Volume 65, Issue 3, 2021
Journal of Scientific Research
Institute of Science,
Banaras Hindu University, Varanasi, India.

Use of the Natural Pigments of Red Beet Root Pomace (*Beta vulgaris* L.) to Develop a Mycological Stain: An eco-friendly Alternative Substitute

Payel Sutradhar* and Chandrika Bhattacharya

Department of Microbiology, Institute of Genetic Engineering, Badu, Kolkata-700128
payel.sutradhar@gmail.com*, bhattacharyac78@gmail.com

Abstract: **The deleterious effects of synthetic dyes evoked great enthusiasm in many scientists to search for eco-friendly natural colorants for morphological study and identification of microorganisms. The aim of the study was to develop a mycological staining reagent from the red pigments extracted from beet root pomace (*Beta vulgaris* L.). Beet root rich in water soluble betalains was extracted with distilled water at 40°C.The conventional easy and economical wet mount procedure was followed to prepare the fungal slides. Glycerol was used as a hygroscopic agent which did not affected the colour of the extract. In the current investigation the extract was evaluated as a staining reagent to study the morphology of the test fungal samples viz. *Rhizopus* sp., *Microsporum gypseum* and *Aspergillus niger*. The study revealed that the use of citric acid buffer intensified its red colour potentially at pH 5.This novel staining reagent was proved to be reliable and safe with satisfactory visual clarity of the characteristic fungal structures. In conclusion, this ecofriendly alternative method of staining by such non-toxic vegetal active principle provided a promising way to support environmental sustainability.**

Index Terms: ***Beta vulgaris* L, beet root red, mycological stain, betalains, Wet mount procedure, *Rhizopus sp., Microsporum gypseum, Aspergillus niger*.**

I. INTRODUCTION

Now a days the availability of the plant products has greatly increased the consumer preferences for using naturals dyes to minimize the health hazards caused by synthetic and inorganic dyes.

Staining process is an important requirement for the microbiological laboratories for the study and identification of the microbes which involves various inorganic synthetic reagents (Rymbai, H. et al. , 2011). Plant products therefore can provide a natural biodegradable resource for such chromogenic pigments.Beetroot (*Beta vulgaris* L.) is the succulent, tuberous herb which represent the main source for the natural red dye. The main component of this natural red dye is betanin which are not found in plants containing anthocyanin pigments based upon their molecular structure. The pigment stability is influenced by enzymes, temperature, oxygen and pH (Sarkar et al., 2015).

Betanin is the most abundant of the dyes and was first isolated by Schudel in 1918 (Pucher GW et al., 1938). The major commercially exploited betalain crop is red beetroot which contains two major pigments, namely betanin (a red betacyanin) and vulgaxanthine I (Gengatharan et al., 2015). This red Betanin is majorly present by 75–95% and vulgaxanthin I, which is the principal pigment of yellow betaxanthin group. Another yellow pigment, betalamic acid, derived directly from cleavage of betanin (Singh et al., 2014; Chhikara et al., 2019). These biocolorants are advantageous for their water solubility nature and thus widely used for food colouring. Their antioxidant and radical scavenging properties for protection against certain oxidative stress-related disorders are remarkable (Strack et al., 2003). Beside their uses in food industries as food colorant, betanin has been reported to be used in staining of various substances like wool and thread. Also, it has been used in staining of helminth parasites, ova of intestinal nematode, buccal smears, as well as applied as a fluorescent dye in tissue staining (Udonkang, M. I. et al., 2018).

Cotton blue widely applied to stain the chitin present in the fungal cell walls should be cautiously used due to its hazardous property (Shedbalkar, U. et al., 2008). The earlier works with

* Corresponding Author

aqueous extracts of beetroot dye reported that it contains slightly acidic betalain pigments which can be easily used as hematological stain (Udonkang, M. I. et al., 2018). Thus the present study aimed to replace the conventional synthetic stain (cotton blue) by the natural red dye extracted from beet root pomace to stain fungal species. The use of this eco- friendly, non toxic harmless natural dye could be a novel staining reagent in mycology.

II. MATERIALS AND METHODOLOGY

A. Collection of plant materials

100 grams fresh beet roots (*Beta vulgaris* L.) were procured from the local market of Dumdum area (Kolkata, India). The samples were collected and washed thoroughly with water to remove any dirt. The beet roots were peeled off and sliced into small pieces.

B. Extraction of crude natural dye

Double distilled water extraction process was followed. 50 grams of the fresh sliced beet root samples were mixed with 100 ml (0.5 g/ml) of double distilled water and heated to 40 °C for 30 minutes in a water bath. The red extract was separated, cooled and further proceeded for purification steps.

C. Purification and preparation of the stain

The purification of the extract was done by a filtration process followed by centrifugation. The aqueous extract was filtered using Whatmann filter paper No.1 and centrifuged at 3000 rpm for 15 minutes to get rid of all the solid debris (Cheng, C. W et al. , 2014). The supernatant was transferred into an amber coloured bottle and stored at 4°C for further steps. 20ml of this purified extract was mixed with 20ml glycerol. As earlier reported beet root extract is stabler at pH 5 and citric acid is one of the best solvent for the extraction of the active principles (Sturzoiu, A. et al., 2011). In this work the resulting solution was adjusted to pH 5 by using citric acid buffer for the better stability of the dye.

D. Preparation of test cultures

Frozen glycerol stock cultures of the fungus viz. *Rhizopus* sp., *Microsporum gypseum* and *Aspergillus niger* were collected from Microbiology department, Institute of Genetic Engineering (Badu, Kolkata). The samples were reactivated by subculturing on the slants of YEPD agar (Cappuccino J G et al., 1996). The freshly grown pure cultures were stored at 4º C for subsequent staining procedures and morphological study.

E. Staining procedure and microscopy

Staining of the fungal samples were done by following a similar method of wet mount preparation (Leck, A., 1999). A drop of the prepared extract was placed on a clean and dry slide. By using an inoculating wire, sufficient amount of sample was placed on the drop and teased carefully to make a thin film of the fungus. Coverslip was placed gently, avoiding air bubbles and kept undisturbed for about 5 minutes. The preparation was examined under microscope (B1 series Motic microscope) initially with low power (10x) for screening and then 40x for the detailed morphological study.

III. RESULTS AND DISCUSSION

The findings of the present study evaluated the beet root pomace aqueous extract as a potent fungal stain. Staining of microbial cells is a very important procedure in microbial identification. Cells need to be fixed and stained to increase contrast, and to study morphological structures under microscope. The increasing environmental awareness is reducing the deleterious effects of hazardous inorganic or synthetic dyes by replacing them with alternative eco-friendly dyes or stains (Adeyemo, S. M. et al.,2017). Plants being the principally natural resource for such myriad vegetal active principles, they can be easily isolated and evaluated for staining microorganisms. The earlier studies also reported efficient staining results with beet root extracts to study the samples of Platyhelminthes and helminth parasites (Al-Amura et al., 2012; Kumar, N. et al., 2015). This natural extract was also used in fluorescence cell imaging and textile dyeing (Das, A. et al., 2017; AL-Khateeb et al., 2019). Beet root is also reported to stain buccal smear in exfoliative microscopic anatomy (Singnarpi, S. et al, 2017). Thus the present work was attempted to use the extract of *Beta vulgaris* L. to stain the fungal cells and study their structural features. Cotton blue is an acidic dye binds to chitin which is a positively charged polysaccharide and is the main component of fungal cell wall (Roy, J. C et al., 2017). The conventional method generally used to stain fungus is wet mount method by lactophenol cotton blue reagent. This reagent involves harmful chemicals viz. cotton blue that binds chitin of the fungal cell wall and phenol kills the fungus which is very corrosive (Leck, A. , 1999). Mfoniso I. Udonkang et al. reported that the aqueous extracts of beetroot dye stained most basic tissue structures because they contain slightly acidic betalain pigments (Udonkang, M. I. et al., 2018). Thus in the present study, the basis of choosing beet root extract as a natural colour to stain fungal cell wall was with the agreement of their statement.

The study preliminary resulted a dark red coloured extract when the sliced beet roots were subjected to double distilled water extraction method. Beetroot Vacuoles are store houses of water soluble pigments and can be extracted by disrupting the membranes possibly by the application of detergents, solvents, heat shock, etc. (Singh, A et al, 2017). As the maximum stability of these pigments could be in the range of 40ºC -50ºC (Attia, G. Y et al., 2013), the extraction process was performed by heating the beet root samples at a temperature of 40ºC. The resulted extract was further purified by filtration and centrifugation to exclude all the present debris from the extract. The maximum

retention of the coloured pigment is at pH 5 (Attia, G. Y et al., 2013). Weak acid solutions also can be efficient as a solvent for extraction (Sturzoiu, A et al., 2011). Thus in the current work the pH of the resulted purified aqueous crude extract of beet root was adjusted to pH 5 by citric acid buffer.The red colour of the extract showed to be more intensified at this acidic pH.The glycerol was used similarly as the conventional method which showed least discolouration of the extract .The beet root extract possess antimicrobial activity as reported by Deshmukh et al. , 2018 .Therefore this was also advantageous for using beet root extract as a stain in the present work.

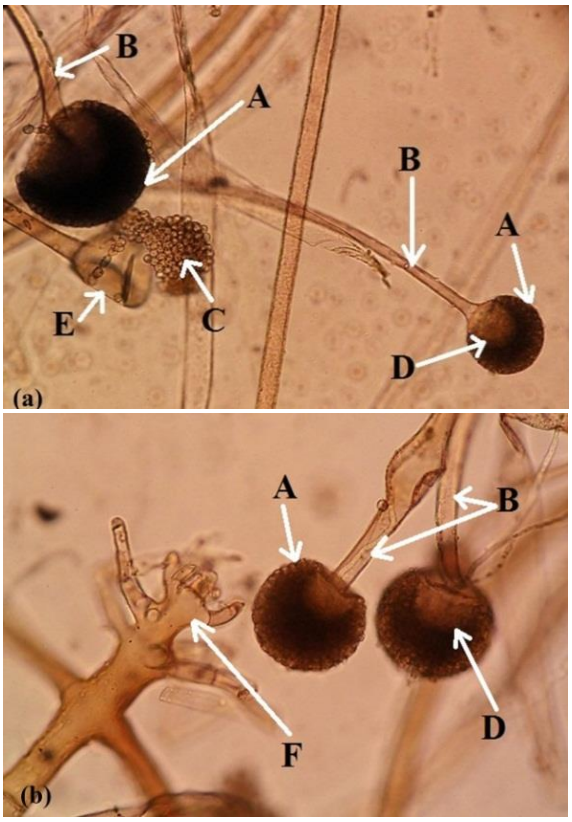

Fig: 1(a,b): Photomicrograph of *Rhizopus* sp. stained with prepared beet root extract stain: A-Sporangium ; B-Sporangiophore ; C-Spores ; D-Columella ; E Collapsed columella ; F-Rhizoids

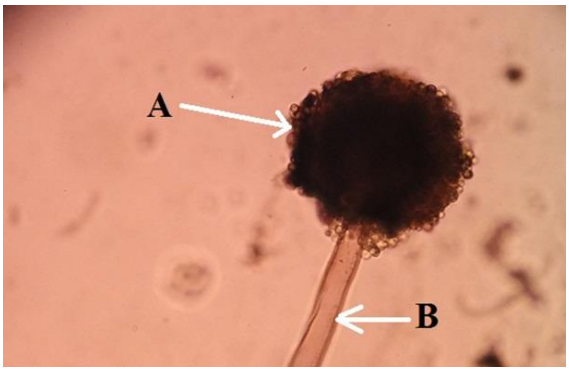

Fig 2: Photomicrograph of *Aspergillus niger* stained with prepared beet root extract stain showing A- condial head with conidia; B- conidiophore

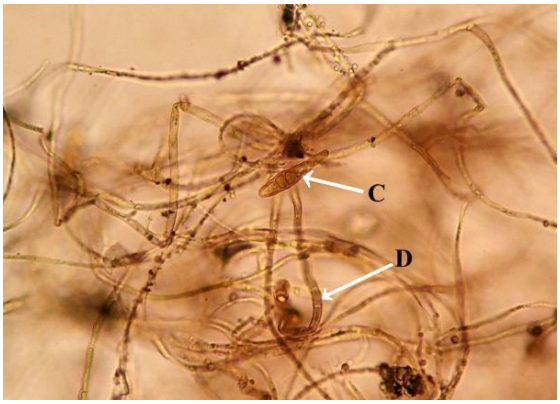

Fig 3: Photomicrograph of *Microsporum gypseum* stained with prepared beet root extract stain *showing :* C- macrospore and D- septate hyphae

Fig 4: Beet root stain imparted red colour to the macrospore of *Microsporum gypseum*, clearly showing its fusiform structure with typical 5 cells.

The photomicrographs of the stained fungal samples, observed under 10X and 40X showed satisfactory degrees of staining with the crude natural stain prepared from the beetroot pomace extract. The stained mount of *Rhizopus sp*. showed differentiated reddish coloured rhizoids, and sporangiophores distinctly with sporangium. Spores were observed clearly adhered to invaginated or collapsed columella whereas some sporangium were seen as intact globular structures (Fig 1a, b). Faint red colour was imparted to the *Aspergillus niger*, which showed a mature conidiophore or radiated conidial head with rough walled conidia (Fig 2). The septate mycelium of *Microsporum gypseum* were stained reddish and showed distinctive fusiform macrospore with a thin wall contained typically 5 cells (Fig 3, 4). The prepared stain successfully helped to observe the fungal structures with a good clarity. The identifying structures of the test fungus was in agreement with the structures described by O.P sharma, 1989. Hence, this

alternative method of staining with natural dye from *Beta vulgaris* L. could replace the use of synthetic dyes thereby minimizing their environmental and health hazards.

CONCLUSION

The results of this study evidenced that natural extract from *Beta vulgaris* L. potentially replaced the conventional synthetic dye in wet mount procedure in mycology.The present study concluded the beet root extract was safe ,eco-friendly , economically feasible , more reliable and biodegradable non hazardous colorant which could be potentially applied as a fungal stain for their morphological study .The work did not required any specialized technique for extraction and application of the natural stain.The stability of the prepared stain was also satisfactory and rendered clear visibility of the fungal morphology similar to conventional synthetic dye (cotton blue).More fungal samples are suggested to stain and visually study their identifying structures by this alternative method before transfer to the entrepreneurs for commercial production of this natural stain. The present study with the crude extract of the beet root was found to be much potent to stain or colour the fungal cell walls. Hence the work also recommends to stain various fungal species with the chemically isolated and purified active principle present in the same extract in further scope.

ACKNOWLEDGEMENT

The authors are thankful to Institute of Genetic Engineering (IGE), Badu, West Bengal, India for providing all necessary facilities in the laboratory.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Adeyemo, S. M., Akinloye, A. J., & Adekanmi, G. B. (2017). The Use of Plant Dyes for Microbial Staining and Identification: An Eco-friendly and Non-Toxic Alternative Method. *Journal of Advances in Biology & Biotechnology*, 1-10.

Al-Amura, M. F. (2012). Staining technique for helminth parasites by use red beet (beta vulgaris l.) Extract. *Basrah Journal of Veterinary Research.*, *11*(1), 283-292.

AL-Khateeb, D. S. M. (2019, September). Extraction Dyes From Two Natural Plants Olive Leaves and Beta vulgaris And The Uses In dyeing Textile. In *Journal of Physics: Conference Series* (Vol. 1294, No. 6, p. 062109). IOP Publishing.

Attia, G. Y., Moussa, M. E. M., & Sheashea, E. R. (2013). Characterization of red pigments extracted from red beet (Beta vulgaris L.) and its potential uses as antioxidant and natural food colorants. *Egyptian Journal of Agricultural Research*, *91*(3), 1095-1110.

Cappuccino J G and Sherman N. (1996). *Microbiology - A Laboratory Manual*, (The Benjamin/Cummings Publishing Co., Inc., Menlo Park, California).

Cheng, C. W., Saad, S. M., & Abdullah, R. (2014). Alternative staining using extracts of hibiscus (Hibiscus rosa-sinensis L.) and red beet (Beta vulgaris L.) in diagnosing ova of intestinal nematodes (Trichuris trichiura and Ascaris lumbricoides). *Eur J Biotechnol Biosci*, *1*(5), 14-8.

Chhikara, N., Kushwaha, K., Sharma, P., Gat, Y., & Panghal, A. (2019). Bioactive compounds of beetroot and utilization in food processing industry: A critical review. *Food Chemistry*, *272*, 192-200.

Das, A., De, D., Ghosh, A., & Goswami, M. M. (2017). Improvement in Cell Imaging by Applying a New Natural Dye from Beet Root Extraction. *arXiv preprint arXiv:1710.08119*.

Deshmukh, G. P., Inka, P., & Sindhav, R. (2010). Application of beetroot as natural coloring pigment and functional ingredient in dairy and food products. *Int J Curr Microbiol Appl Sci*, *7*, 2010-2016.

Gengatharan, A., Dykes, G. A., & Choo, W. S. (2015). Betalains: Natural plant pigments with potential application in functional foods. *LWT-Food Science and Technology*, *64*(2), 645-649.

Kumar, N., Mehul, J., Das, B., & Solanki, J. B. (2015). Staining of Platyhelminthes by herbal dyes: An eco-friendly technique for the taxonomist. *Veterinary world*, *8*(11), 1321.

Leck, A. (1999). Preparation of lactophenol cotton blue slide mounts. *Community Eye Health*, *12*(30), 24.

Pucher GW, Curtis LC, Vickery H: The red pigment of the root of the beet (*Beta vulgaris*) I. The preparation of betanin. J Biol Chem 1938; 123: 61–70

Roy, J. C., Salaün, F., Giraud, S., Ferri, A., Chen, G., & Guan, J. (2017). Solubility of chitin: solvents, solution behaviors and their related mechanisms. *Solubility of polysaccharides*, *10*.

Rymbai, H., Sharma, R. R., & Srivastav, M. (2011). Bio-colorants and its implications in health and food industry–a review. *International Journal of Pharmacological Research*, *3*(4), 2228-2244.

Sarkar, T., Sen, M. K., & Nihar, S. (2015). Extraction of natural pigment from beet root and proper packaging of that red dye: A review. *J Agric Eng Food Technol*, *2*(2), 116-8.

Sharma, O. P. (1989). *Textbook of fungi*. Tata McGraw-Hill Education.

Shedbalkar, U., Dhanve, R., & Jadhav, J. (2008). Biodegradation of triphenylmethane dye cotton blue by Penicillium ochrochloron MTCC 517. *Journal of Hazardous Materials*, *157*(2-3), 472-479.

Singh, A., Ganesapillai, M., & Gnanasundaram, N. (2017, November). Optimizaton of extraction of betalain pigments from beta vulgaris peels by microwave pretreatment. In *IOP Conf Ser Mater Sci Eng* (Vol. 263, p. 032004).

Singh, H. B., & Kumar, A. B. (2014). *Handbook of natural dyes and pigments*. Woodhead Publishing India Pvt Limited.

Singnarpi, S., Ramani, P., Natesan, A., Sherlin, H. J., & Gheena, S. (2017). Vegetable stain as an alternative to H and E in exfoliative cytology. *J Cytol Histol*, *8*, 469.

Strack, D., Vogt, T., & Schliemann, W. (2003). Recent advances in betalain research. Phytochemistry, 62, 247e269.)

Sturzoiu, A., Stroescu, M., Stoica, A., & Dobre, T. (2011). Betanine extraction from beta vulgaris-experimental research and statistical modeling. *UPB Sci Bull, Series B*, *73*(1), 145-56.

Udonkang, M. I., Inyang, I. J., Ukorebi, A. N., Effiong, F., Akpan, U., & Bassey, I. E. (2018). Spectrophotometry, physiochemical properties, and histological staining potential of aqueous and ethanol extracts of beetroot on various tissues of an albino rat. *Biomedicine hub*, *3*(3), 1-10.

***