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SCREENING OF PIGMENT PRODUCING FUNGI FROM FLORAL WASTE AS A PROSPECTIVE ANTI-MICROBIAL TOOL

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ABSTRACT

Floral waste management in Indian purview is seemingly diffused. The main contributors of floral waste are religious offerings spanning across PAN India level. Certainly, floral waste appropriately referred to as temple waste is either dumped into adjoining surface waters and streams or is being piled up with municipal solid waste for its further processing. Predominantly, floral waste consists of *Tagetes* sp. Our preliminary study is inspired by the fact that floral waste has an immense potential to develop value added products through microbiological interventions by utilizing the natural attenuation property of autochthonous microbes. This led to planning of bio-prospective study aimed to screen indigenous pigment producing fungal isolates from *Tagetes* sp. with diversified applications in healthcare, textile, food and pharmaceutical industries. Two promiscuous isolates with pigment producing efficacy were screened by Solid State Fermentation (SSF) and Submerged Fermentation (SmF) and were identified as *Mucor* sp. and *Penicillium* sp. Briefly, an attempt was made to ascertain anti-microbial efficacy of *Bacillus* sp. by Disc Diffusion Assay through Minimum Inhibitory Concentration (MIC) against pigment produced by both the fungal isolates with Chloremphenicol, Amoxycillin and Vancomycin as positive controls. A significant ($p<0.05$) anti-microbial efficacy was observed in both the cases (17 mm and 11 mm) with respect to positives controls. These preliminary findings have led to emergence of new vistas considering potential of myco-pigments as novel bio-therapeutics.

KEY WORDS : Anti-microbial, Antibiotics, *Mucor, Penicillium*, *Tagetes* sp.

INTRODUCTION

Plants and microorganisms are considered potential sources of natural pigments (Sen *et al*., 2019). Plant derived pigments include anthocyanins, beta carotenes, betalains, and chlorophyll (Fernández-López *et al*.2020). Until recently, microorganisms such as bacteria and fungi have been explored for production of natural pigments (Kalra *et al.,* 2020). Noteworthy is the fact, that commercial scale production of high quality pigments within a narrow time frame has necessitated need of microbial pigments as natural coloring agents (Lagashetti *et al.,* 2019). Amongst micro-organisms, fungi have been explored for their potential pigment producing efficacy (Akilandeswari and Pradeep, 2016) belonging to genera *Aspergillus, Monascus, Paecilomyces* and *Penicillium* (Mendez *et al*., 2011). In our previous study, we have explored the role of *Aspergillus* sp., in sulfate removal from dairy effluents (Sharma *et al*., 2022). Biochemically, these myco-pigmemts are quinones, flavonoids, melanins and azaphilones, that form part of the aromatic-polyketide chemical group (Venil *et al.,* 2020), and has been described widely for their medicinal and dyeing properties (Teixeira *et al.,* 2012). Attributing to the commercial aspect of myco-pigments, the most promising include red coloured anthraquinones derived from *Penicillium oxalicum* (Caro *et al.*, 2017), blood-red coloured naphthoquinone from *Cordyceps unilateralis* (Nematollahi *et al.*, 2012), Monascorubramin from *Monascus* sp. (Mostafa and Abbady, 2014) and yellow-tinted riboflavin obtained from *Ashbyagosspi* (Tuli *et al.*, 2015). *Fusarium oxysporum* synthesizes pink-tinted anthraquinone (Gessler *et al.*, 2013) and *Fusarium verticillioides* generates yellow-tinted Naphthoquinone (Boonyapranai *et al.*, 2008). Table 1 elucidates a lucid account of myco-pigments and their diversified industrial applications.

MATERIALS AND METHODS

Sampling and processing of floral waste

Floral waste (5 kg) was collected from Shiv Temple, Mullana- Ambala and transported to the laboratory in accordance with the standard procedures. Floral waste primarily comprised of flowers belonging to genera *Tagetes* sp. These were segregated, washed dried and compartmentalized (separated into different components) (Singh *et al.*, 2017).

Mycological screening of floral waste for pigment production efficacy

For the above study, a 4 tier strategic study was planned to screen for pigment producing fungi from floral waste (*Tagetes* sp.) with a potential to exert anti-microbial efficacy (Figure 1).

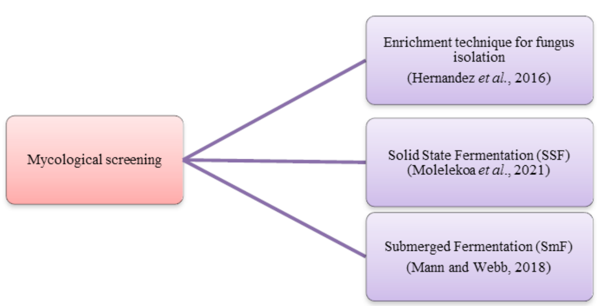

Fig. 1. Strategic mycological study layout

Enrichment technique: Briefly, the technique was based on enriching the minimal media with segregated parts retrieved from *Tagetes* sp. Using the conventional culture technique, aseptically; petals and sepals were transferred onto Potato Dextrose Agar (PDA) with the following composition. This was followed by incubation at room temperature for 3-4 days for visible mycelial growth.

Characterization of screened fungal isolates: Identification of fungal isolates was carried out based on their morphological, colonial and microscopic characteristics (Cappuccino and Shermann, 2009).

Solid State Fermentation (SSF): The screened fungal isolates were processed for SSF for enhanced pigment production in accordance with protocols devised by Molelekoa *et al.*, (2021). Briefly, substrate used for the pigment production was partially dried *Tagetes* sp. floral components with a *precursor seed.* On daily basis, visible mycelial growth was observed following pigment production.

Submerged State Fermentation (SmF): The strain with best pigment production in SSF was progressed for SmF for further anti-microbial assays based on the protocol devised by Manan and Webb, (2018). Briefly, 250 ml Potato Dextrose Broth (PDB) per strain was enriched by sepals and petals to exert an inducible effect for pigment production. Qualitatively, pigment production was monitored. Under aseptic conditions, the pigment (5ml) was decanted and spinned at 3000 rpm for 10 minutes. The supernatant was carefully decanted and used for further antimicrobial studies.

Antimicrobial activity of selected pigment against screened bacterial isolates (Based on qualitative screening) by Disc Diffusion Assay

Bacterial isolates were also screened from floral components of *Tagetes* sp. by culture dependent approach. Characterization of predominant bacterial isolate was carried out according to Bergey's Manual of Systematic Bacteriology (Cappucino and Shermann, 2009). For antimicrobial screening, we followed a protocol devised by Saravanan and Radhakrishnan, (2016). Briefly, 1 mm discs were prepared from sterile Whatmann no. 42 filter paper and were immersed in partially purified supernatant (pigment). This was followed by drying discs in oven for 50 ^{o}C for 2 hours. Lawn culture of *Bacillus* sp. (Obtained from our parallel bacteriological study) was prepared and 5 discs were evenly placed. This was followed by incubation at 37 ^{o}C for 24-48 hours. Minimum Inhibitory Concentration (MIC) was calculated with respect to known standards. Distilled water was used as negative control and Chloremphenicol (30 mcg), Vancomycin (30 mcg) and Amoxycillin (30 mcg).

RESULTS AND DISCUSSION

Sample collection and processing

Our study was primarily aimed at floral waste management through biotechnological interventions, which certainly may lead to circular economy model! Floral waste comprising primarily of *Tagetes* sp. was collected from Shiv Temple, Mullana, District Ambala Similar reports are suggestive of *religious places* being considered paramount source of floral waste. In a recent study conducted in Anand Town, Gujarat; 20 temples were selected and routinely floral waste was collected for value added product formulation. Likewise, Banaras, one of the holiest cities of the country, has no policy for the disposal of the tones of waste. Every day waste material weighing 3.5–4.0 ton is left as such (Mishra, 2013). During entire course of our study, primarily, *Tagetes* sp. was predominantly collected. Briefly, floral waste aptly referred to as temple waste was subjected to sequential processing.

This methodological approach was inspired by the study conducted by Singh *et al*., (2017). Briefly, the similar research was focused on value added product development leading into "waste to wealth" model. The research was focused at development of products like pigments, phyto-pharmaceuticals, bio-fertilizers to name a few. Petals have been found to possess characteristic patterns for pigments as reflected in FTIR spectral analysis (Waghmode *et al.,* 2016). It had been also reported that enormous amount of flower waste is produced in temples of India which can be utilized in making dyes for dyeing of cotton, wool and silk on industrial scale (Vankar *et al.,* 2009). They used (marigold) *Tagetuserecta* petals which mainly consists of carotenoids-lutein and flavonoid-patuletin, these colorants have been identified, isolated and used for dyeing textiles. Likewise, a similar study conducted GC-MS analysis and revealed presence of petals as main component of pigment production (Perumal *et al.,* 2012).

Mycological screening of floral waste for pigment production efficacy

Enrichment technique

Enrichment technique for fungal isolation from

Table 1. Myco-pigments with diversified industrial applications

(*Source:* Anugraha and Thomas, 2021)

Fungi	Soil habitat	Pigment	Colour	Activity and uses	Reference
Mucorbridgeri	Eucalyptus tree plantation in Lucknow, India	Melanin	Black	Antioxidant activity	Kumar *et al.,* 2011
Mucorniger	Women's Christian College premises, Chennai India	Aspergillin	Black Brown	Antimicrobial activity, Textile dyeing	Anchanadevi, 2014
Mucorsclerotiorum	Amazonia Brazil	Neoaspergillic acid	Yellow	Antibacterial Activity	Teixeira *et al.,* 2012
Mucor versicolor	Mud of the South China Sea, China	Asperversin	Yellow	Antifungal activity	Li *et al.,* 2018
Fusarium oxysporum	Not specified	Anthraquinone	Pink/ violet	Antibacterial Activity, Textile dyeing	Gessler *et al.,* 2013
Fusarium verticillioides	Chiang Mai, Thailand.	Naphthoqu-inone	Yellow	Antibacterial activity	Boonyapranai *et al.,* 2008
Isariafarinose	Nilgris region of Western Ghats of Tamil Nadu, India	Anthraquinone	Red	Textile dyeing	Velmurugan *et al.* 2010b
Penicillium herquei	Not specified	Atronenetin	Yellow	Antioxidant activity	Takahashi and Carvalho, 2010
Penicillium oxalicum	Not specified	Anthraquinone	Red	Anticancer effect in food and pharmaceuticals Textile Dyeing	Atalla *et al.,* 2011
Penicillium sclerotiorum	Amazon soil, Brazil	Sclerotiorin	Yellow to orange	Antimicrobial activity	Celestino *et al.,* 2014

different components of *Tagetes* sp. was based on our understanding of *inducible mechanism* of indigenous microbes which exhibit a property of *natural attenuation* exhibiting *gratuitous metabolism* (Sharma *et al.*, 2019). During our course of study, we found petals segregated from *Tagetes* sp. exerted an inducible effect on pigment production. The same has been reflected in our previous studies conducted on fungal laccases which exerted an impact on bio-decolorization of textile effluents (Sharma *et al.*, 2017). Enrichment technique has been used to derive pigments from marine fungi like *Penicillium* sp., *Talaromyces* sp., *Fusarium* sp., *Mucor* sp., *Trichoderma* sp. *Dreschlera* sp., and *Paecilomyces* sp. for applications in textiles, healthcare and agriculture (Duffosse *et al.*, 2014).

Characterization of screened fungal isolates

Fungi are well documented among the widely recognized pigment producing microorganisms and provide an alternative to synthetic colorants (Hoeksma *et al.*, 2019). Primarily, we had 3 fungal isolates qualitatively screened from two samples FW_X and FW_Y Based on morphological, colony and microscopic investigations, they belonged to genera *Penicillium* sp., *Mucor* sp. in line with mycological investigations studies conducted in Western Ghats. Microbes have advantages of versatility and productivity over higher forms of life in the industrial-scale production of natural pigments and dyes. Fungi contain several anthraquinone compounds and pigments such as delphinidin, melanin and volatile organic compounds (VOC's) which have been identified as their secondary metabolites (Saravanan and Radhakrishnan, 2016). In nature, pigments are synthesized in microorganisms as secondary metabolites and are not often found in all types of microorganisms. Bio-prospecting and screen out 15 selected ascomycetes fungal strains, originating from terrestrial and marine habitats belonging to seven different genera (*Penicillium*, *Talaromyces*, *Fusarium*, Mucor, *Trichoderma*, *Dreschlera*, and *Paecilomyces*) (Lebeau *et al.*, 2017).

Solid State Fermentation (SSF)

SSF is suitable for low-moisture fermentation procedures using fungus and microorganisms.

Washed and dried petals (50 gm) obtained from *Tagetes* sp. were used as a substrate for SSF. Under aseptic conditions, a loop ful of pure culture each of *Penicillium* sp., *Mucor* sp. (actively growing mycelial mesh) were inoculated in round bottom flask (250 ml) as mono-culture. This was followed by incubation under controlled physiological conditions for optimal pigment production. Briefly, growth conditions were optimizedas follows:

- Aeration: Static
- Temperature: Room Temperature
- pH: 7
- Duration: 5-7 days

Until recently, fungi have emerged as producers of natural pigments., five filamentous fungi; *Penicillium multicolour, P. canescens, P. herquie, Talaromyces verruculosus and Fusarium solani* isolated from soil primarily with orange, green, yellow, red and brown pigments, respectively, when cultured on a mixture of green waste and whey were tested. The culture media with varying pH (4.0, 7.0 and 9.0) were incubated at 25 °C for 14 days under submerged and solid-state fermentation conditions. Optimal conditions for pigment production were recorded at pH 7.0 and 9.0 while lower biomass and pigment intensities were observed at pH 4.0 (Molelekoa *et al.*, 2021).

Submerged Fermentation (SSF)

Molasses and broths are examples of free-flowing liquid substrates used by SmF. Another advantage of this method is that product purification is simplified. SmF is largely employed in the liquid extraction of secondary metabolites.

Penicillium sp. and *Mucor* sp. were progressed for SmF aiming at enhanced pigment production. Quantitatively, an approximation close to 7 ml of crude pigment was obtained from 50 gm of pre seeded petal waste for both the fungal isolates after 7 days of prospective SSF. Briefly, 250 ml Potato Dextrose Broth (PDB) per strain was supplemented with 7 ml of pigment extracted from SSF for both strains in addition to loopful of actively growing mycelial mesh of respective fungal strains. Two main process parameters of aeration and pH were optimized keeping other physiological parameters same as that of SSF. This was in harmony with findings of Pratap *et al.*, (2017) who consistently highlighted the significance of using green whey as an important substrate. Fungal (SmF) is carried out on complex heterogeneous media such as whole-wheat flour, wheat bran, soybean hulls and rape seed meal, which are constituted of various soluble and insoluble solid particles. The estimation of the course of fungal growth during such fermentation, conducted using either SmF or SSF, is hindered by the presence of insoluble particles (Scotti *et al.,* 2001).

Antimicrobial activity of selected pigment against screened bacterial isolates (Based on qualitative screening) by Disc Diffusion Assay

Fungal pigments are stable at various temperatures. Pigments from *Monascus purpureus, Isaria* sp., *Emericella* sp., *Fusarium* sp., and *Penicillium* sp. used for the dyeing pre-tanned leather samples that were found stable at high temperatures (Velmurugan *et al.,* 2010a). Briefly, our experimental set up comprised of three set of experiments including two control groups (positive and negative as an indicator organism to be tested for anti-microbial (bacterial) efficacy of crude pigment extracted from *Mucor* sp. namely $CRPG_A$ and *Penicillium* sp. $CRPG_F$B *Bacillus* sp. is a ubiquitous in nature which almost occupies every possible ecological niche (Sharma and Dwivedi, 2017). Through disc diffusion assay, MIC was calculated to preliminarily validate anti-microbial efficacy of fungal derived pigments. For $CRPG_A$, an inhibitory effect on *Bacillus* sp. was found to be significant ($p<0.05$) with respect to both controls. In line with MIC calculation, $CRPG_A$ exhibited a promising anti-bacterial activity (17 mm) Likewise; $CRPG_F$ also exhibited bi-efficacious anti-bacterial activity (13 mm) corroborating to significant findings with respect to both positive and negative control. Positive controls except Amoxycillin, Cephalosporin and Vancomycin exhibited a resistant pattern, indicating to *Antibiotic Resistance.* Antibiotic resistance is an emerging public health challenge which is attributed to antibiotic misuse, over-use and prescription related errors (Sharma and Sharma, 2021). A study was aimed to assess antibacterial activity by disc diffusion method (Saravanan and Radhakrishnan, 2016). Ethyl acetate extract of strain MF5 showed maximum of 22 and 17 mm of zone of inhibition against *Bacillus* sp., and *Staphylococcus aureus* respectively. The ethyl extracts showed activity against test pathogens; whereas other solvent doesn't show any activity. The pigment compound was extracted by ethyl acetate and activity was tested by disc diffusion method.

CONCLUSION

Environmental degradation is a major threat confronting the world. The rapid increase in the volume of waste is one of the major phenomena leading to environmental crisis. Management of solid waste is one of the biggest problems that we are facing today. The safe and environmentally harmonious management of solid wastes becomes a major issue in many cities of developing nations. Enormous production of solid wastes coupled with poor management system, results in a significant environmental degradation. In India at most of the religious places a huge tonnage of solid waste is generated largely during functions, worships, ceremonies and festivals.

With sound planning, future environmental degradation due to improper management of waste can be prevented. Solid Waste Management is one of the essential services provided by municipal authorities in different states of our country. The proper disposal of solid waste derived from any source depends on management practices.

Recycling of wastes should be given priority in waste management practices and land disposal should be avoided as far as possible. Recycling and reuse of solid waste helps to reduce the problem of waste disposal. As we intend to maximize the efficiency of resources to meet our growing needs, let us not neglect the fact that waste products have magnificent potential waiting to be harnessed. Our preliminary study concluded utilization of floral waste through biotechnological interventions, seemingly important to attain environmental sustainability and establishment of a value proposition model for different industrial applications including mainstream healthcare as reflected in our findings based on anti-microbial assays. Additionally, industries such as textile where dyeing and printing are the mainstream users of synthetic dyes.

That being said, fungal derived pigments necessarily project an immense potential in textile and leather printing clusters. Apart from these mainstream industrial applications, value added products of agricultural importance like bio-compost, bio-pesticides and bio-based inoculums could also be explored. Handmade paper industry also finds an immense usage of floral trash through tools of biopulping utilizing bacterial and fungal laccases, peroxidases and other redox enzymes of industrial significance. We, hereby believe, our pilot study leaves behind an immense potential to take it forward in terms of technology transfer and establish a sustained circular economy model. Floral waste utilization would eventually be beneficial to the society as people would get to live in a cleaner and a healthier environment. The "green temple concept" can prove to be helpful in Government policy formulation for waste management and in promoting sustainable development approach towards temples.

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Conflict of Interest

Authors declare none competing interests

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