular interval of time. Infusion is the process of soaking the samples in solvents for a short period of time with heat (or) cold temperature. Decoction is the process for extracting heat-stable compounds, hard plants materials (e.g., roots and barks) and usually resulted in more oil-soluble compounds. It uses the principle of both maceration and infusion. Percolation is the dried powdered samples are packed in the percolator, added with boiling water and macerated for 2 h. The percolation process is usually done at a moderate rate (e.g., 6 drops/min) until the extraction is completed before evaporation to get a concentrated extract. The advantages of the traditional methods are easy and simple. Proper management of the waste is needed. Alteration in temperature and choice of solvents enhance the extraction process, reduce the volume needed for extraction and can be introduced in the maceration technique. Disadvantages are large volume of solvents is used, and the solvents used in the soaking process play a critical role. Conventional methods include alcoholic extraction includes the use of alcoholic solvents to extract the pigment by placing it in temperature below the boiling point of the solvent used for extraction. Alkaline extraction[11] includes sodium hydroxide acts as a chemical compound to extract the pigment from the plant source.

Modern methods of extraction include microwave-assisted extraction[12], ultrasound-assisted extraction, supercritical fluid extraction, surfactant-assisted extraction, and enzyme-assisted extraction. Microwave-assisted extraction is a selective method which utilizes the microwave as a source for energy to separating the analyte from the crude sample. Conduction plays a major role in which the microwave radiation to interact with the sample of interest causes polarization. This method is more specific for the polar molecules and the solvents having high dielectric constant. Advantage of this method is the reduction in extraction time and the solvent volume as compared to the conventional methods. The major disadvantage is optimization of the conditions for the extraction of desired compounds from the crudes. Ultrasonic wave assisted extraction involves the use of sound energy ranging from 20 KHZ to 2000 KHZ. It produces a mechanical effect of cavitation on the cell wall indirectly increase the permeability and enhance the extraction. Benefits of UAE are a reduction in extraction time and solvent and solvent consumption. It also affects the phytochemicals to form the free radicals. Supercritical extraction method uses the supercritical fluid for the extraction process. Most commonly used supercritical fluid is CO. It also reduces the extraction time, but the cost of equipment is very high when compared to others. Surfactant-assisted extraction uses the surfactants or detergents for enhancing the extraction process. Surfactants may be chemical or from a plant source. Chemical surfactants such as CTAB, Sodium deodecyl sulfate, and Triton X-100 are used. Most commonly used surfactant from the plant source is saponin. It is used along with the solvent to enhance the extraction process.

MATERIALS AND METHODS

Isolation of Enzyme Producers

The termites were collected from the wood store. Termites were placed in a mortar and pestle with the help of sterile distilled water the microbial consortia was obtained. The sample obtained from the process was exposed to serial dilution for the cellulase producers.

Medium Used for the Enzyme Producers

Media for cellulase producers

0.03%$MgSO_4$, 0.2%$K_2H_2PO_4$, 1%glucose, 0.25% $(NH_4)SO_4$, 1%Peptone for 100 g base.

Production media for cellulase producers

Overnight grown culture was inoculated in 4% glucose, 0.75% peptone, 0.01% $FeSO_4$, 0.5% KH_2PO_4, and 0.5% $MgSO_4$ at pH 7 with 37°C.

Screening of Enzyme Producers

The isolates were confirmed by the formation of zone of clearance when it is stained by Congo red and flooded with NaCl solution.

Enzyme Purification

For the enzyme purification, the culture was centrifuged at 5000 rpm for 20 min. The supernatant was collected and placed in an ultra-filtration set up having the molecular cutoff 30 KDa [Figuere 1]. Both the retentate and permeate exposed to the enzyme activity and the optical density was measured.

Identification of Cellulase Activity

After ultrafiltration, both the retentate and permeate were tested for cellulase with the plate containing the composition of 0.5 g KH_2PO_4, 0.25 g $MgSO_4$,2 g CMC, 15 g agar, and 0.2 g Congo red.

Figure 1: Purified cellulase from the *Brevibacillus borstelensis*

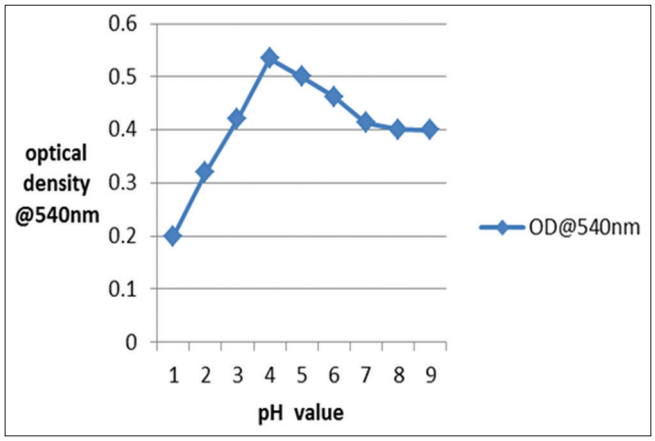

Figure 2: Optimization of pH for the cellulase activity

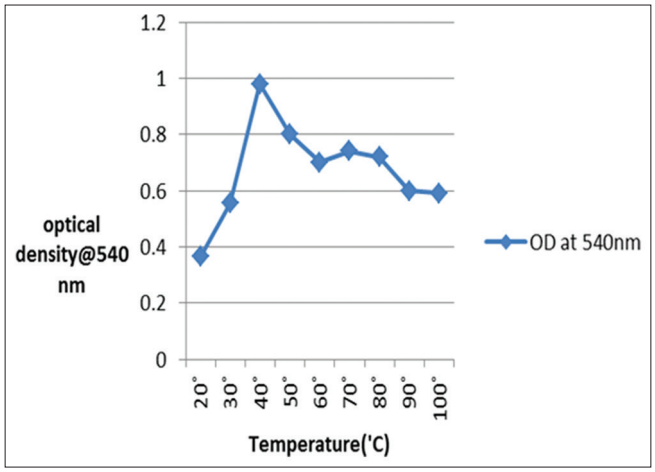

Figure 3: Optimization of temperature for the cellulase activity

Enzyme Assay for Specific Activity

Specific activity was checked by the DNSA method. 50 µl of the crude enzyme with 3 ml of 0.5% CMC in 50 mM phosphate buffer was added with 3 ml of DNSA reagent. Then, it was placed in a water bath at 60°C for 15 min. Optical density was measured at 570 nm.

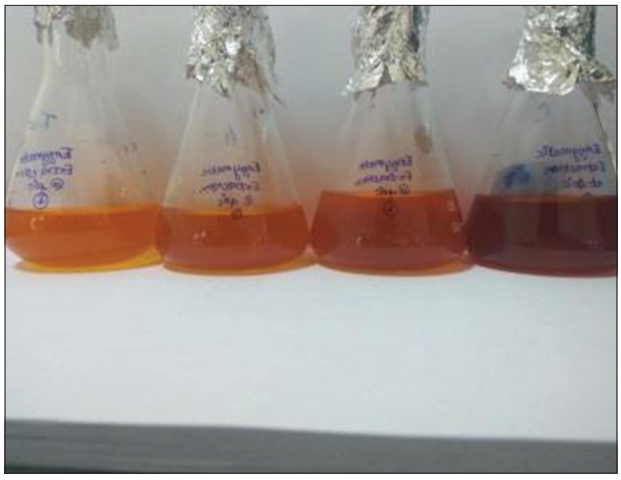

Figure 4: Extraction of pigment by cellulase from Henna leaves

Figure 5: Extraction of pigment by hot water

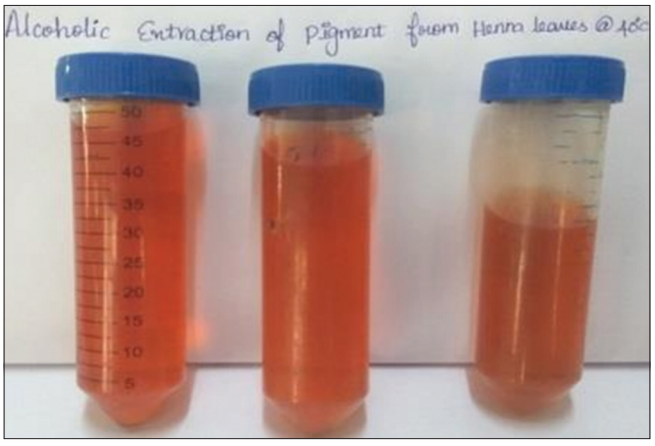

Figure 6: Extraction of pigment by Ethanol

Extraction of Natural Pigment

Hot water extraction

To the 20 g of Henna leaves, 100 ml of distilled water was added and boiled up to 100°C to extract the pigment [Figure 2].

Cellulolytic Extraction of Pigment

Optimization for Enzyme Activity

The pH and temperature were determined for the enzyme activity by varying the pH from 2, 3, 4, 5, 6, 7, 8, and 9 and the temperature from 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C. The optimum condition for the stability of cellulase was about pH 4 [Figure 3] and temperature 40°C [Figure 4]. To the 20 g of Henna leaves, the cellulase along with the buffer was added to the material and incubated in the optimum condition for the extraction. After 1 h, the extracted pigment was collected [Figure 5].

Ethanolic Extraction of Pigment

To the 20 g of Henna leaves, 100 ml of 70% ethanol was added to the leaves extract the pigment [Figure 6].

Antibacterial Activity of Extracted Pigment

The extracted pigment was tested for the antibacterial activity against *Escherichia coli* and *Aspergillus niger* using nutrient agar as a medium.

RESULTS AND DISCUSSION

Identification and Isolation of Enzyme Producers

The enzyme producing organisms are plated in the CMC media. Formation colonies indicate that the isolated enzyme producers can produce the enzymes by the use of carbon source and their substrates.

Screening of Enzyme Producers using Congo Red

Thus, the plates flooded with the Congo red and sodium chloride showed in the following figures. Congo red acts as an indicator which binds with the colonies formed in the plate based on the prepared concentration. When it is flooded with NaCl, it removes the colonies.

Enzyme Production

With the production media, the enzyme was produced by the enzyme producers in the 250ml conical flask. The culture was centrifuged at 10,000 rpm for 20 min. After centrifugation, the supernatant was collected in a beaker. The collected supernatant was checked for the enzyme activity by DNSA method taken as crude value. Then, the remaining supernatant was subjected to ultrafiltration. Both the retentate and permeate were collected and checked for the enzyme activity. Permeate shows the maximum activity of about 3.22 µmol/ml min.

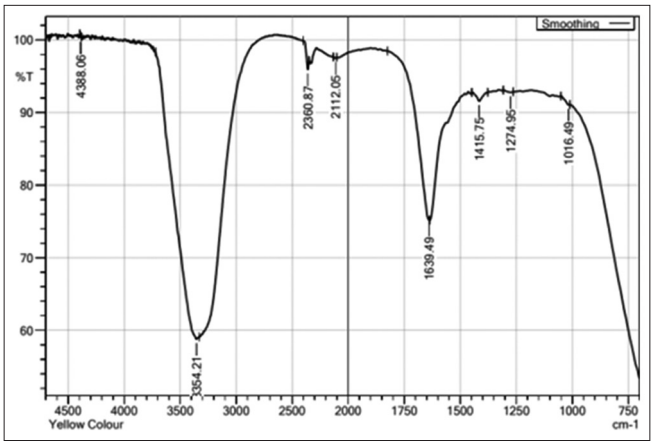

Figure 7: Fourier-transform infrared result for the extracted pigment from Henna leaves

Table 1: Antibacterial activity of extracted pigment

Organism used	Zone of inhibition (mm)
E. coli	10±0.01
Aspergillus niger	12±0.03
Control	-

A. niger: Aspergillus niger, E. coli: Escherichia coli

Pigment Extraction

About 1000 ml of pigment was obtained from the hot water, and cellulolytic extraction of Henna leaves with the optimized parameters. The maximum wavelength was observed at 445 nm for the extracted pigment using UV-Vis spectrophotometer.

Characterization of Extracted Pigment

Figure 7 shows the result of the characterization of extracted pigment. Observed peaks are 3354.21 for O-H alcoholic stretch, 2360.87 for C-C carboxylic acid stretch, 1639.49 for C=C stretch of alkene group, 1415.75 for S=O sulfate group, 1274.95 show C-Oalkyl-arylstretch, and 1016.49 for C-O stretch.

Antibacterial Activity of Extracted Pigment

From Table 1, the antibacterial activity of extracted pigment for *E. coli* and *A. niger* and the zone of inhibition were measured.

CONCLUSION

The pigment extracted from the Henna leaves by various methods such as hot water extraction, cellulolytic extraction, and ethanolic extraction was characterized using Fourier-transform infrared, and wavelength was determined as 445 nm.

Cellulolytic extraction has more efficiency of extracting pigment from Henna leaves. The antibacterial activity was measured by the zone of inhibition and the extracted pigment against for both of *E. coli* and *Aspergillus niger*.

REFERENCES

1. Sivarajasekar N, Baskar R. Optimization, equilibrium and kinetic studies of basic red 2 removal onto waste *Gossypium hirsutum* Seeds. Iran J Chem Chem Eng 2018;37:157-69.
2. Karthik V, Sivarajasekar N, Padmanaban VC, Nakkeeran E, Selvaraju N. Biosorption of xenobiotic reactive black B onto metabolically inactive *T. harzianum* biomass: Optimization and equilibrium studies. Int J Environ Sci. Technol 2018;2018:1-12.
3. Sivarajasekar N, Ramasubbu S, Maran JP, Priya B. Cationic dyes sequestration from aqueous phase using biosurfactant based reverse micelles. In Recent Advances in Chemical Engineering. Singapore: Springer; 2016. p. 67-74.
4. Muthusaravanan S, Sivarajasekar N, Vivek JS, Paramasivan T, Naushad M, Prakashmaran J, *et al*. Phytoremediation of heavy metals: mechanisms, methods and enhancements. Environ Chem Lett 2018;2018;1-21.
5. Sivarajasekar N, Mohanraj N, Sivamani S, Moorthy IG, Kothandan R, Muthusaravanan S. Comparative Modeling of Fluoride Biosorption onto Waste *Gossypium hirsutum* Seed Microwave-Biochar using Response Surface Methodology and Artificial Neural Networks. In Intelligent Computing, Instrumentation and Control Technologies (ICICICT), International Conference on IEEE; 2017. p. 1631-5.
6. Sivarajasekar N, Mohanraj N, Sivamani S, Maran JP, Moorthy IG, Balasubramani K. Statistical optimization studies on adsorption of ibuprofen onto *Albizia lebbeck* seed pods activated carbon prepared using microwave irradiation. Mater Today 2018;5:7264-74.
7. Ferbiyanto A, Rusmana I, Raffiudin R. Characterization and identification of cellulolytic bacteria from gut of worker *Macrotermes gilvus*. Hayati J Biosci 2015;22:197-200.
8. Badri BM, Burkinshaw SM. Dyeing of wool and nylon 6.6 with henna and lawsone. Dyes Pigm 1993;22:15-25.
9. Sowbhagya HB, Sampathu SR, Krishnamurthy N. Natural colorant from marigold-chemistry and technology. Food Rev Int 2004;20:33-50.
10. OHair JA. Inadequacy of Gram's Iodine Assay to Detect Cellulase Degradation during Screening for Lignocellulolytic Bacteria and Fungi. Tennessee: Tennessee State University; 2014.
11. Azmir J, Zaidul IS, Rahman MM, Sharif KM, Mohamed A, Sahena F, *et al*. Techniques for extraction of bioactive compounds from plant materials: A review. J Food Eng 2013;117;426-36.
12. Butnariu M. Methods of analysis (extraction, separation, identification and quantification) of carotenoids fromnatural products. J Ecosys Ecography 2016;6:193.

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