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Isolation, Identification, and Application of Pigment-Producing Actinobacteria from Stingless Bee Hives for Handicraft Production

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Abstract

Actinobacteria are distributed in natural habitats and produce a vast variety of natural pigments that are different in colour shades and applied in textile industry and others. The extracted actinobacterial pigments are used as eco-friendly natural dyes and colour and non-toxic to living organisms and environments compared with chemical or synthetic colours. In order to produce actinobacterial pigments for handicraft making, eight strains of the pigmented producing actinobacteria were isolated from stingless bee hives. Based on deep colour shades presented, 4 actinobacterial strains were chosen to prepare various colour for handicrafts, including yellow, violet, green, and pink. To identify actinobacterial strains selected, phylogenetic identification was carried out. The phylogenetic results indicated that strain C2 phylogenetically shared the 16S rDNA sequence 99.6 % similarity with its closest species, namely *Streptomyces cellulosae*. Strain C4 had the phylogenetic relationship close to *Streptomyces californicus* (99.9% similarity of 16S rDNA). Strain E1 was a closest member belonged to *Streptomyces chartreusis* (99.9% 16S rDNA sequence similarity), and strain E2 was phylogenetically closely related to *Streptomyces aureoversilis* with 99.2% 16S rDNA sequence similarity. To produce the handicraft colour, we cultivated four actinobacterial strains on broken-milled rice, and actinobacterial pigments were extracted using ethyl acetate. The crude extracts obtained were mixed with white flower clay that was used for handicraft making, and the artificial clay flowers were made. This study is the first to report the use of natural colours obtained from pigment-producing actinobacteria in handicrafts in Thailand and other countries. Future research will explore the application of actinobacterial pigments in various fields, including fine arts, the ceramic industry, and others.

Keywords**:** *pigment-producing actinobacteria; actinobacterial pigments; clayed flower; stingless bee; handicraft*

1. Introduction

Actinobacteria are the microbes classified into Domain Bacteria and are distributed in natural ecosystems, including terrestrial and aquatic habitats (Narsing Rao & Li, 2022; Yaradoddi et al., 2022; Hazarika & Thakur, 2020; Anandan, Dharumadurai & Manogaran, 2016). In recent decades, many researchers have reported that actinobacteria produce several kinds of secondary metabolites that were utilized for production of biotechnological products applied in agricultures, environments, industries, medicines and pharmaceuticals (Urtgam & Thurnkul, 2021; Ramesh et al., 2020; Abraham & Chauhan, 2018; El-Naggar & El-Ewasy, 2017; Shivlata & Satyanarayana, 2017; Abd-Elnaby et al., 2016; Anandan et al., 2016; Jadhav & Kulkarni, 2014; Chakraborty et al., 2015; Manikkam et al., 2015; Kramar et al., 2014; Karuppiah et al., 2013; Stankovic et al., 2012; Vijayabharathi et al., 2011; Amal et al., 2011; Zhang et al., 2006).

Actinobacteria associated with stingless bees have been described (Menegatti, et al., 2020; Suphaphimol, et al., 2020; Cambronero-Heinrichs et al., 2019; Rodríguez-Hernández et al., 2019). *Streptomyces* is a genus naturally distributed and has a symbiotic relationship with stingless-bees, namely *Tetragonisca angustula* (Cambronero-Heinrichs et al., 2019). *Streptomyces* associated with stingless bee were applied in industrial utilization, including textile industry. However, application in fine arts and handicraft applications has no report. Therefore, we are pioneer group for application of actinobacterial pigments in handicraft production in Thailand.

Pigmented producing actinobacteria, namely *Streptomyces* and non-*Streptomyces*, are of interestfor many applications, such as dyes for textile industry (Kramar & Kostic, 2022; Naligama et al., 2022; Vasanthabharathi & Jayalakshmi, 2020; Chakraborty et al., 2015; Amal et al., 2011). They produce pigments presented with different colour shades and tones, especially yellow, violet, gray, pink, orange and green colours (Kramar & Kostic, 2022; Usman et al., 2017; Malik et al., 2012). Most reports have focused on the textile industry, including actinobacterial dyes for natural fibers dyeing (silk, wool, cotton, hemp fiber and mixed fibers made from banana and cotton fibers) (Chen et al., 2021; Urtgam & Thurnkul, 2021; Wan et al., 2014; Amal et al., 2011).

The handicrafts industry commonly uses the synthetic or chemical colours that are toxic to living organisms and cause environmental pollution (Yadav, Tripathi & Tripathi, 2022; Murcia Mesa et al., 2021; Ajò et al., 2019; Konstadakopulos, 2008). Additionally, synthetic colours are imported from Europian and other countries, leading our country to spent a lot of money on these imports, resulting in trade deficit (Hagan & Poulin, 2021; Ferreira et al., 2004; Gilbert & Cooke, 2001). To solve these problems, natural colour, including actinobacterial pigments obtained from pigmentproducing actinobacteria isolated from natural habitats such as stingless bees and other habitats, in Thailand, are alternative choices for handicraft making that had important benefits for eco-friendly and green industries, especially handicraft industry (Yadav et al., 2022; Nurcahyanti et al., 2021; Indrayani & Triwiswara, 2020). The goal of actinobacterial pigments application in this study is to produce and scale-up actinobacterial pigments production from lab to pilot scales within a decade under cooperative works between university and private sectors.

2. Objectives

The aims in this study are described as follows: 1) isolation and identification of actinobacteria associated with stingless bee found in Thai natural habitats; 2) evaluation of extraction procedures used for actinobacterial pigment preparation; 3) application of extracted actinobacterial pigments for the production of artificially clayed flowers.

3. Materials and methods

3.1 Isolation of actinobacteria from stingless bee hives

Actinobacteria associated with stingless bee hives were isolated by the procedure as described following: 1 g of stingless bee hives was microbially enriched with 50 mL of sodium caseinate broth (SCB) (2.0 g skimmed milk, 2.0 g glucose, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g $FeSO_4.7H_2O$, 1.0 g sodium propionate, 1.0 L distilled water) and incubated at 30 ^oC at 120 rpm for 24 h. Then, the medium enriched actinobacterial growth was pipetted onto sodium caseinate agar (SCA) and actinobacteria were isolated by spread plate technique. The experimental plates were incubated at 30 ^oC for 5-7 days. The actinobacterial colonies were purified and collected according to the standard protocols and they were used for next studies.

3.2 Phylogenetic identification of actinobacteria isolated from stingless bee hives.

Based on phylogenetically 16S rDNA sequence analysis, The collected strains of actinobacteria associated with stingless bee hives were identified. Genomic DNA was extracted from actinobacterial cells using BioFact^{TM} Genomic DNA prep Kit (Biofactory, Korea) followed by the protocol as described by the company. The chromosomal DNA of the actinobacteria was amplified by PCR with universal primers for actinobacteria to obtain the 16S rDNA (Lane, 1991) with BioFact™ *Taq* DNA Polymerase (Biofactory, Korea). The purified 16S rDNA was collected by BioFact™ Gel & PCR Purification System (Biofactory, Korea) before sequencing was done by the protocol of Bionics (Korea). Phylogenetic identification and tree based on partial 16S rDNA sequence analysis was done and constructed by the Neighbor-joining method in the package of MegaX (Kumar et al., 2018). Morphologically, colonial and cell morphologies were studied (Williams, Hinnebusch & Donahue, 1989) and used in polyphasic approach that were concentrated on phylogenetic and morphological results.

3.3 Extraction of actinobacterial pigments

Actinobacteria were cultivated by the protocol of Abraham & Chauhan (2018) on medium containing broken- milled rice as carbon and energy sources. The protocol was briefly described as follows: 40 g of broken- milled rice was put into 250 mL Erlenmeyer flask before soaking with tap water for 30 min. The soaking water was rinsed and autoclaved. After that, actinobacterial inocula with an initial cell and spore concentration equivalent to McFarland No.0.5 (1.5 x 10^8 CFU/mL) were used. 1mL of actinobacterial inocula were inoculated onto the sterile media and incubated at 30 ºC for 7 days before actinobacterial pigment extraction. The actinobacterial mycelia obtained were prepared the crude pigments by soaking and extraction with 100 mL ethyl acetate at room temperature for 2 days. Therefore, the supernatant containing the extracted pigments was evaporated by rotary evaporation.

3.4 Application of actinobacterial pigments in artificially clayed flower ingredients

Actinobacterial pigments obtained from previous steps were used as component of artificial clay for handicraft flower production in the 95-99:1-5 ratio (white colour-based clay:actinobacterial pigments ingredient) before homogeneous clay was finally prepared. The prepared clay was used for production of artificially clayed flowers.

4. Results

4.1 Isolation of actinobacteria from stingless bee hives

In this study, a total of 8 actinobacterial strains were isolated from stingless bee hives. All strains of actinobacteria were cultivated on broken-milled rice in order to evaluate the pigment production and assess the deep-shade colours of the obtained actinobacterial pigments. Based on the deep-shade colours presented, 4 actinobacterial strains, namely C2, C4, E1 and E2, were selected for actinobacterial pigments production in the next step. Colony morphologies of 4 selected strains of pigment-producing actinobacteria were shown in figure 1. Four actinobacterial strains cultivated on the SCA medium different shades of actinobacterial pigments, including yellow (C2), violet (C4), blue (E1) and orange (E2).

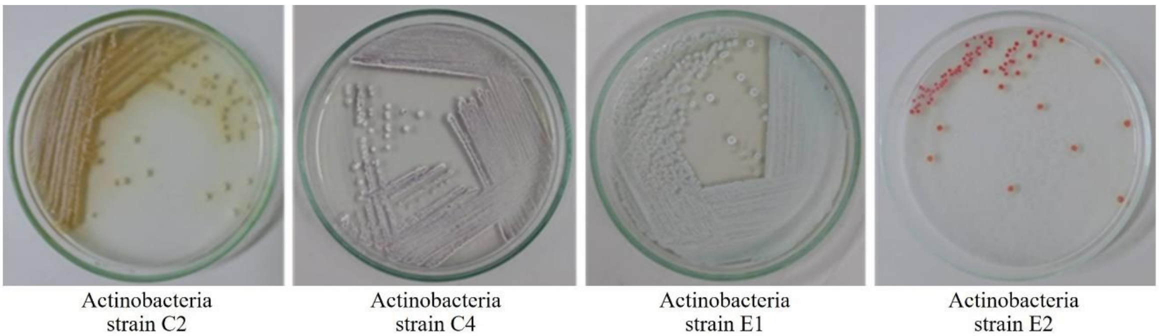

Figure 1 Colony of actinobacteria C2, C4, E1 and E2 on sodium caseinate agar

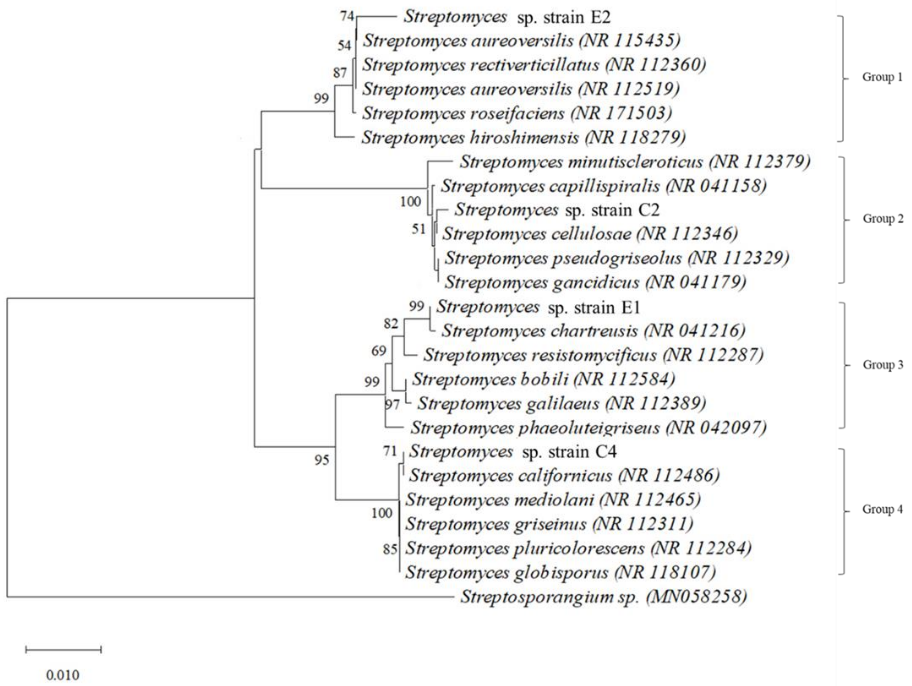

Figure 2 Phylogenetic tree of 4 *Streptomyces* spp. strains C2, C4, E1 and E2 constructed by Neighbor-Joining statistics method with Tamura 3-parameter based on the alignment of 16S rDNA nucleotide sequences and other *Streptomyces* species. *Streptosporangium* sp. was assigned as the out-group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale bar corresponds to 0.01 substitutions per nucleotide position.

4.2 Phylogenetic identification of actinobacteria isolated from stingless bee hives.

Based on partial 16S rDNA sequence analysis, 4 strains previously selected were identified. They shared phylogenetically sequences with their closest species as follows: strain C2 phylogenetically shared the 16S rDNA sequence 99.6 % similarity with its closest species, namely *Streptomyces cellulosae*; strain C4 had the phylogenetic relationship closed to *Streptomyces californicus* (99.9% similarity of 16S rDNA), strain E1 was a closest member belonged to *Streptomyces chartreusis* (99.9% 16S rDNA sequence similarity), and strain E2 was phylogenetic strain closed to *Streptomyces aureoversilis* on the basis of 99.2% 16S rDNA sequence similarity. A phylogenetic tree was constructed (Figure 2)

Morphologically, a total of 4 actinobacterial strains were gram-positive. Two mycelium types, namely substrate and aerial mycelia, were found. They shared morphological characters with the genus *Streptomyces*.

Based on polyphasic characteristics, they belonged to *Streptomyces*, and close to 4 phylogenetic relatives as mentioned above.

4.3 Extraction of actinobacterial pigments

Ethyl acetate was used as a solvent to extract the actinobacterial pigments grown on broken-milled rice. After extraction step, the crude pigment extracts were statically put onto the room temperature for 2 days in order to obtain the deep-shade of actinobacterial pigments. The results indicated that four actinobacterial strains cultivated on the medium containing broken-milled rice yielded different shades of actinobacterial pigments, including yellow (C2), violet (C4), green (E1) and pink (E2) as shown in Figure 3.

Streptomyces sp. strain C2 | *Streptomyces* sp. strain C4 | *Streptomyces* sp. strain E1 | *Streptomyces* sp. strain E2

Figure 3 Concentrated actinobacterial pigments

Figure 4 The materials made in this study before production of flowers (A), and artificially clayed flowers were made from actinobacterial pigments clay mixed with the white colour-based ingredients (B)

4.4 Application of actinobacterial pigments in artificially clayed flower ingredients

To produce artificially clayed flowers, the white colour-based ingredient was mixed with the actinobacterial pigments extracted from a total of 4 actinobacterial strains used in this study. The ratios designed were 95-99:1-5. The colour shades were yellow, violet, green and pink. When we mixed the yellow-and pink-actinobacterial pigment, the orange-colour shade was obtained. The artificially clayed flowers were made from these materials as shown in Figure 4.

5. Discussion

A total of 8 actinobacterial strains were isolated and collected from stingless bee (*Tetragonilla collina* Smith, 1857) hives. After phylogenetic and morphological identification, they belonged to the genus *Streptomyces* that was concomitant with many scientific reports as follows: *Streptomyces* were isolated from stingless bee (*Trigona* and *Partamona*) hives (Morais, Calaça & Rosa, 2013; Promnuan et al., 2013).

A total of 4 strains were selected on the basis of pigment production and deep-colour shades. Initially, they were identified phylogenetically. The results showed that they belonged to *Streptomyces* and shared phylogenetic relationship with several *Streptomyces* species, namely C2 closed to *S. cellulosae* (99.9% 16S rDNA sequence similarity, C4 had the phylogenetic relationship closed to *S. californicus* (99.9% similarity of 16S rDNA), E1 was a closest member belonged to *S. chartreusis* (99.9% 16S rDNA sequence similarity), and E2 closed to *S. aureoversilis* on the basis of 99.2% 16S rDNA sequence similarity. As we known, *Streptomyces* is a common actinobacterial species found in terrestrial habitats, including soils, plant and animal materials, and stingless bee (Promnuan, Promsai, & Meelai, 2020; Rodríguez-Hernández, et al., 2019; Promnuan et al., 2013).

Morphologically, a total of 4 actinobacterial strains were gram-positive. Two mycelium types, namely substrate and aerial mycelia, were found. Comparative studies indicated that all strains shared phenotypic characteristics, especially morphological and cultural characteristics of *Streptomyces* (Goodfellow et al., 2012).

Actinobacterial strains were cultivated on medium containing broken-milled rice as carbon and energy sources. Cells obtained from cultivation were used for pigment extraction with ethyl acetate. The actinobacterial pigment shades obtained from 4 strains, namely C2, C4, E1 and E2, were yellow, violet, green and pink. However, the pigments produced from these strains cultivated on SCA were yellow, violet, blue and orange colours compared with the same strains cultivated on broken-milled rice as previously mentioned. We thought that the chromophoric groups of actinobacterial pigments were modified when we cultivated these strains in different kinds of media. The colour shades of actinobacterial pigments were also changed after ethyl acetate extraction due to the chromophoric groups of actinobacterial pigments were structurally affected by the solvent used. The colours obtained from extraction were evaluated and the pigments produced from 4 strains of actinobacteria were selected for next study based on the coloured shades: yellow, violet, green and pink.

Application of actinobacteria, especially *Streptomyces* relatives, have been reported in many scientific publications and efforts are being made to up-scale them for industrial use (Khushboo et al., 2022; Kumar et al., 2022; Al-Dhabi et al., 2019). The species closed to our 4 strains, including *S. cellulosae, S. californicus, S. chartreusis* and *S. aureoversilis*, were applied and evaluated for medical, pharmaceutical, agricultural and industrial utilization, including biocontrol agent, anti-virus, anti-fungi, anti-bacterial, anti-biofilm, anti-fouling, anti-cancer, strepchazolins A and B, tunicamycin, calcimycin, DyP-type peroxidases, anti-oxidants, chitinase, lipase (Xu et al., 2022; Yayci et al., 2022; Abo-Zaid, Matar & Abdelkhalek, 2021; Al-agamy et al., 2021; Arend & Bandow, 2021; Hamed, Abdrabo & Youssif, 2021; Zulfa et al., 2021; Abo-Zaid et al., 2020; Singh & Dubey, 2020; Ortega et al., 2019; Werten et al., 2019; Boran, 2018; Rani et al., 2018; Tan et al., 2018; Widdick et al., 2018; Yang et al., 2017; Sripiroj, Tanasupawat, & Suwanborirux, 2008).

As mentioned above, we did not find application of actinobacterial pigments in art and folk handicraft, including production of artificially clayed flowers as we described in this publication. Importantly, our works are results of innovative research and we are the pioneer in this field in Thailand. Benefits of this research are to solve the problems, such as use of synthetic or chemical colour in handicrafts industry that is toxic to living organisms and caused of environmental pollution (Yadav et al., 2022; Murcia Mesa et al., 2021; Ajò et al., 2019; Konstadakopulos, 2008). Synthetic colours, including acrylic colours, are imported from European and other countries, leading our country to spend a lot of money on these colours contributing to a trade deficit (Hagan & Poulin, 2021; Ferreira et al., 2004; Gilbert & Cooke, 2001). The actinobacterial pigments are alternative choices for handicraft making that had important benefits for eco-friendly and green industries, especially handicraft industry (Yadav et al., 2022; Nurcahyanti et al., 2021; Indrayani & Triwiswara, 2020). In the future, we must research in depth, especially modification of materials containing actinobacterial pigments that presented the different shades of clay-colours. Upscaling of actinobacterial cultivation and production of coloured clay are essential for SMEs entrepreneur in Thailand. Additionally, pigment characterization should be analyzed by physical and chemical procedures, such as FT-IR and LC-MS.

6. Conclusion

A total of 8 strains of actinobacteria associated with stingless bee hives were isolated and tested in order to selected the strains produced the colour shades of pigment when they were cultivated on the designed media. Then, the 4 strains were selected and phylogenetically identified. They closely related to 4 species of *Streptomyces*, namely *S. cellulosae*, *S. californicus*, *S. chartreusis* and *S. aureoversilis*, that shared 16S sequence similarities to be 99.2-99.9%. Phenotypically, they shared morphological and cultural characteristics with *Streptomyces*. After ethyl acetate extraction of the actinobacterial cells cultivated on the media containing broken rice mill as carbon and energy sources, the actinobacterial pigments collected were yellow, violet, green and pink. These colours were used as ingredients of the clays used in production of artificial clay flowers that were potentially produced SMEs products of Thailand.

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