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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Optimization and characterization of red pigment production from an endophytic fungus, *Nigrospora aurantiaca* CMU-ZY2045, and its potential source of natural dye for use in textile dyeing

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Abstract

Some of the most important natural pigments have been produced from fungi and used for coloring in food, cosmetics, textiles, and pharmaceutical products. Forty-seven isolates of endophytic fungi were isolated from *Cinnamomum zeylanicum* in northern Thailand. Only one isolate, CMU-ZY2045, produced an extracellularly red pigment. This isolate was identified as *Nigrospora aurantiaca* based on morphological characteristics and the molecular phylogenetic analysis of a combined four loci (large subunit and internal transcribed spacer of ribosomal DNA, β-tubulin, and translation elongation factor 1-alpha genes). The optimum conditions for red pigment production from this fungus were investigated. The results indicated that the highest red pigment yield was observed in the liquid medium containing glucose as a carbon source and yeast extract as a nitrogen source, at a pH value of 5.0 and at 27 °C with shaking for 5 days. The crude red pigment revealed the highest level of solubility in methanol. A fungal red pigment was found to have high stability at temperatures ranging from 20 to 50 °C and pH values at a range of 5.0–6.0. Based on liquid chromatography-mass spectrometry analyses, the red pigment was characterized as bostrycin. The extracted pigment was used for the textile dyeing process. Crude fungal red pigment revealed the highest staining ability in cotton fabrics and displayed excellent fastness to washing, which showing negative cytotoxicity at the concentrations used to cell culture. This is the first report on bostrycin production from *N. aurantiaca*.

Keywords Bostrycin · Fungal pigment · Solubility · Stability · Textile dye

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Introduction

Pigments are used for coloring in food, cosmetics, textiles, and pharmaceutical products and can attract the attention of consumers to specific products (Kumar et al. 2015). Nowadays, consumer concern has increased regarding the potential long-term toxicity of synthetic pigments in food processing, cosmetics, pharmaceuticals, and the textile industries due to their carcinogenicity, hyperallergenicity and other potential toxicological problems (Osman et al. 2004; Unagul et al. 2005). Hence, the interest in natural pigments derived from plants and microorganisms continues to increase and many research efforts have been made to replace synthetic pigments with natural pigments (Akilandeswari and Pradeep 2016; Malik et al. 2012). Microbial pigments are advantageous over plants in term of their high availability, stability and yield, low residues, and easy harvesting

(Durán et al. 2002; Joshi et al. 2003; Venil and Lakshmanaperumalsamy 2009). Microorganisms including many types of bacteria, fungi, algae, and protozoa are recognized as potential sources for various pigments, e.g., carotenoids, flavins, melamins, quinines, and more specifically monascin, violacein, phycocyanin, or indigo (Dufossé et al. 2005; Torres et al. 2016). Many microbial pigments not only act as coloring agents, but also possess antioxidant, anti-inflammation, and antimicrobial activities (Venil and Lakshmanaperumalsamy 2009). However, the limitations of high-yield production of microbial pigments on a large-scale can be affected by various microbial strains, the fermentation process, cell growth, and nutrition content (Dufossé et al. 2014; Joshi et al. 2003; Panesar et al. 2015). To defeat these constraints, selection of the appropriate strain, mutations or genetic engineering techniques, and the selection of the appropriate fermentation process can all help to significantly improve pigment production yields, as well as the selection of suitable substrates for cultivation (Arumugam et al. 2014; Mapari et al. 2005).

Filamentous fungi have been identified as potential pigment-producing microorganisms (Babitha et al. 2007). Many fungi in the genera *Alternaria*, *Aspergillus*, *Ashbya*, *Blaskeslea*, *Curvularia*, *Epicoccum*, *Fusarium*, *Monascus*, *Nigrospora*, *Penicillium*, and *Trichoderma* have been reported as pigment producers (Akilandeswari and Pradeep 2016; Dufossé et al. 2014; Sharma et al. 2012; Torres et al. 2016). Generally, fungal pigments have been used as food colorants, e.g., ankaflavin, monascin, monascorubin, and rubropunctatin, from the *Monascus* species, Arpinkred™ (anthrachinone-type) from *Penicillium oxalicum*, β-carotene, and lycopene from *Blakeslea trispora*, and riboflavin from *Ashbya gossypii* (Dufossé et al. 2014). The application of fungal pigments in the dyeing of cotton, silk, and wool has been evaluated (Chadni et al. 2017; Mabrouk et al. 2011; Nagia and El-Mohamedy 2007; Perumal et al. 2009; Sharma et al. 2012; Velmurugan et al. 2010; Hinsch et al. 2015; Palomino et al. 2017). In this study, endophytic fungi were isolated from cinnamon plants, and the pigment-producing strains were screened. The appropriate pigment-producing strain was selected and the optimal culture conditions (liquid culture medium, temperature, pH, carbon and nitrogen sources, and cultivation period) for pigment production were determined. The values associated with the solubility, stability, and cytotoxicity of the crude pigment were evaluated. The fungal pigment was purified and identified. Moreover, the crude pigment extract was applied for multifiber dyeing. Therefore, the knowledge acquired from this study could lead to the development of pigments production from endophytic fungi as textile colorants, which may replace the toxic colorants that are currently being used.

Materials and methods

Fungal isolation

Endophytic fungi were isolated from healthy mature leaves and stems of cinnamon plants (*Cinnamomum zeylanicum* L.) collected from the University of Phayao, Phayao Province, Thailand, in February 2008. The cinnamon leaves and stems were washed in running tap water for 15 min and cut into small pieces (5 mm^2 leaf and 1 mm length stem). The cut samples were triple surface-sterilized (70% ethanol for 1 min, 2% sodium hypochlorite for 3 min and 95% ethanol for 30 s) under a laminar flow hood. Twenty-five leaf and stem pieces were placed on potato dextrose agar (PDA) supplemented with 50 ppm of chloramphenicol and 35 ppm of Rose Bengal. The plates were incubated at 25 °C in darkness. The fungi growing out from the tissue samples were transferred to fresh PDA plates. The pure cultures were preserved on PDA slants for the short-term and 20% glycerol at − 20 °C for the long-term preservation.

Identification of pigment-producing endophytic fungus

Morphological studies Conventional morphological characters were used to tentatively identify the selected pigment-producing endophytic fungi. Colony characteristics including aerial mycelium, density and pigment production were recorded. Micromorphological characteristics were examined using a light microscope (Olympus CX51, Japan). Size data of the anatomical features are based on at least 50 measurements of each structure.

Molecular studies Molecular techniques were used to confirm the identification of pigment-producing endophytic fungi. Genomic DNA was extracted from 1-week-old fungal mycelia on PDA (1–5 mg) using a DNA Extraction Mini Kit (FAVORGEN, Taiwan) following the manufacturer's protocol. The large subunit (LSU) and internal transcribed spacer (ITS) of ribosomal RNA, β-tubulin (TUB) and translation elongation factor 1-alpha (TEF1) genes were amplified with primers and annealing temperatures following the previous studies (Table 1). Polymerase chain reaction (PCR) was performed in 20 μL reaction containing 1.0 μL DNA template, 1.0 μL of each forward and reverse primers, 10.0 μL 2X Quick Taq HS DyeMix (TOYOBO, Japan), and 7 μL deionized water, and the following thermal conditions: 94 °C for 2 min, followed by 35 cycles of 94 °C for 2 min, the temperatures dependent on the amplified gene (Table 1) for 1 min and 72 °C for 1 min, and a final 72 °C for 10 min on a peqSTAR thermal cycler (PEQLAB Ltd., UK). PCR products were

Table 1 Details of primers, annealing temperature, and the obtained product size of the amplification of gene targets in this study

Region	Primer name	Orientation	Annealing temperature (°C)	Reference	Obtained product size (bp)
LSU	LROR	Forward	52	Vilgalys and Hester (1990)	888
	LR5	Reverse	52	Vilgalys and Hester (1990)	
ITS	ITS5	Forward	50	White et al. (1990)	556
	ITS4	Reverse	50	White et al. (1990)	
TUB	Bt-2a	Forward	52	Glass and Donaldson (1995)	402
	Bt-2b	Reverse	52	Glass and Donaldson (1995)	
TEF1	EF1-782F	Forward	56	Carbone and Kohn (1999)	414
	EF2	Reverse	56	O'Donnell et al. (1998)	

purified using a PCR clean up Gel Extraction NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The purified PCR products were directly sequenced. Sanger sequencing was carried out by 1ST Base Company (Kembangan, Malaysia) using the PCR primers mentioned above. Sequences were used to query GenBank via BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For phylogenetic analysis, the sequences obtained from this study and from previous studies along with sequences from GenBank database were used. The multiple sequence alignment was carried out using MUSCLE (Edgar 2004). A maximum likelihood (ML) phylogenetic tree was constructed using RAxML v7.0.3 (Stamatakis 2006), applying the rapid bootstrapping algorithm for 1000 replications using the GTRGAMMA model. *Arthrinium arundinis* and *Ar. sacchari* were used as the outgroup. The ML trees were viewed with TreeView32 (Page 2001). Clades with bootstrap values (BS) ≥ 70% were considered as significantly supported (Hillis and Bull 1993). Bayesian phylogenetic analyses were carried out using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method in MrBayes version 3.2 (Ronquist et al. 2012), under a GRT + I + G model. Markov chains were run for 1,000,000 generations, with six chains and random starting trees. The chains were sampled every 100 generations. Among these, the first 2000 trees were discarded as the burn-in phase of each analysis and the resulting trees were used to calculate Bayesian posterior probabilities. Bayesian posterior probabilities (PP) ≥ 0.95 were considered as significant support (Alfaro et al. 2003).

Optimization of fungal pigment production

Fungal cultivation and pigment yield estimation Three fungal mycelial plugs (5 mm in diameter) obtained from the periphery of the growing colony on PDA at 30 °C for 1 week were transferred into a 25-mL of liquid medium in each 125-mL Erlenmeyer flask after being autoclaved at 121 °C for 15 min. Cultivation was performed in the dark at 30 °C with shaking at 125 rpm on a reciprocal shaker. After incubation, the cultures were centrifuged at 11,000 rpm for 15 min to harvest the supernatant. The fungal mycelia were dried at 30 °C for 48 h and maintained in desiccators for 20 min before being weighed. Only the secreting pigment was considered in this study. Analysis of pigment production was carried out by measurement of the maximum level of absorbance (λ_{max}) of pigments using a spectrophotometer (BOEGO spectrophotometer model S-220 UV/VIS, Germany). Pigment yield in the supernatant was estimated following the method described by Tseng et al. (2000) in which the wavelength of its absorbance maxima was expressed in absorbance unit (AU). Three replications were performed for each treatment.

Effect of culture liquid medium Five different liquid media were used in this experiment; potato dextrose broth (PDB; Conda®, Spain), Czapek Dox broth (CDB; Signma-Aldrich, USA), glucose yeast extract medium (GY; glucose10 g, yeast extract 2 g), malt extract medium (MM; malt extract 20 g), and oatmeal medium (Difco™, USA). In all media, the volume was adjusted to 1000 mL with distilled water and the pH was adjusted to 6.0 using 1 N HCl and 1 N NaOH. After being inoculated, the culture media were incubated in darkness with shaking at 30 °C for 1 week. The culture liquid medium that presented the highest yield of the pigment was selected for further experiments.

Effect of temperature In this experiment, a fungal culture was inculcated in the selected liquid medium that had been obtained from previous experiments. A pH value of 6.0 was achieved and the culture was incubated in darkness at 20, 25, 27, 30, 35, 40, and 45 °C for 1 week. The temperature that presented the highest yield of the pigment was selected for further experiments.

Effect of carbon and nitrogen sources In this experiment, GY medium was selected based on the highest amount of production yield of the pigment. For each treatment glucose was

replaced with alternative carbon sources; maltose, fructose, sucrose, soluble starch, carboxy methyl cellulose (CMC), lactose, and mannitol. Beef extract, peptone, malt extract, typtone, yeast extract, urea, $(NH_4)H_2PO_4$, and KNO_3 were assayed as the nitrogen source. The pH of all media was adjusted to 6.0 before being autoclaved. The inoculated flasks were incubated in darkness at a selected temperature. The carbon and nitrogen sources that presented the highest yield of the pigment were selected for further experiments.

Effect of initial of pH The initial pH of the selected suitable components of the medium that had been obtained from previous experiments was adjusted from 3.0 to 9.0 in each flask before being autoclaving. After inoculation, cultures were incubated at temperature that presented the highest pigment yield for 1 week. The initial pH of culture media that presented the highest yield of the pigment was selected for further experiments.

Effect of cultivation period The effect of the incubation period on fungal pigment production was determined. The suitable components of the medium and pH that had been obtained from previous experiments were used in this experiment. Cultivation was performed in darkness at a suitable temperature with shaking for 1 week. The fungal biomass was harvested and pigment yield was measured.

Fungal pigment fermentation and extraction

This experiment was conducted in 1000-mL Erlenmeyer flasks containing 300 mL of GY medium and at a pH of 5.0 under optimized conditions. Ten fungal mycelial plugs (5 mm in diameter) obtained from the periphery of the growing colony on PDA at 30 °C for 1 week were transferred into the medium after being autoclaved. The inoculation medium was incubated for up to 1 week at 27 °C and then shaken at 125 rpm on a reciprocal shaker. The same conditions were maintained throughout the experiment.

After fermentation, the culture media were filtrated through Whatman's No. 1 (Whatman, UK) filter paper. The culture filtrate was extracted using two volumes of ethyl acetate according to the method described by Arumugam et al. (2014). The ethyl acetate fraction was evaporated until dryness using a rotary evaporator (BUCHI Rotaryvapor™ model R-210/215, Switzerland). The crude extracts were kept at − 20 °C before being used in further experiments.

Characterization of crude fungal pigment

Solubility test The solubility of the pigment was evaluated using various types of solvents; distilled water, absolute methanol, 95% ethanol, 0.1 M HCl, 0.1 M NaOH, acetone, ethyl acetate, and 0.1 M alum. The solubility test was performed following the method described by Ahmad et al. (2012) with some modifications. Ten milliliters of the solvents were added to 15 × 150 mm test tubes containing 1 mg of the pigment extract. The solutions were mixed by vortex and kept in darkness at room temperature (25 ± 2 °C). After 12 h at room temperature, the λ_{max} of each solution was scanned (400–650 nm) by a spectrophotometer and the total pigment yield at 505 nm was recorded. The color names and codes of each solution were recorded using the Methuen handbook of color (Kornerup and Wanscher 1967). Three replications were performed for each treatment.

Stability test The stability of the fungal pigment toward pH, temperature, light, and being autoclaved was evaluated according to the methods of Velmurugan et al. (2010) and Ahmad et al. (2012) with some modifications. The pH value of the pigment concentration that was used was adjusted to between 4 and 11 by either 1 M NaOH or 1 M HCl at 0.1 mg/mL in methanol with a total volume of 10 mL in 15 × 150 mm test tubes. The effect of pH on the pigment's stability was recorded after the pH adjustment and the samples were incubated at room temperature for 2 h. With regard to the stability of the pigment solution, a pH value of 6.0 (the highest pigment yield) was incubated in a water bath at 20, 25, 30, 35, 40, 50, 60, and 80 °C for 5 h. The degree of stability under sunlight (11:00 am to 4:00 pm with a light intensity of 12,000 to 55,000 lx), UV light (Philips 15 W, 1 m distance), and fluorescent light (Philips 18 W, 1 m distance, light intensity 2000 lx) for 5 h accompanied by autoclave conditions was investigated. The λ_{max} of each treatment was scanned; the total pigment yield at 505 nm was recorded and compared with the control treatment. The stability percentage of pigment at 505 nm was calculated according to the following formula. The percentage of stability = (As / Ao) × 100, where As is the absorbance of pigment after treatment and Ao is the absorbance of pigment before treatment. Each treatment was performed in triplicate.

Fungal pigment purification

Sample preparation and column chromatographic separation The crude extract of the pigment was dissolved in absolute methanol and loaded into Sephadex™ LH-20 (GE Healthcare Bio-Science AB, Sweden) columns. Absolute methanol was used as an eluent. Column eluted fractions that were read through a spectrophotometer between 450 to 550 nm were pooled together. To obtain high purity of the compound, an additional preparative thin layer chromatography (TLC) step was incorporated. The pigment fraction was concentrated and applied to a preparative TLC plate (20 cm × 20 cm, thickness 0.25 mm, Silica gel G F254; Merk, Germany). The running solvent was a mixture of 2% methanol in dichloromethane. The target band of the red pigment was scrapped off, dissolved

in methanol, and separated from silica through filtrations with Whatman's No. 1 filter paper. The samples were concentrated and dried using a rotary evaporator.

Liquid chromatography-mass spectrometry analyses The purified pigment was analyzed using HPLC (LC-20 AD binary pump system, Shimadzu, Japan) coupled with high-resolution mass spectrometry (LTQ Orbitrap XL; Thermo Fisher Scientific, Waltham, MA). Four microliters of the pigment samples were injected into the L-Column2 ODS (2.1 × 150 mm, 3 μm; Chemicals Evaluation and Research Institute, Japan) and the column was heated to 40 °C. The gradient elution was applied. Solvent A consisted of 0.1% formic acid and solvent B consisted of acetonitrile containing 0.1% formic acid. The mobile phase began with A: B = 80: 20 to 20: 80 for 10 min at a flow rate of 0.2 mL/min. The MS data was obtained in ESI-positive and negative mode using the following parameters: source voltage, 4500 V (positive mode) and 4000 V (negative mode); capillary voltage, 10 V (positive mode) and − 20 V (negative mode); and capillary temperature of 400 °C. The MS spectrums in both the positive and negative modes were used to search for the possible structures as described below.

Identification of the purified fungal pigment was confirmed with a bostrycin standard (AdipoGen Life Science, USA) by LC/MS/MS analysis. LC/MS/MS was performed with 3200 QTRAP (AB Sciex, Framingham, MA) equipped with Prominence UFLC (Shimadzu, Japan). Four microliters of the pigment sample were injected to the Mightysil RP-18GP Aqua (150 × 2.0 mm, 5 μm) column heated at 40 °C. The gradient elution was applied as mentioned above. The retention time was determined and MS/MS data was obtained from ESI in the positive ion mode (ion spray voltage, 5000 V; temperature, 300 °C; N_2 as both curtain gas (set to 12 arbitrary units) and collision gas (set to "high"); collision energy, 30 V; scan range, m/z 100–1200; scan speed, 4000 Da/s; declustering potential, 10 V).

Searching of the structure of pigment by MetFrag MetFrag (http://msbi.ipb-halle.de/MetFrag/), a freely accessible web application, can automatically assign possible structures from a chemical compound database such as KEGG, PubChem, or ChemSpider (Wolf et al. 2010). The possible candidates were ranked by comparison of their in silico fragmentations against the measured MS fragments. The molecular formula and the fragmentation mass spectrum of the pigment were submitted to MetFrag. The search was performed for negative ions with an accuracy of 10 ppm.

Textile dyeing

Textile substrate The SDC Multifiber SLW (SDC Enterprises Ltd., UK) was used in this study. The multifiber substrate was in the form of a 10-cm wide wrap stripe comprised of a 25 mm wide band of silk, cellulose, cotton, nylon, polyester, acetate, and wool.

Dyeing procedure The dye solution was prepared by dissolving 5-mg pigment extract in 5 mL absolute methanol and adjusted to 50 mL by distilled water. The initial pH of the dye solution was adjusted to 6.0 using 1 N CH_3COOH and 1 N Na_2CO_3 for the color red. The dyeing process was done following the method of Kramar et al. (2014) and Sharma et al. (2012) with some modifications. Dyeing was carried out by shaking a multifiber substrate in 100-mL steel beakers containing a 50 mL of dye solution at 35 °C for 1 h. After dyeing, samples were thoroughly washed with warm distilled water (35 °C) to remove the unfixed pigment, then soaked in 0.1% $CuSO_4$ solution for 10 min and rinsed with tap water. The samples were air dried at room temperature. The color name and code of each sample was recorded. Five replications were made.

Characterization of dyed textile

Color fastness testing The color fastness of the dyed multifiber substrate with respect to washing was evaluated according to the standard ISO 105-C01 (1981) by washing the dyed samples in a water bath containing 0.5% (*w*/*v*) of standard detergent (SDC Enterprises Ltd., UK) at 40 °C for 30 min. After washing, samples were rinsed for 10 min and dried at 60 °C. The color name and code of each sample was recorded. The surface color of the samples was measured using a Hunter Lab Mini Scan XE Plus colorimeter (Hunter Association Laboratory, Inc., USA).The L* (lightness), a* (red/green), and b* (yellow/blue) values were measured in the CIELab color space. The differences in color value (ΔE*) were determined between the dyed samples and the samples that were subjected to the washing fastness test. The color name and code of the samples were recorded. Experiments involving each sample were performed in five replicates.

In vitro cytotoxic assay

In vitro cytotoxic assay was performed using the human embryonic kidney cell (HEK 293T). The MTT assay was used according to the methods described by Carmichael et al. (1987). Cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Thermoscientific, USA) until covering 70% of the T-25 flask area. The cell line was plated overnight in 96-well plates with 1×10^4 cells for each well. The crude red pigment was treated at various concentrations. After 48 h, 10 μL of 5 mg/mL MTT solution (Sigma-Aldrich, USA) was added to each well and incubated for 4 h. To dissolve the formazan crystals, 0.04 N HCl in isopropanol was added and the absorbance at 570 nm was

measured using a EZ Read 2000 microplate reader (Biochom, Germany). The percentage of cell viability and the concentration for 50% lethality (LC_{50}) were calculated.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) by SPSS program version 16.0 for Windows and Tukey's test was used for significant differences ($P < 0.05$) between treatments.

Results

Fungal isolation and identification of pigment-producing isolate

Twenty isolates were obtained from stem tissues and 27 isolates were obtained from leaf tissues. Only one fungal isolate (CMU-ZY2045) obtained from leaf tissue could produce an extracellularly red pigment. Colonies of isolate CMU-A109 on PDA grew to 80–85 mm at 25 °C in the darkness after 1 week. Colonies were flat, edge entire, floccose at the center with gray aerial mycelia, becoming black with age in the center and produced a soluble red pigment (Fig. 1A). Hyphae were pale brown, smooth, branched, septate, 1.5–4.5 μm. Conidiophore reduced to conidiogenous cells. Conidiogenous cells were pale brown, monoblastic, discrete, doliform, ovoid, or ampulliform, 7.5–12 × 6–8 μm. Conidia were ellipsoidal, dark brown to black, shiny, smooth surface, 12–15 × 9–15 μm (Fig. 1B). Based on morphological observations, this fungal isolate was identified as *N. aurantiaca* (Wang et al. 2017). A pure culture was deposited in the Culture Collection of the SDBR Laboratory, Faculty of Science, Chiang Mai University, which a partner of Thailand Bioresource Research Center (TBRC), Thailand, under number SDBR-CMU350.

Molecular methods were used to confirm the identity of the isolate CMU-ZY2045. The LSU, ITS, TUB2, and TEF1 sequences of this fungal isolate were deposited in GenBank database under accession number KY048408, KY048409, MK388224, and MK388225, respectively. The combined LSU, ITS, TUB, and TEF1 dataset consisted 19 isolates with the final alignment dataset comprised 2867 characters including gaps (LSU, 1–853; ITS, 854–1469; TUB, 1467–2301; and TEF, 2302–2867). A phylogram of combined LSU, ITS, TUB, and TEF sequences is showed in Fig. 1C. Our phylogenetic results assigned isolate CMU-ZY2045 to *N. aurantiaca*. The species was clearly distinguished from other *Nigrospora* species and formed a sister taxon to *N. vesicularis* with BS support of 100% and PP value support of 1.0. Therefore, isolate CMU-ZY2045 was identified as *N. aurantiaca* based on molecular characteristics.

Optimization of fungal pigment production

The wavelength of maximum absorbance was scanned in a range of 300 to 650 nm. The results showed that the wavelength of maximum absorbance of red pigment in the supernatant was observed at 505 nm; therefore, indication of the total pigment yield was estimated at this wavelength. Red pigment and fungal biomass yields of *N. aurantiaca* CMU-ZY2045 in various liquid media are shown in Fig. 2A. The results indicated that the significantly highest pigment yield (3.652 ± 0.174 AU_{505}) was observed in GY medium followed by PDB (3.217 ± 0.122 AU_{505}) and OM medium (1.550 ± 0.163 AU_{505}), while the highest biomass was found in PDB (4.97 ± 0.78 mg/mL). Temperature affected both pigment production and fungal growth (Fig. 2B). The highest pigment yield (3.591 ± 0.480 AU_{505}) was determined at 27 °C, which was the optimum temperature. However, a fungus could not grow at 40 and 45 °C.

After 1 week of incubation, the pigment and fungal biomass yield responses to various carbon sources were observed (Fig. 2C). A fungus that produced the highest pigment yield was observed in glucose at 3.598 ± 0.210 AU_{505}, but no significant differences were observed when compared with fructose (3.282 ± 0.307 AU_{505}). Glucose was chosen as a suitable carbon source based on its availability at low cost. Both the lowest pigment and biomass yields were observed when using CMC as substrate. The results showed that the yeast extract was the best nitrogen source for pigment production and fungal growth (Fig. 2D). Our results indicated that the initial pH medium affected pigment production and fungal growth (Fig. 2E). The significantly highest pigment yield (4.042 ± 0.154 AU_{505}) was found in GY medium at pH 5.0, followed by pH 6.0 (3.734 ± 0.098 AU_{505}) and pH 7.0 (3.665 ± 0.213 AU_{505}). The effect of the incubation period on pigment production was investigated for this fungus. The results indicated that the high amount of fungal pigment yield was produced when fungus was grown in the stationary phase, which then slowly decreased (Fig. 2F). The highest pigment yield (4.215 ± 0.148 AU_{505}) was found at 5 days after incubation. Therefore, the use of GY medium (carbon and nitrogen sources were glucose and yeast extract, respectively), at a pH value of 5.0 and incubation at 27 °C for 5 days made up the optimum conditions for the pigment production of this fungus.

Fungal pigment fermentation and extraction

The evaluation of pigment production in 1000-mL Erlenmeyer flasks containing 300 mL of GY medium was observed at the optimal fermentation conditions (pH 5.0, temperature 27 °C). After 1 week of inoculation, the highest pigment yield (4.302 ± 0.238 AU_{505}) was obtained and the liquid culture was filtrated. The extraction of the culture filtrates produced yields of crude pigment at 238.75 ± 42.20 mg/L with five replications.

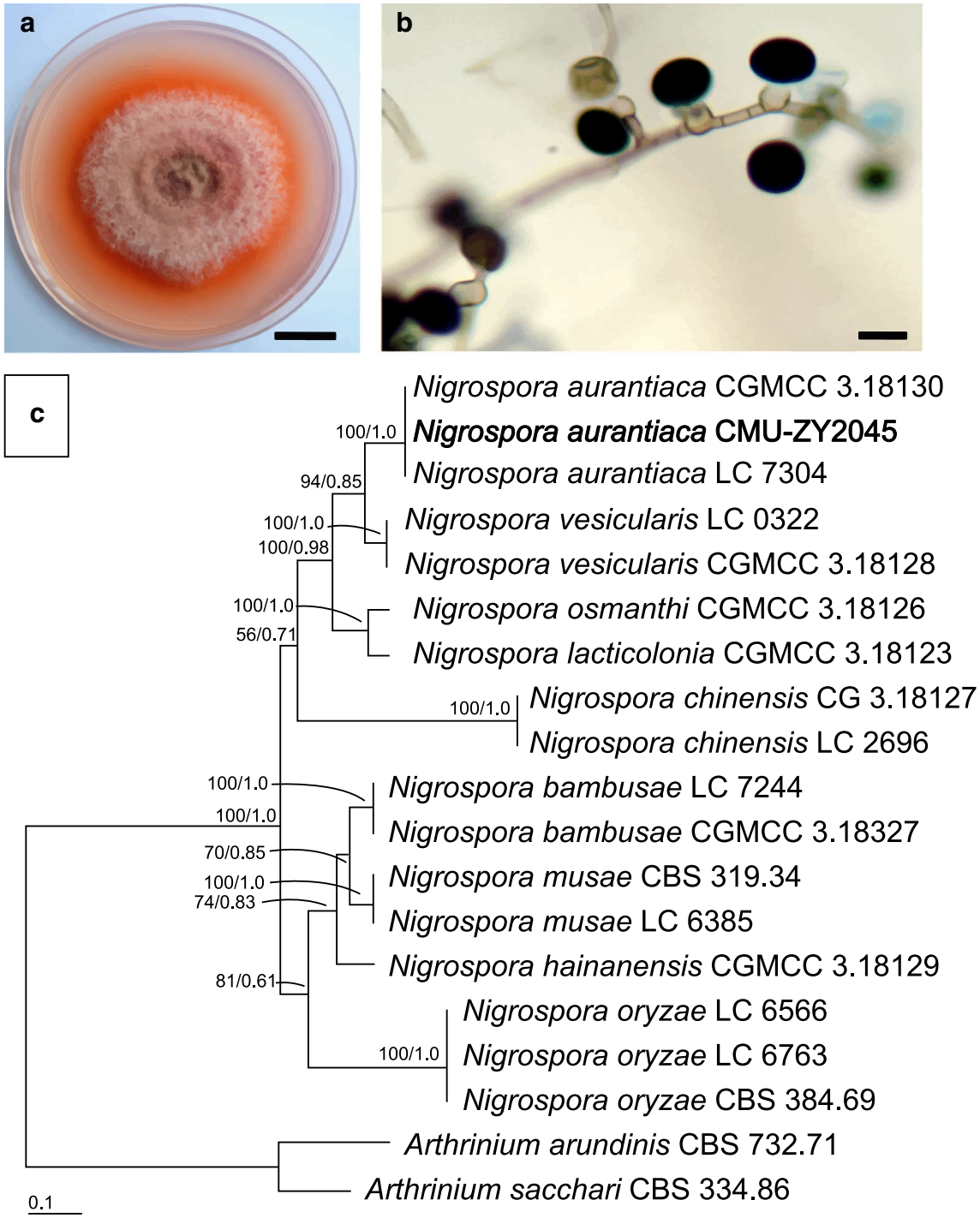

Fig. 1 Morphological characteristics and phylogenetic tree of *Nigrospora aurantiaca* CMU-ZY2045. A. Colony on potato dextrose agar at 25 °C for 1 week. B. Conidiophore and conidia. C. Phylogram derived from maximum likelihood analysis of a combined three-gene loci (LSU, ITS, TUB, and TEF1) from *Nigrospora aurantiaca* CMU-ZY2045 and related species. *Arthrinium arundinis* and *Arthrinium sacchari* were used as the outgroup. The numbers above branches represent maximum likelihood bootstrap percentages (left), Bayesian posterior probabilities (right) and the species described in this study is shown in bold. Bar A = 10 mm, B = 10 μm, C = the number of nucleotide substitutions per site

Characterization of crude fungal pigment

Solubility test The solubility of the crude fungal pigment extract in different solvents was evaluated and the results are shown in Supplemental Table S1. The solubility of the crude fungal pigment extract varied and appeared in red color in absolute methanol, 95% ethanol and acetone with a maximum absorption level at 505 nm. It was found that both the highest solubility ability was found in absolute methanol. The solubilization in distilled water, 0.1 M HCl and ethyl acetate showed

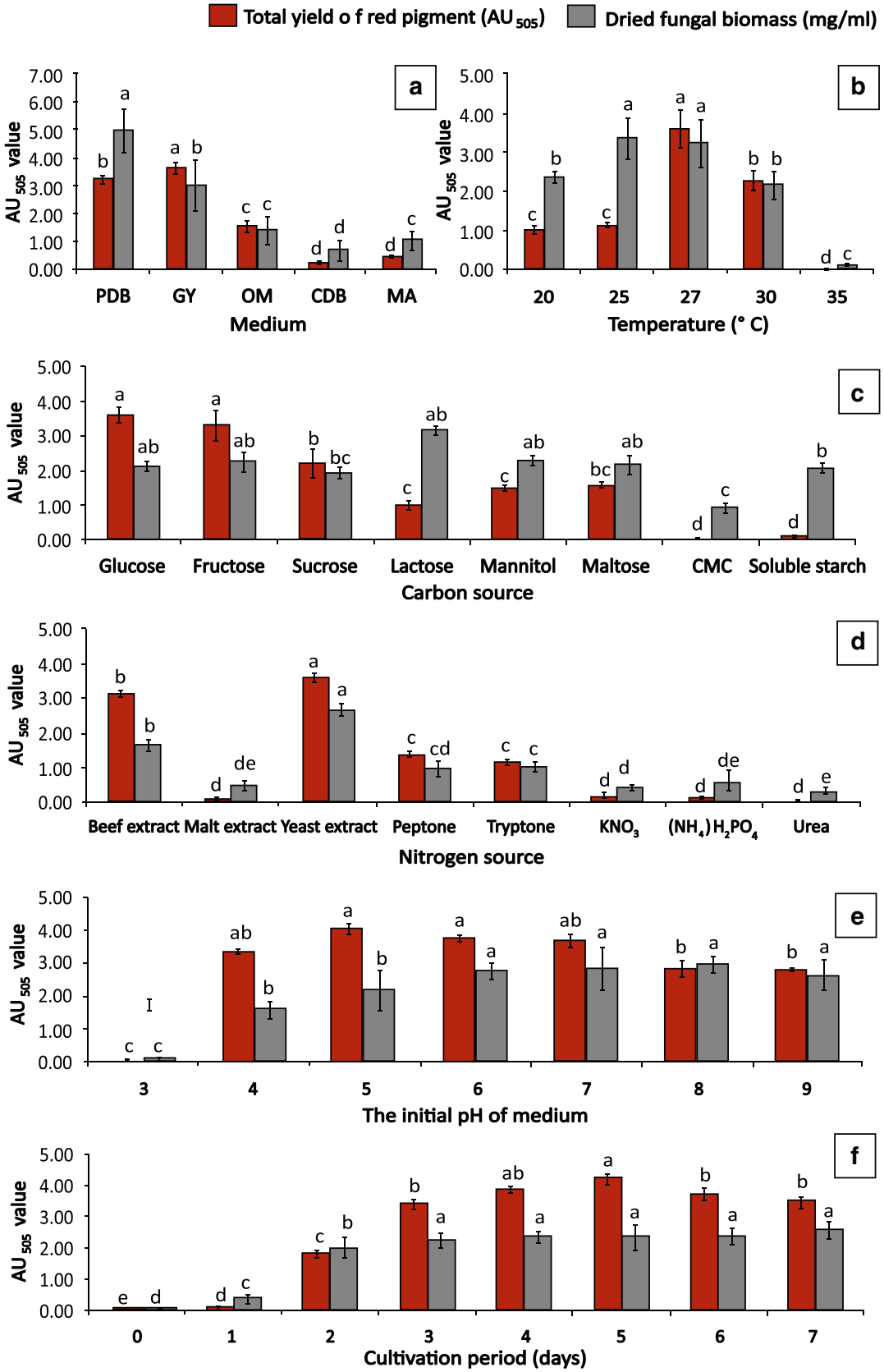

Fig. 2 Effects of liquid medium (A), temperature (B), carbon source (C), nitrogen source (D), the initial pH of liquid medium (E), and cultivation period (F) on fungal red pigment and fungal biomass productions by *Nigrospora aurantiaca* CMU-ZY2045. The results are means of three replicates ± SD. Different letters above each bar in the same parameter indicate the significant difference ($P < 0.05$)

an orange-red color. However, the solubilization in 0.1 M NaOH showed a bluish-violet color with a maximum absorption value of 600 nm, while a pink color was observed in 0.1 M alum showing a maximum absorption value at 565 nm.

Stability test The crude fungal pigment extract was subjected to various treatments to test its stability and the results are presented in Supplemental Table S2. For the pH stability test, the initial color of this pigment was red (pH 6.4, λ_{max} 505 nm, yield 0.984 ± 0.030 AU_{505}), and different shades of colors resulted when the pigment extract was adjusted to different pH values. At pH values of 3, 4, 5, 6, and 7, the red color was observed with a maximum wavelength of 505 nm, except for the pH value of 7. The highest percentage of stability was found at pH 6.0 (99.6 ± 0.4%), followed by pH 5.0 (98.3 ± 1.1%) and pH 7.0 (97.3 ± 0.5%). At all other pH values, different colors such as bluish-red (pH 8.0 and 9.0), purplish-red (pH 10.0), and reddish-violet (pH 11.0) were observed with the maximum wavelength values at 540, 545, and 590 nm, respectively. The pigment stability was tested at different temperatures ranging from 20 to 80 °C, and the pigments that were subjected to all tested temperatures showed no changes in color. A high level of stability at low temperatures (20–50 °C) was observed. The pigment subjected to sunlight, UV, fluorescent lights, and autoclaved conditions showed no changes in color. The precipitation of the pigment was not observed at the bottom of the tubes after all stability tests. The red pigment from this fungus revealed high percentage values of stability at various temperatures and under sunlight. Our study indicates that the red pigment produced by *N. aurantiaca* CMU-ZY2045 can be used as a textile colorant.

Fungal pigment purification and identification

The scheme for purification of the red pigment is shown in Fig. 3. One band was observed in the TLC having R_f values of 0.65. A final yield of the purified fungal red pigment was 1.6 mg from 25 mg of the red pigment fraction. LC/MS analysis of the pigment was performed. The MS spectrum in a positive mode showed a peak at *m/z* 337.0925, which corresponded to $C_{16}H_{17}O_8$ ($[M+H]^+$, calcd 337.0918), in the negative mode, *m/z* 335.0753 ($C_{16}H_{15}O_8$, $[M-H]^-$, calcd 335.0761) as it had appeared. Therefore, the molecular formula was confirmed as $C_{16}H_{16}O_8$. In the negative mode, MS/MS analysis of *m/z* 335.0753 $[M-H]^-$ showed nineteen product ions (Supplemental Table S3). To search for the structure of red pigment in the chemical database, in silico fragmenter MetFrag was performed. The molecular formula and the fragmentation mass spectrum of the red pigment were submitted to MetFrag. When applying KEGG, PubChem, and ChemSpider as the chosen database, the MetFrag search resulted in 2 hits, 81 hits, and 63 hits, respectively. The most probable forms of red pigment with chemical structures were altersolanol A, altersolanol D, or bostrycin.

Previous studies reported that altersolanol A, altersolanol D, and bostrycin resulted in yellow, orange-yellow, and red pigments, respectively (Fuoillaud et al. 2016; Suemitsu and Nakamura 1981; van Eijk 1975; Yagi et al. 1993). Therefore, a fungal red pigment in this study was compared with the bostrycin standard using the LC/MS/MS technique. Under relevant conditions, the retention times of a purified fungal red pigment showed a peak at 14.0 min, which corresponded to a bostrycin standard with a maximum absorption value of 504 nm (Fig. 4A and C). The identification of the fungal red pigment was confirmed by co-injection with a bostrycin standard. Moreover, it was found that the fungal red pigment and bostrycin standard showed the same fragmentation of mass spectrum (Figs. 4B and D). Therefore, the fungal red pigment was considered as bostrycin based on the comparisons of MS profile and a retention time with bostrycin standard.

Characterization of dyed textile

Color fastness testing The color name, code, fastness, and total color difference values of the dyed multifiber substrates before and after being washed are presented in Supplemental Table S4. The results revealed that the differences in the stained colors varied for the different types of fiber substrates (Fig. 5). Silk, cotton, and nylon fabrics displayed a pastel red color, cellulose fabric displayed a pale red, color and the acetate fabric was pink. However, both the polyester and wool fabrics presented a pinkish color with slight staining. After the washing test, silk and cellulose fabrics showed a pale red color, whereas cotton and nylon fabrics showed a pastel red color and the acetate fabric appeared pink. The polyester and wool fabrics were white indicating no staining. The irregular staining in the cellulose, cotton and acetate fabrics was not changed after being washed. It was found that all the dyed fabrics after washing were lighter than the dyed fabrics before being washed because an increase in the L* value in the washed fabrics was observed. The A* value was decreased in the dyed fabrics after being washed. Consequently, the red shade of the washed fabrics was lighter than the unwashed fabrics. The ΔE* values ranged from 1.32–4.80, while the ΔE* values in the cotton fabrics were lower than the other fabrics. Our study indicated that the red pigment produced by *N. aurantiaca* CMU-ZY2045 can produce a higher degree of staining in cotton fabrics than in other fabrics.

In vitro cytotoxic assay

The viability percentage of HEK 293T in each selected concentration is shown in Supplemental Fig. S1. The results indicated that cell viability percentage was decreased when the concentration of the crude red pigment was increased. The viability percentage of HEK 293T cell at a concentration of 0.1 mg/mL in methanol (a concentration used in textile

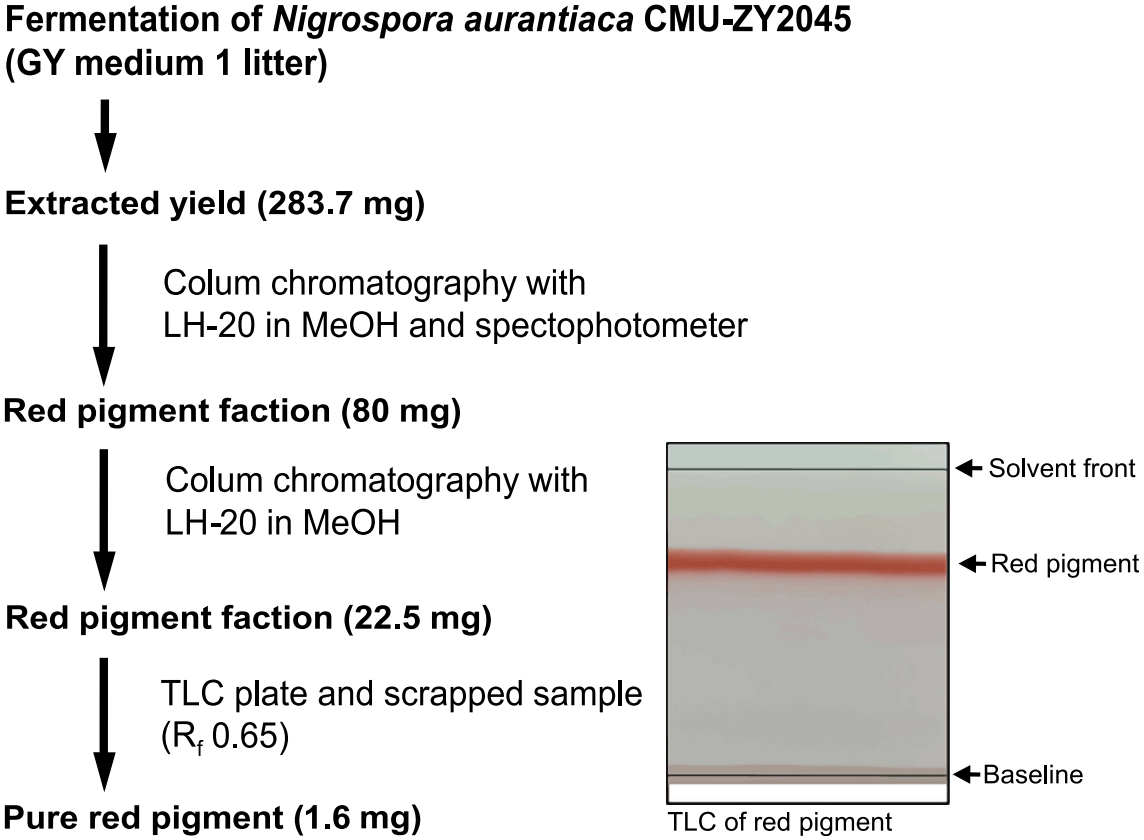

Fig. 3 Scheme of the isolation and purification of red pigment produced by *Nigrospora aurantiaca* CMU-ZY2045

dyeing) was 89.06 ± 7.50%, which was considered non-significant with regard to methanol and the untreated cell treatments. It could presumptively indicate that it is a non-toxic concentration by comparing control as the viability was above 85%. The LC_{50} of the crude red pigment was observed at 2.34 mg/mL.

Discussion

Endophytic fungi are natural sources in the production of secondary metabolites, e.g., antibiotics and pigments (Babitha et al. 2007; Qui et al. 2010; Torres et al. 2016). In the present study, an endophytic fungus, *N. aurantiaca* CMU-ZY2045 isolated from cinnamon trees produced an extracellularly red pigment. The results were similar to those of previous studies which found that pure cultures of fungal genera, e.g., *Blakeslea*, *Eurotium*, *Fusarium*, *Isaria*, *Monascus*, *Nigrospora*, *Paecilomyces*, *Penicillium*, and *Talaromyces* could secrete the red pigment in both solid and liquid media (Arumugam et al. 2014; Chadni et al. 2017; Cho et al. 2002; Lebeau et al. 2017; Méndez et al. 2011; Qui et al. 2010; Torres et al. 2016; Unagul et al. 2005; Velmurugan et al. 2010).

In this study, the optimum conditions for the highest yield of red pigment production from *N. aurantiaca* CMU-ZY2045 was obtained at 5 days of cultivation in GY medium used with carbon and nitrogen sources as glucose and yeast extract, respectively, at a pH of 5.0 and incubation at 27 °C. This result was supported by the findings of several previous studies, which reported that fungal growth and pigment production yields were greatly influenced by cultivation conditions (e.g., cultivation medium, temperature, pH, carbon and nitrogen sources, mineral, aeration, and type of fermentation) and the optimum conditions for pigment production were not homologous for the fungal species and strains (Chadni et al. 2017; Cho et al. 2002; Kumar et al. 2015; Méndez et al. 2011; Pandey et al. 2018; Pradeep et al. 2013; Sharma et al. 2012; Souza et al. 2016; Unagul et al. 2005). Notably, the optimum conditions for red pigment production from *Nigrospora* sp. NIOT was found at 6 days of cultivation in PDB using soluble starch as carbon source and peptone as nitrogen source, a pH of 6.0 and incubation at 30 °C (Arumugam et al. 2014). Zhou et al. (2015) found that the cultivation of *Monascus ruber* in liquid medium at a pH of 5.4 using corn flour and NH_4Cl as the carbon and nitrogen sources, respectively and being incubated at 30 °C for 1 week gave the highest yield of red pigment production. In addition, previous studies suggested that the optimal physical conditions and suitable nutrients for fungal pigment fermentation could improve the pigment production yields significantly on an industrial production scale (Arumugam et al. 2014; Kumar et al. 2015; Mapari et al. 2005). Additionally, Tudor et al.

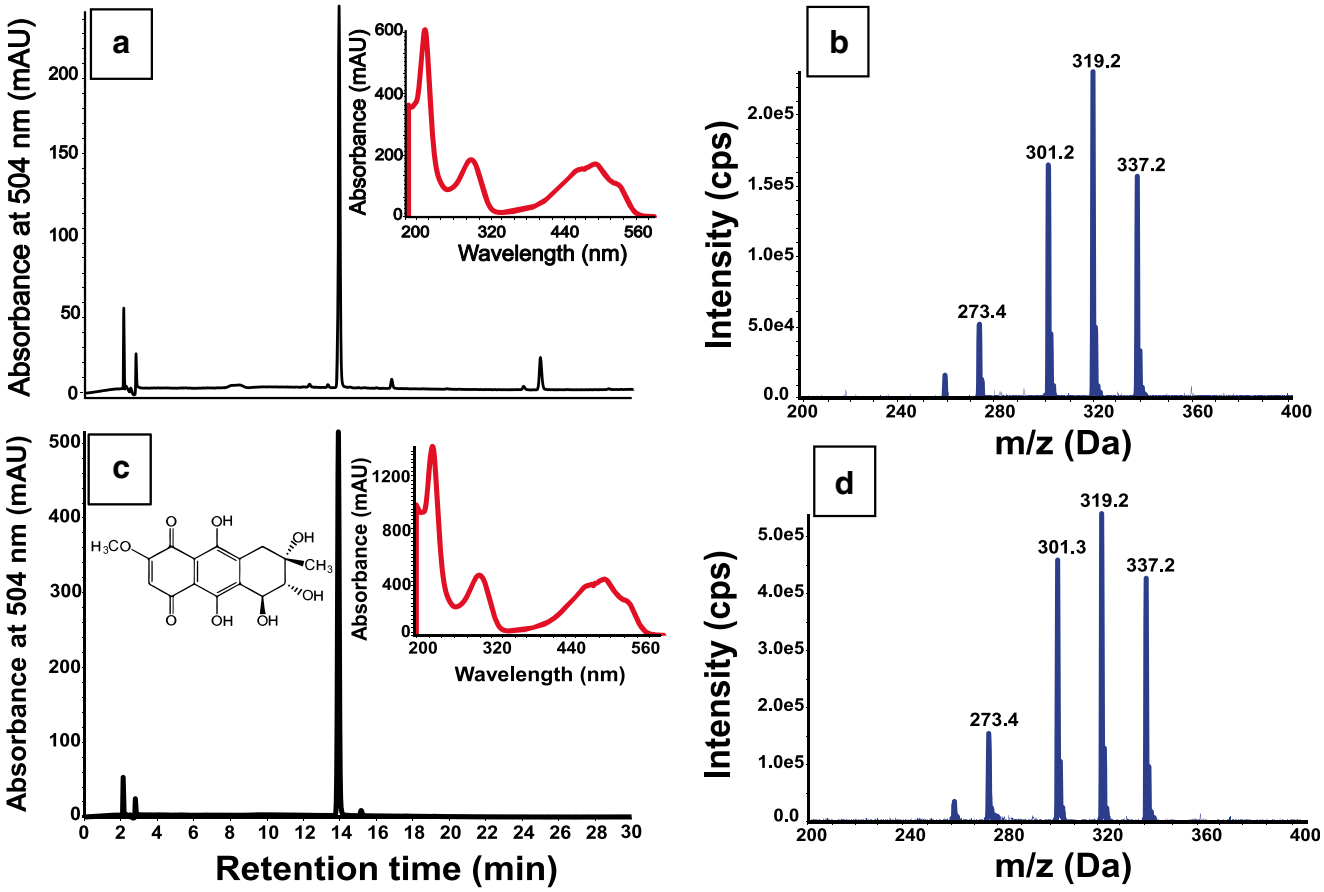

Fig. 4 LC/MS/MS data of red pigment produced by *Nigrospora aurantiaca* CMU-ZY2045 (A and B) and bostrycin standard (C and D)

(2013) reported that pH values did not only affect the fungal growth and pigment production, but also the intensity of the pigment production.

The solubility of crude red pigment of *N. aurantiaca* CMU-ZY2045 varied in different solvent types, and the highest solubility was found in methanol. This result is similar to those of previous reports where the solubilization ability of the crude microbial pigment was dependent upon various solvent types (Narendrababu and Shishupala 2017; Rokade and Pethe 2016). Souza et al. (2016) reported that pigment production from *Epicoccum nigrum*, *Lecanicillium aphanocladii*, and *Pe. flavigenum* was soluble in ethyl acetate, but pigments production from *As. aureolatus*, *As. sydowii*, *As. keveii*, and *Pe. chermesinum* were insoluble in ethyl acetate. Orange-red pigment production occurred from *Aspergillus* showed the highest solubility in ethyl acetate (Narendrababu and Shishupala 2017). In addition, the solubilization ability was influenced by the composition of the polarity substances in the crude pigment, for which polar substances tend to dissolve in polar solvents and non-polar substances dissolve in non-polar solvents (Latha and Jeevaratnam 2010; Robinson et al. 2014; Rokade and Pethe 2016; Souza et al. 2016).

This study found that the color stability of fungal red pigment is highly stable at low temperatures (20–50 °C) and in a more acidic pH range than the basic pH (pH 5.0–6.0). This result was supported by several previous studies that reported that the stability of the fungal pigment was influenced by different conditions such as pH, heat, light, and chemical additives (Kaur et al. 2017; Poorniammal et al. 2010; Torres et al. 2016). Sastrawidana et al. (2016) found that red pigment produced from *Pe. purpurogenum* was stable at 30–80 °C and at pH values in a range of 4.0–8.0. A red pigment produced from *Penicillium* sp. HSD07B was stable between pH values of 2 and 10, and temperatures of 10–100 °C (Hailei et al. 2011). The stability of fungal pigments toward different processing conditions, additives, and pH values make up the major factors affecting the selection and application of fungal pigments (Robinson et al. 2013; Sharma et al. 2012; Torres et al. 2016; Wongjewboot and Kongruang 2011).

The fungal red pigment characterized in this study that was identified as bostrycin was originally isolated from the fungus, *Alternaria* and *Ar. phaeospermum* (van Eijk 1975). It has also been found in other fungi, e.g., *Al. eichorniae*, *Al. porri*, *Al. solani*, *Bostrychonema alpestre*, *Dermocybe splendida*, *Fusarium* sp. PSU-F14, *Fusarium* sp. PSU-F15, *Nigrospora oryzae*, *Nigrospora* sp. 1403, *Nigrospora* sp. 407, *Nigrospora* sp. MA75, *Phomopsis juniperovara*, *Stemphylium botryosum*, and *Xylaria* sp. 2508 (Deshmukh et al. 2014; Huang et al.

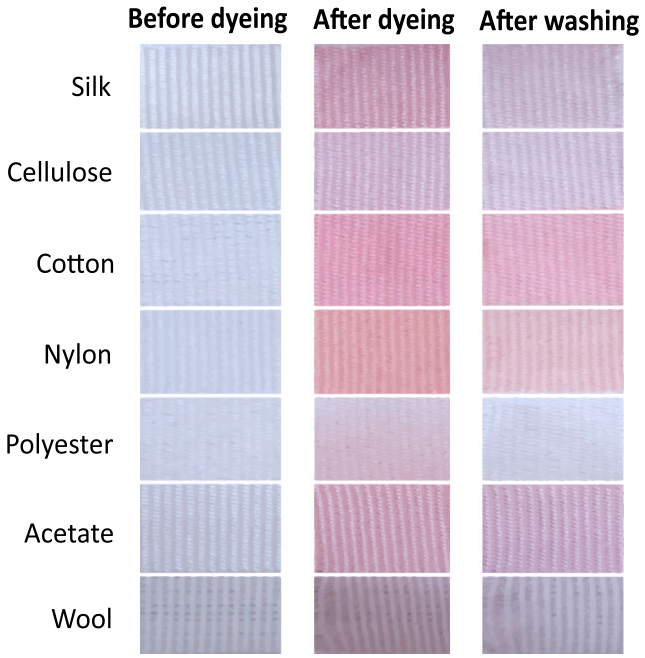

Fig. 5 Multifiber fabric dyed with crude red pigment produced by *Nigrospora aurantiaca* CMU-ZY2045

2014; Thomson 1987; Trisuwan et al. 2010; Wheeler et al. 1975; Xia et al. 2011; Yang et al. 2012). However, this is the first report on bostrycin produced by *N. aurantiaca*. Bostrycin has important biological activities, including antibacterial and phytotoxic activities (Charudattan and Rao 1982; van Eijk 1975; Wheeler et al. 1975). This compound has also been shown to possess anticancer activities by the inhibition of the growth of lung, prostate, and gastric cancer cells (Chen et al. 2010, 2011; Lin et al. 2008). Moreover, bostrycin has the potential to be used in food processing as a safe color fixative agent (Huang et al. 2017).

The present study revealed that the red pigment produced by *N. aurantiaca* CMU-ZY2045 could be used as a textile dye. The red pigment showed the highest strain in cotton fabric, which was expressed by color fastness after being washed. Similarly, previous studies indicated that the fungal pigment can be exploited as one of the natural sources of textile dyes (Babitha et al. 2007; Hernández et al. 2019; Nagia and El-Mohamedy 2007; Sharma et al. 2012; Hinsch et al. 2015; Palomino et al. 2017). In addition, the resulting color of the fabrics after staining and washing varied with both different fabric types and fungal pigments (Kramar et al. 2014; Mabrouk et al. 2011; Poorniammal et al. 2010; Sharma et al. 2012). For examples, the reddish-brown and red pigments produced by *Al. alternata*, *As. niger*, *Talaromyces verruculosus* and *Trichoderma virens* displayed the highest degree of staining and excellent fastness to washing in cotton fabrics (Anchana devi 2014; Chadni et al. 2017; Devi and Karuppan 2015). However, the pigments from *Curvularia lunata*, *Pe. chtysogenum*, *Pe. italicum*, and *Pe. regulosum* showed the highest degree of staining in wool fabric and revealed effective color washing fastness (Mabrouk et al. 2011; Sharma et al. 2012).

In conclusion, an endophytic fungus, *N. aurantiaca*. CMU-ZY2045 isolated from the leaf tissues of the cinnamon plant effectively produced red pigment in vitro. The highest red pigment yield was observed in the glucose yeast extract liquid medium with glucose as a carbon source, yeast extract as a nitrogen source, a pH value of 5.0, and at 27 °C with shaking for 5 days. Red pigment extract was stable in temperatures of 20–50 °C and pH values of 5.0–6.0. The staining of cotton fabrics using this fungal red pigment extract has excellent fastness to washing and the concentration used revealed a negative level of cytotoxicity. The fungal red pigment was identified as bostrycin. Thus, our results suggest that the red fungal pigment extract has the potential to be used in textile processing as a safe natural dye.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors

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