EFFECT OF THE CRUDE PIGMENT EXTRACT FROM ACTINOBACTERIA ISOLATED FROM STINGLESS BEE, *TETRAGONILLA COLLINA* (SMITH, 1857), ENTRANCE NEST ON NATURAL DYEING FIBERS

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Abstract

Actinobacterial dyes are alternatives for natural fiber dyeing. In this study, the strain C4 isolated from stingless bee entrance nest was evaluated for use as a dye in the textile dyeing process. Morphologically, the strain C4 colonies grown on sodium caseinate agar (SCA) produced water-soluble violet-colored pigment. To identify the actinobacterial strain C4, phylogenetic identification was based on the partial 16S rDNA sequence analysis. The result showed that the strain was identical to *Streptomyces californicus* (NR 115432) (100% sequence identity). To scale up actinobacterial dye production, strain C4 was cultivated on media supplemented with broken-milled rice as the carbon and energy source. Following actinobacterial growth, the pigment was extracted by 70 % (v/v) ethanol to obtain the actinobacterial dye. The 5 natural fibers, silk, wool, cotton fiber, hemp fiber, and the mixed banana and cotton fiber, were soaked in the actinobacterial dyes for 12 hr after which color measurement was done, following the CIELAB system and the Standard Color Chart R.H.S for natural fibers.

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The dyeing results indicated that silk showed the highest CIELAB value indicating that it is the most suitable natural fiber for actinobacterial dyeing. It is suggested that the use of actinobacterial dye in the textile industry should be evaluated in future.

Keywords: Actinobacterial pigment, Stingless bee entrance nest, Dyeing, Natural fibers

Introduction

Woven fabric is a traditional product of folk culture found in Thailand using chemical dyes in the dyeing process of both small and large textile producers. These dyes, however, are a cause of environmental pollution, especially water pollution and have also shown toxic effects for some users who wore clothing made from woven fabric dyed with chemical dyes. In Thailand, silk and cotton are the usual natural fibers used in the textile industry. Additionally, wool, hemp fiber and mixed fibers comprised of banana and cotton fiber, are used as industrial fibers in the textile industry. To avoid the toxicity and environmental impact of chemical dyes, natural dyes obtained from actinobacteria, fungi and other microorganisms can be used instead of chemical dyes.

In our study, the strains of actinobacterial dyes that we identified in stock cultures produced pigments in a variety of color shades. These stock cultures were selected for the production of actinobacterial dyes used in this study.

Actinobacteria are a gram-positive bacterial group distributed in nature in both terrestrial and aquatic ecosystems. Their diverse morphologies are different from other bacteria and have beneficial characteristics, especially the biosynthesis of secondary metabolites. These non-growth associated products demonstrate a vast variety of biological activities, including antibacterial, antifungal, anticancer, antioxidant, insecticidal, immunosuppressive, as well as an inhibitory effect of enzyme activities that have been evaluated in medical, pharmaceutical, agricultural and industrial uses. Additionally, some publications have reported the application of actinobacterial dyes in food and textile industries (Meng-Xi et al., 2021; Ramesh et al., 2020; Abraham & Chauhan, 2018; El-Naggar & El-Ewasy, 2017; Manikkam et al., 2015; Karuppiah et al., 2013; Stankovic et al., 2012; Vijayabharathi et al., 2011; Amal et al., 2011; Zhang et al., 2006). Actinobacteria, especially the *Streptomyces* genus, are the major group of microorganisms that produce certain bioactive compounds distinct from other microbial groups (Subramani & Aalbersberg,

2012; Kurtböke, 2012; Kurtboke, 2010). Actinobacteria microorganisms have been used as alternative dyes primarily in the food and textile industries (Urtgam & Thurnkul, 2021; Chakraborty et al., 2015; Kramar et al., 2014; Kulkarni et al., 2014; Amal et al., 2011). Several publications have mentioned the beneficial properties of actinobacterial dyes, such as the pastel and earth-tone dyes extracted from actinobacteria that have been used in textile fibers. The benefits of actinobacterial dyes are low cost, more easily produced, and faster, than other natural dyes obtained from animal, plant, and mineral sources. Additionally, actinobacterial dyes can be continuously produced in plants in large-scale production, such as solid-state fermentation in the laboratory and also on a pilot scale. Importantly, actinobacterial dyes are non-toxic to the environment and other living organisms (Kramar & Kostic, 2022).

In this study, we isolated the actinobacterial strain C4 from stingless bee entrance nest collected in Sukhothai province. The actinobacterial colonies were violet-colored with a gray edge, and produced water soluble violet-colored pigment on sodium caseinate agar (SCA) (Thurnkul et al, 2022). To identify the actinobacterial species, a phylogenetic approach was applied, and some phenotypic characteristics were also determined. To test the potential use in the textile industry, the CIELAB system and the Standard Color Chart R.H.S were applied with five natural fiber types; silk, cotton, wool, hemp, and mixed fiber comprising both banana and cotton fibers.

Materials and Methods

The actinobacterial strain C4 was isolated from the stingless bee entrance nest collected in Sukhothai province (Thurnkul et al, 2022). The strain C4 was isolated by cross streaking on sodium caseinate agar (SCA) (Urtgam & Thurnkul, 2021) supplemented with 1.0 g/L of propionic acid (sodium salt) and incubated at 30°C for 5-7 days.

Production and Extraction of Actinobacterial Dye

The methodologies for dye production and extraction were described by Urtgam & Thurnkul (2021). Following the protocol we soaked 30 g of broken-mill rice with tap water in 250 mL of Erlenmeyer flask, and incubated the soaked rice at room temperature for 30 min. The water was then completely removed. Then, the rice was sterilized by autoclaving at 121°C for 15 min. The actinobacterial strain C4 was prepared as spore and cell suspension inoculums at the same initial cell concentration as the standard solution

of McFarland No.0.5 (1.5 x 10^8 CFU/ml). The inoculums were measured at 625 nm at 0.08-0.1 values. And 1 mL of spore suspension was pipetted into the sterile broken-mill rice and 10 mL of sterile distilled water was added, and incubated at 30°C for 5-7 days. Then, after drying at 100°C for 60 min, strain C4 was grown on the sterile broken-mill rice before grinding. The C4 dye was ground, preserved in sterile plastic bags and kept at room temperature until used.

The actinobacterial pigment was extracted with 30 mL of 70 % (v/v) ethanol using 5 g of the ground C4 in a 250 mL Erlenmeyer flask and shaken at 120 rpm, at room temperature for 60 min. The experimental flask was then kept at a static condition until flocculation. The supernatant was rinsed and used for natural fiber dyeing.

Dyeing of the Natural Fibers

The silk, wool, cotton, hemp fiber, and mixed banana and cotton fibers, were dyed with the actinobacterial C4 dyes extracted previously. The protocol was, briefly: 0.3 g of each fiber was immersed with 30 mL of the actinobacterial C4 dye, and incubated at 120 rpm on a rotary shaker for 30 min. Each of the soaked fibers was twisted and dried at room temperature for 12 hr, and the dyeing step was repeated. The fibers were then washed with tap water and air-dried at room temperature for 12 hr. The evaluation of dyeing potential was observed with a Color Meter followed by the CIELAB System and Standard Color Chart R.H.S.

Actinobacterial identification

Species identification of the strain C4 was phylogenetically carried out. Genomic DNA was extracted with a BioFact™ Genomic DNA Prep Kit (Biofactory, Korea) and the purified DNA template was collected. The partial sequence of the 16S rRNA gene was amplified using the extracted C4 DNA as a template. The universal primers used were 27F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1492R (5'-GTT ACC TTG TTA CGA CTT-3') as described by Lane (1991). The PCR product was amplified using BioFact™ *Taq* DNA Polymerase (Biofactory, Korea) with polymerase chain reaction (Mullis et al. 1986), and purified by BioFact™ Gel & PCR Purification System (Biofactory, Korea). The partial 16S rDNA sequence was determined by the protocols described by the Bionics company (Korea). The blast homology search was applied for the species delineation between the unknown 16S rDNA of the C4 strain and the same gene of the phylogenetic relatives.

The phylogenetic evolution was evaluated and represented as a phylogenetic tree constructed by the Neighbor-joining method in the MegaX (Saitou & Nei, 1987; Felsenstein, 1985; Tajima & Nei, 1984; Kumar et al., 2018). The bootstrap analysis was done with 1,000 replicates. Phenotypically, morphological and cultural characteristics of strain C4 were studied, including Gram'stain and colony morphology.

Results

Production of actinobacterial dyes and natural fiber dyeing

The actinobacterial strain C4 isolated from the stingless bee entrance nest (Figure 1A, 1B) was cultivated on SCA as carbon and energy sources for 5 days at 30°C until the violet pigment found on the C4 colonies (Figure 2A) was diffused onto the cultivated substrate (Thurnkul et al, 2022). Then, the violet-colored broken-mill rice covered by C4 mycelia was dried by heat air and ground. The pigments were extracted with 70 % (v/v) ethanol and applied for natural fiber dyeing. The dyed natural fibers were colored by the Color Meter in the CIELAB system compared with Standard Color Chart R.H.S. The results showed that silk fiber had a violet-tone and the color brightness was better than all of the other dyed natural fibers (Figure 2B and Table 1). Because these pigments have been shown to chemically bond to textile materials and form bonds with functional groups of fibers, they could be considered as appropriate and useable dyes for textile dyeing (Kramar & Kostic 2022).

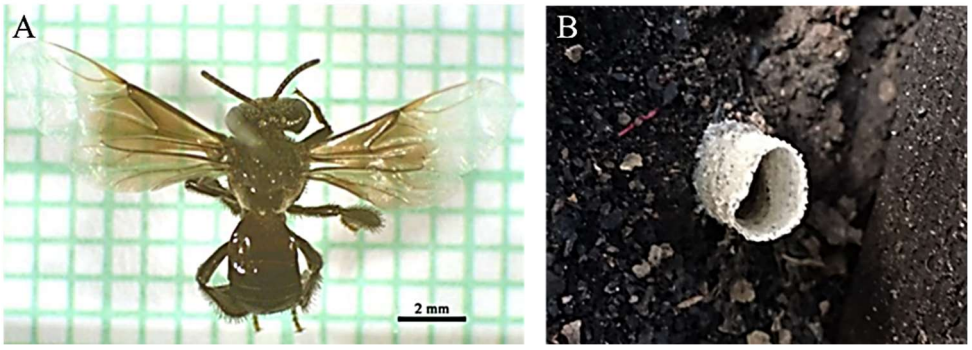

Figure 1 Characters of *Tetragonilla collina* (Smith, 1857) (A) and stingless bee entrance nest (B)

Figure 2 Five natural fibers dyed with actinobacteria strain C4 dye; un-dyed silk (A), dyed silk (B), un-dyed wool (C), dyed wool (D), un-dyed cotton fiber (E), dyed cotton fiber (F), un-dyed cotton fiber (G), dyed cotton fiber (H), un-dyed the mixed banana and cotton fibers (I), dyed mixed banana and cotton fibers (J)

Table 1 Color values and standard colors of 5 natural fibers stained with actinobacteria strain C4 dye.

Natural fibers	L	a*	b*	Standard Color Chart R.H.S
un-dyed silk	92.31	-0.06	5.80	White group 155D
dyed silk	73.03	10.76	-	Purple group 76B
un-dyed wool	79.48	1.34	16.25	Yellow-white group 158B
dyed wool	74.04	8.25	-	Purple group 76C
un-dyed cotton fiber	87.73	1.00	12.68	Yellow-white group 158D
dyed cotton fiber	75.05	8.46	-	Purple group 76C
un-dyed Hemp fiber	67.67	2.68	12.69	Gray-brown group 199D
dyed Hemp fiber	63.75	6.04	-	Purple group 78D
un-dyed the mixed banana and cotton fibers	84.16	2.12	13.84	Yellow-white group 158D
dyed the mixed banana and cotton	74.60	7.85	-	Purple group 76C

Remark L determine the lightness between 0 – 100: 0 is interpreted as dark/black, and 100 is interpreted as light/white. a* determine the red/green color: + interpreted as red, – interpreted as green. b* determine the yellow/blue color: +interpreted as yellow, –interpreted as blue. Standard Color Chart R.H.S A-D (A represents the darkest shade)

Identification of the actinobacterial strain C4

Based on the partial sequence analysis of 16S rDNA with 1392bp length, the actinobacterial strain C4 (OQ848239) was close to *Streptomyces californicus* strain CSSP711 (NR 115432) (100 % sequence similarity). Certain species of *Streptomyces* closely related species to strain C4 are *Streptomyces floridae* (99.9% sequence similarity) *Streptomyces puniceus* (99.9% sequence similarity) *Streptomyces globisporus* (99.8% sequence similarity) and *Streptomyces albovinaceus* (99.8% sequence similarity). To show the evolutionary relationship between the actinobacterial strain C4 and the closest and close relatives, a phylogenetic tree was constructed (Figure 3).

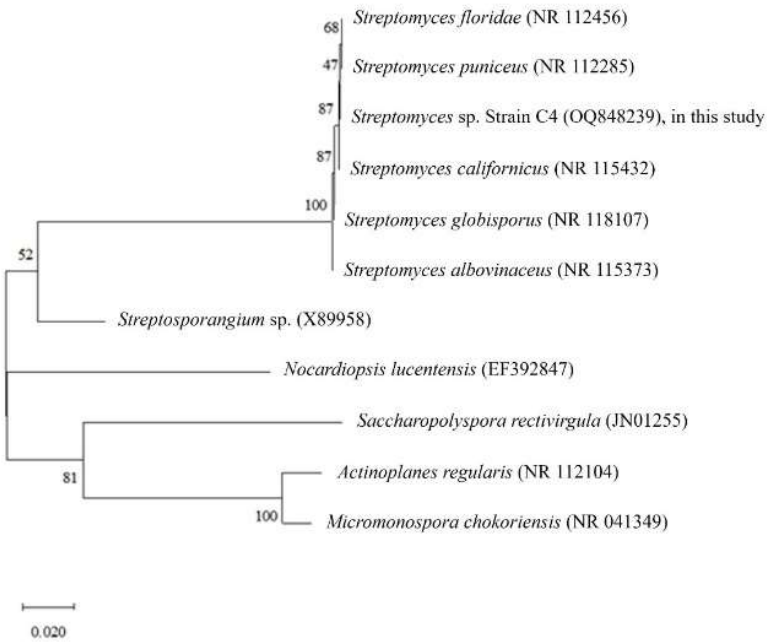

Figure 3 Phylogenetic tree of *Streptomyces* sp. strain C4 (OQ848239) conducted with Neighbor-Joining statistics method and Tamura-Nei based on the alignment of 16s rDNA partial sequences with other *Streptomyces* species. Five bacterial 16s rDNA partial sequences of *Actinoplanes regularis, Micromonospora chokoriensis, Nocardiopsis lucentensis, Saccharopolyspora apinosa,* and *Streptosporangium* sp. Were assigned as the out-group. The percentage of replicate trees in which the associated taxa clustered together in 1000 replicates of the bootstrap test are shown next to the branches. The scale bar corresponds to 0.02 substitutions per nucleotide position.

Phenotypically, colonies of strain C4 are flat and smooth, with a violet center surrounded by a gray edge (Figure 4A). The substrate and aerial mycelia were produced. The violet-colored pigment was diffusible onto the media cultivated as described previously. The spore chains were rectiflexibiles (Goodfellow et al., 2012) (Figure 4B).

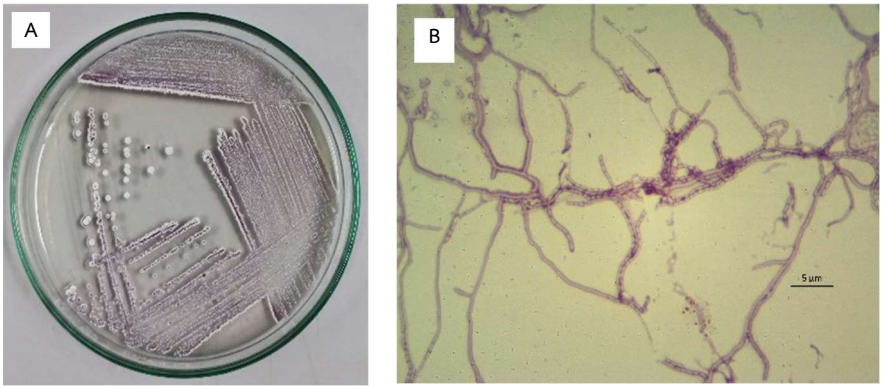

Figure 4 Colony of actinobacteria strain C4 on SCA at 30°C for 7 days (A) and conidia under the light microscope (1000X) (B)

Discussion

The actinobacterial strain C4 is microscopically related to the colonial characteristics of the *Streptomyces* genus. It produces the violet-colored pigment with substrate and aerial mycelia on SCA media supplemented with the broken-mill rice as carbon and energy sources. The broken-mill rice is the cheapest and most easily found, and could be used as the nutritional source for the cultivation of strain C4. The pigments were extracted by organic solvent, particularly 70% (v/v) ethanol. The actinobacterial C4 dye obtained was applied for natural fiber dyeing of the 5 fiber types used in this study were silk, wool, cotton fiber, hemp fiber, and banana blended with cotton fiber. The degree of brightness was different, depending on the type of natural fiber. The silk and wool, which are protein-based fibers; showed a higher colored degree of fiber dyeing than other fibers used.

The actiniobacterial strain C4 shares morphological and cultural characteristics with *Streptomyces californicus*, especially from the colonies that produced the violet-colored and water-soluble pigments (Goodfellow et al., 2012). Additionally, the partial

16S rDNA sequence of strain C4 had 100% identity equality with *S. californicus*. Based on polyphasic approaches, the actinobacterial strain C4 was closest to *S. californicus*. Therefore, a multigene analysis should be undertaken in future research to confirm the species identification. *S. californicus* has recently been reported in biotechnological applications, such as medical (antibacterial and antifungal drugs, antioxidants, immunostimulants) (Al-agamy et al., 2021; Hamed et al., 2021; Tan et al., 2018; Gozari et al., 2016; Jussila et al., 2003; Thrum et al., 1967), agricultural (growth inhibitors of plant pathogens), environmental and industrial applications (antibiofilm agent, antifouling agents, biosurfactant, and enzymes used in textile and food industries) (Singh & Dubey, 2020; Chi et al., 1990; Ryu et al., 1990; Hussein & El-Gammal., 1976).

Many publications have reported on the color properties of actinobacterial strains. For example, Urtgam & Thurnkul (2021) isolated the actinobacterial J10 strain from wasp soil samples. The J10 strain produced yellow colored water-soluble pigments that were cultivated on SCA and incubated at 30°C for 5 days. After dyeing, yellow colored silk was obtained. Chakraborty et al. (2015) collected mangrove soils to isolate the actinobacteria. Their phylogenetic results indicated that the isolated strain was *Streptomyces fradiae* VITIPMSB. This particular strain produced pink-colored pigments, and these researchers, whose focus was on cotton and wool dyeing, and cosmetic ingredients, cultivated the actinobacterial strain for pigment production appropriate for these applications. Kulkarni et al. (2014) isolated *Kocuria flava* HO-9041 for pigment production, and used the yellow-colored actinobacterial dyes for silk and wool dyeing. Kramar et al. (2014) isolated *Streptomyces* strain NP2 and NP4 from soil samples collected in Serbia. These strains produced blue and red diffusible pigment on starch casein agar (SCA). After ethyl acetate extraction, the blue and red dyes extracted from actinobacterial NP2 and NP4 were applied for synthetic fiber dyeing. Amal et al. (2011) isolated *Streptomyces virginiae* and the brown- and yellow-colored dyes obtained could be used for wool dyeing.

Streptomyces dyes have the potential for use in textile industries (Janković et al., 2023; Kramar & Kostic, 2022; Kramar et al., 2014) where natural and synthetic fibers can be dyed with actinobacterial dyes. The beneficial characteristics of actinobacterial dyes are known as environmentally friendly for textile applications.

Conclusion

The actinobacterial strain C4 , isolated from stingless bee entrance nest, was phylogenetically identified as *Streptomyces californicus.* This strain shares morphological and cultural characteristics with this species. After dyeing the natural fibers silk, wool, cotton fiber, hemp fiber and banana blended with cotton fibers, violet colored fibers were obtained. These fibers are the primary fibers used in the textile industry (Janković et al., 2023; Kramar & Kostic 2022; Chen et al., 2021; Kramar et al., 2014; Amal et al., 2011). In the future, advanced research in actinobacterial dyes production, including genetic manipulation and process optimization, should be a focus for the evaluation of the use of these fibers in commercial applications.

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