Extraction and optimisation of red pigment production as secondary metabolites from *Talaromyces verruculosus* and its potential use in textile industries

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**ABSTRACT**

Textile dyes and effluents are considered as one of the worst polluters of our priceless water sources and soils. New sources of natural pigments are getting particular research interests due to the toxicity produced by synthetic colouring agents. Plant sources are being explored extensively for natural pigments but inadequate yield of those sources hampered the progression. Apart from the enormous antibacterial applications, fungi may provide a readily available alternative source of natural pigments. Here, we isolated a fungal strain from spoiled mango which is capable of producing pigments suitable for textile dyeing. The spoiled mangoes were selected as a source of different fungi. Among them one particular fungal isolate was selected for its visible production of secondary metabolites. Molecular identification using internal transcribed spacer sequencing revealed the fungi as *Talaromyces verruculosus* strain. The growth and pigment production of the fungi was optimised to obtain highest yield. Extracted pigment was applied to cotton fabric following a standard dyeing procedure for natural pigment. Adequate colour yield and negative cytotoxicity result suggested that the fungi source of pigment could be a potential replacement for hazardous synthetic dyes.

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*Talaromyces verruculosus*; secondary metabolite; fungal pigment; ITS sequencing; textile dye

1. Introduction

Secondary metabolites are structurally heterogeneous low-molecular-mass molecules that are not essentially required to ensure growth of the particular organism that produces them. A lot of microorganisms produce a variety of secondary metabolites including carotenoids like soil dwelling bacteria and fungi (Berdy 2005). It is obvious that evolution of these secondary metabolites over hundreds of millions of years occurred because microorganisms used them as chemical signals for communication, to defend their habitat or to inhibit the growth of competitors (Brakhage 2011). Fungi produce a large number of secondary metabolites, which have roles in a range of cellular processes such as transcription, development and intercellular communication. In addition, many of these compounds have been found to have important applications, for instance, as antibiotics or immunosuppressant (Brakhage 2013), food colourant, etc. The secondary metabolites from fungi found their applications as anti-rejection drugs, enzyme activity blocking agent, cholesterol lowering agent, prebiotics, food additives replacing sugar, toxins, antineoplastic drugs, anti-cancer drug (Somjaipeng et al. 2015), antimicrobials, production of organic acids (Roberts et al. 2016), proteins, vitamins, biofuels, dye effluent treatment (Porri et al. 2011) and in pigment production (Arai et al. 2015; Demain & Martens 2016). Carotenoids obtained from several unique fungi species have obtained special attention as colourants considering their advancements towards commercialisation (Schweiggert & Carle 2016). Pigments from fungi could be considered as an important alternative of traditional synthetic dyes and pigments which may fall in the broad classification of carotenoids, as the concern has become high for their harmful effects like hampering the environmental and human health (Khan et al. 2013). Specifically, textile industry that uses a lot of dyestuff, along with the effluents of different process, is polluting our soil and water sources. They are also listed as mutagenic, carcinogenic, allergic and cytotoxic agents posing threats to all life forms (Khandare & Govindwar 2015). Recent research trends which are focused on pigments for textiles reveal significant advancements. Archana Devi (2014) isolated *Trichoderma* sp. and *Aspergillus* sp.
from soil samples and grown in Potato Dextrose Broth (PDB) for production of pigment. Cotton and silk fabrics were dyed and cotton showed better results (Anchana Devi 2014). Pigment extracted from *Sclerotinia* sp. was used to dye cotton yarn for different shades, which were pretreated with different mordants (Perumal et al. 2009). Water-soluble and salt-stable red pigment was extracted from *Isaria farinose*, which was suitable for textile applications (Velmurugan et al. 2010a). A comprehensive review mentioned several fungi pigments of different colours which have potential textile colouration applications (Panesar et al. 2015). In addition, there are reports that several coloured pigments are produced in different fungi species including *Monascus purpureus*, *Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides*, *purpureus*, *Isaria spp.*, *Emericella spp.* and *Fusarium spp.* (Velmurugan et al. 2010b, 2010c). Soil and other organic sources have been the most common source for separating the fungal strains for producing pigments (Dos Reis Celestino et al. 2014). In this subcontinent, mango could be a potential source of fungus as it is prone to spoilage (Singh et al. 2000) and several varieties of fungi could be found in or on the surface of post-harvested spoiled mangoes (Pernezny & Simone 2000). In the current project, we have isolated and identified a fungal strain from the spoiled tissue of mango which produces red pigment. We have optimised the growth condition as well as optimum environment for pigment production from this fungus. We also have designed an approach for their optimal extraction and application in cotton fabric colouration.

2. Materials and methods

2.1 Chemicals

For culture and different experimental test, potato dextrose agar (PDA) media were obtained from HiMedia Laboratories Pvt. Ltd, India. For molecular identification, TIANamp Genomic DNA Kit (TIANGEN Biotech Beijing Co. Ltd), TIANquick Midi Purification Kit (TIANGEN Biotech Beijing Co. Ltd) and BigDye terminator version 3.1 cycle sequencing kit were used. Cycle sequencing was carried out in an ABI Prism DNA sequencer. All other chemicals and reagents used in the current studies were of high quality.

2.2 Sampling and fungi isolation

For isolation of fungal strain associated with mango spoilage, different varieties of ripe mangoes such as Mollica, Langra, Gopalvog, Lokkhonvog, Alphonso, Lokhna, Totapuri, etc. were collected from the local market and Fruit Research Centre, Rajshahi, Bangladesh. Collected mangos were allowed to spoil at room temperature. PDA media were used for isolation and pure culture of fungi. Mycelia from pure cultures were examined under Optika digital microscopes (Italy) and identified by comparing their morphological and culture characteristics (Barnett & Hunter 1998; Hamd et al. 2013). From the identified fungal isolates, one fungal strain was selected which causes Black rot diseases in ripe mango. The selection was done on the basis that it was producing red pigments. Subculture of the fungi was performed on PDA media and was incubated at 28 ±2°C for 7 days (Figure 1).

2.3 Molecular identification of selected fungi

The fungal isolate was grown in 250-ml culture flasks containing 50 ml of the PDB media. Each flask was inoculated with 5-mm-diameter plug excised from a 7-day-old colony and incubated for 7 days at 28 ±2°C at pH 6.5 to obtain mycelium for DNA extraction. Fungal DNA isolation was carried out using TIANamp Genomic DNA Kit (TIANGEN Biotech Beijing Co. Ltd). The DNA quality was determined...
using agarose gel electrophoresis and spectrophotometrically. Polymerase chain reaction (PCR) product was purified using TIANquick Midi Purification Kit (TIANGEN Biotech Beijing Co. Ltd). Purified PCR product was subjected to gene sequencing using chain termination method using BigDye terminator version 3.1 cycle sequencing kit (Clark & Pazdernik 2013) for confirmed identification with internal transcribed spacer (ITS) primer pair ITS-1 as a forward (5’ TCCGTAGGTGAACCTGCGG 3’) and ITS-4 as a reverse (5’ TCTCCGCTTATTGATATG 3’). The sequence was submitted to GenBank.

2.4. Growth profiling of selected fungal isolate

To check the effect of pH on fungal growth, PDA medium was adjusted with pH 5.0, 6.5, 7.0, 8.0 and 10.0 using hydrochloric acid and sodium hydroxide. After 7 days of incubation of fungi at 28 ±2°C, the plates were observed and dry weights (g/25 ml) of mycelium were measured.

To check the effect of different temperature on the growth of fungi, petri dishes of PDA medium were prepared in sterilised condition and 5-mm-diameter plug was excised from a 7-day-old colony of both fungal isolates and put on the medium of each petri dish. The petri dishes were then wrapped with parafilm and incubated in incubator at temperatures 5°C, 15°C, 30°C, 35°C and 40°C for 7 days. After 7 days of incubation of fungi, the plates were observed and radial growth rates were measured.

About 2% different carbohydrates such as sucrose, fructose, glucose, maltose and starch were added as sole carbon source to the medium instead of dextrose to check the effect of different carbohydrates on the growth of fungi and a control of dextrose was used for comparison. All the mediums were adjusted to pH 6.5. After 7 days of incubation of fungi, the plates were observed and radial growth rates were measured.

To check the effect of organic acids on the growth of fungi, two different organic acids such as citric acid and malic acid were used. For this experiment, potato dextrose liquid medium was prepared and was supplemented with 0.25 %, 0.5%, 1% and 2% (w/v) organic acids A control of potato dextrose liquid medium was used for comparison. All the mediums were adjusted to pH 6.5. After 7 days of incubation, dry weights (g/100 ml) of fungal mycelium were recorded.

The effect of salt on the growth of fungi was observed by adding salt to the PDA media. About 0.5%, 1%, 2%, 4% and 6% (w/v) NaCl were added to the PDA media. All the media were adjusted to pH 6.5. After the incubation period of 7 days, the growth rate was recorded.

2.5. Optimisation of pigment production

To check the effect of pH on the production of pigment, potato dextrose liquid medium was adjusted with pH 5.0, 6.5, 7.0, 8.0 and 10.0 using hydrochloric acid and sodium hydroxide. After sterilisation when the medium became cool in laminar air hood, 5-mm-diameter plug from a 7-day-old colony of fungal isolate was put into the medium of each culture flasks and incubated in an incubator at temperature 28 ±2°C for 14 days. Subsequently, the optical densities of the culture filtrates were measured at 520 nm to know the optimum pH for the production of pigment.

Seven-day-old colony of fungal isolate was taken into the medium of each culture flask and incubated at different temperatures, viz. 5°C, 15°C, 30°C, 35°C and 40°C, for 14 days. The optical density measurement reveals the effect of temperature on pigment production.

Similar approach was followed to observe the effect of different carbohydrates on pigment production as mentioned in Section 2.4. The incubation period was 14 days and the optical density was measured for the interpretation of result.

About 0.25%, 0.5%, 1% and 2% (w/v) organic acids were added to the media containing pigmenting fungi. After 14 days of incubation period, optical density of culture filtrates was measured at 520 nm to find out the effect of organic acids on the production of pigment.

About 0.5%, 1%, 2%, 4% and 6% (w/v) NaCl were added to the media, keeping all other parameters similar to the above-mentioned experiment and then measuring the optical density to know the effect of salt for pigment production by the fungal strain.

2.6. Pigment extraction and dyeing

The fungal isolate was cultured in three different culture flasks. Each culture flask contained 100 ml of liquid culture media. The culture flasks were
incubated in an incubator at temperature 28 ±2°C for 21 days. Then the pigment was extracted. The extraction process of pigment was done in three different ways such as follows:

1. By filtering the fungal culture broth media showing colour.
2. By boiling only the mycelium that was growth in PD broth along with 100 ml distilled water at 98°C and then filtered it.
3. By boiling both the mycelium and the fungal culture broth media at 98°C showing colour together and then filtered.

Upon extraction, the optical density of pigment was measured by spectrophotometry at 520 nm for each process to know which one is better to get more concentrated pigment.

Prior to use the pigment for dyeing fabric, the pigment solution was filtered through Whatmann filter paper no: 4. Cotton fabric, both scoured (textile industry grade) and unsoured (finished, procured from local market), was subjected to dyeing. Ferrous sulphate was used as a mordanting chemical. For this, a set of cotton clothes weighting 1 g was used. About 50 ml of red colour pigment was taken in a beaker and 5% of ferrous sulphate was added into it. Then cloth was immersed into it. The dyeing was carried out for overnight at room temperature. The sample was then washed with cold water and dried in sunlight.

To analyse the fabric properties, percentage absorption of the dyed fabrics was calculated on UV spectrophotometer at 520 nm and the percentage was determined for both fabrics separately using the following formula:

\[
\text{Percentage absorption} \% = \frac{\text{OD before dyeing} - \text{OD after dyeing}}{\text{OD before dyeing}}
\]

2.7. **HPLC analysis of extracted pigment**

Extracted pigment samples were purified and run in Ultimate™ 3000 HPLC, following the method mentioned in a similar research article (Bhardwaj et al. 2007). As Liquid chromatography–mass spectrometry (LC-MS) method is required to have an idea about the chemical structure and MS attachment was not available; the obtained LC curve was compared with some similar coloured natural pigment HPLC analysis.

2.8. **Cytotoxicity test**

Recent research result showed that metabolites of fungus often possess cytotoxic activities against cancer cells and considerably less toxicity against normal living cells (Shah et al. 2014; Hao et al. 2016). However, the cytotoxicity of the pigment produced by this fungus is yet to be tested. Therefore, to ensure safe use for dyeing textile, cytotoxicity test of the red pigment extracted from our target fungus was carried out using the standard procedure (Meyer et al. 1982).

3. **Results and discussion**

3.1. **Identification of fungi**

*Talaromyces verruculosus* was identified under microscope which caused black rot disease of the mango. The colony was characterised by greenish grey colour with white coloured border. The conidiophores (Figure 2) were branched and the metulae and phialides extended from these branches. Conidia were globose (round) to sub-globose (somewhat off-round) in shape.
3.1.1. Molecular identification using ITS sequencing

For molecular identification, ITS region of the fungus was amplified from the genomic DNA using ITS-1 and ITS-4 primers. PCR yielded a ~550-bp product (Figure 3). PCR produce was purified using TIANquick Midi Purification Kit and subjected to DNA sequencing in a ABI Prism platform using Big Dye Terminator version 3.1. To recognise the identity of the strain from ITS sequencing, sequence search was performed using the BLAST standard nucleotide-nucleotide basic local alignment search tool (National Centre for Biotechnology Information sequence-based identification). Greater than 98% homology with the referenced culture was required to confirm the preliminary identification of the test sequence and we found that the nucleotide sequences of fungal isolate had 99% similarity with the reference strain Talaromyces verruculosus previously submitted to the GenBank database (Sequence ID gb|KM278044.1). So our sequencing result confirms the isolated fungal strain as Talaromyces verruculosus. The sequence was deposited to GenBank as MGBAN036 and an accession number was provided (KX268678).

3.2. Growth profiling of T. verruculosus

The optimum pH for the mycelial growth of T. verruculosus was found to be 6.5 which was, however, significantly different from the reported standard pH of 3.5 for the said fungal strain (Goyari et al. 2014). The probable reason was that the result was noted without considering the effect of head-space oxygen content, which can change the growth rate significantly (Samapundo et al. 2007). The fungi grew best when the temperature was maintained at 30°C, which is supported by literature (Goyari et al. 2014). Dextrose was also found to be the most suitable carbohydrate source for the fungi (Devi et al. 2014). As the fungi is able to grow well in acidic conditions (Samapundo et al. 2007), there was no significant change in growth rate inhibition, which was found by applying malic and citric acid (Figure 4).

It has been proved that when cells are exposed to stress such as high temperature, high osmotic pressure, metabolic inhibition, presence of heavy metal and viral infection, production of metabolites such as amino acids by fermentation is affected. The productivity of fermentative products is adjusted automatically, in order to decrease the influence of stress. This reisolates growth of microorganisms and/or production of fermentative products so that the productivity and the yield are improved instead of being lowered (Kawahara et al. 2002). NaCl has the ability to apply stress to the growth of fungi and we found that the increasing concentration of NaCl had a great inhibitory effect on the growth of Talaromyces verruculosus. From the result, it was observed that the concentration of 6% NaCl can inhibit 75% of growth of T. verruculosus.

3.3. Optimisation of pigment production

The maximum yield of the pigment was obtained after 24 days of the incubation. The concentration of the pigment in the liquid media released by the fungi was measured by optical density at 520 nm. The optimum pH was found to be 7.0; which is close to reported one (7-7.5) (Soto-Cruz et al. 2008). Maximum optical density was observed at 30°C, which supports previous research (Babitha et al. 2007). When sucrose was used, the pigment
concentration became maximum. This confirms the research; explaining, how the increment in carbon source enhances pigment production (Arumugam et al. 2015) (Figure 5).

Adding acids interfere with the optimum pH for pigmentation (Soto-Cruz et al. 2008), so the pigment production was reduced as the dosage of malic and citric acid was increased. The pigment production was also hampered due to stress induced by NaCl, which resulted in reduced optical density of pigment in a dose dependent manner.

### 3.4. Pigment extraction and dyeing of fabric

We obtained the highest concentration of pigment in media by boiling mycelia of T. verruculosus and the media in which pigment produced by T. verruculosus is diffused together. The method was in compliance with the previous research works (Velmuirugan et al. 2010b; Sharmala et al. 2012).

Dyeing method was selected on the basis that natural dyes/pigments perform better along with a mordant (Singh & Bharati 2014). Good colour yield was found by using ferrous sulphate as mordant (Sharmala et al. 2012). The un-scoured fabric showed brighter shade than the scoured one (Figure 6).

Duller shade was observed due to the fact that scoured fabric retains some residual NaOH and surfactants (Broadbent 2001). The pigment might be susceptible to alkaline condition, which can alter the original hue (Boerema et al. 2004).

### 3.5. Chromatography analysis of extracted pigment

The peaks obtained in the chromatogram (Figure 7) shows probability of the presence of several impurities. The comparison of the absorbance corresponding to retention time with similar kind of pigments (Cserháti 2006) does not result in any concluding remarks. Further analysis of the pigment should be done using LC-MS, Fourier transform infrared spectroscopy and Nuclear magnetic resonance for confirmation of the chemical structure.
3.6. Cytotoxicity test

To check whether the pigment or dye from *T. verruculosus* is safe to use, brine shrimp lethality bioassay was carried out (Figure 8). The LD$_{50}$ values obtained from brine shrimp lethality bioassay was 56.69 μg/ml for the red coloured pigment produced by *T. verruculosus*. Compared to control (ampicillin trihydrate, LD$_{50}$ 7.21 μg/ml), our pigment showed that the toxicity level is negligible and safer to use. The cytotoxic activity exhibited by the solvent fractions was promising and this clearly indicates that the metabolite is different from the mentioned reference work. Considering the result, it can be recommended for textile application.

**Figure 5.** Effects of different physical and chemical parameters on pigment production by *T. verruculosus*. (A) The effect of pH on the production of pigment by *Talaromyces verruculosus*. (B) The effect of temperature on the production of pigment by *Talaromyces verruculosus*. (C) The effect of carbohydrates on the production of pigment by *Talaromyces verruculosus*. (D) The effect of organic acids on the production of pigment by *Talaromyces verruculosus*. (E) The effect of salt (NaCl) on the production of pigment by *Talaromyces verruculosus*.

**Figure 6.** Cotton fabric dyed using pigment from *T. verruculosus* culture. (A) Scoured. (B) Unscoured. The latter one exhibited better result but duller hue due to the unscoured yellowness of cotton (Broadbent 2001).
4. Conclusion

In this research work, an interesting secondary metabolite from fungal strain *Talaromyces verruculosus* visualised as a red pigment was successfully extracted and used to dye cotton fabric. Cytotoxicity assay using brine shrimp lethality bioassay reveals that it has negligible toxicity and hence safe to use. Further research to obtain commercial colourants from this particular fungal strain and chemical characterisation will open up new avenue in dyeing industry which will eventually help minimising the adverse effects of using synthetic dyes and pigments.

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