

## Original article

# Isolation and Characterization of Endosymbiotic Bacteria Producing Pigments from Dye-Yielding Plants in North Central Timor Regency

Maria Yasinta Moi<sup>1\*</sup>, Ni Putu Yuni Astriani Dewi<sup>1</sup><sup>1</sup>Department of Biologi Education, Faculty of Teacher Training and Education, Timor University

## Abstract

The use of biopigments has emerged as an alternative solution to minimize the risks associated with synthetic dyes. Biopigments, also known as natural dyes, can be extracted not only from plants but also from bacteria. Biopigments are biodegradable, making them more environmentally friendly and safer for health. This research is important to conduct as it aligns with the SDGs on Good Health and Well-Being. The research was carried out in several stages, starting from isolation, followed by 16S rRNA molecular characterization, biochemical activity tests, and pigment extraction and characterization. Out of 15 pure isolates obtained, four showed the highest potential. Based on the results of Gram staining, biochemical testing, and molecular identification, isolate HTB1 was identified as closely related to *Pseudomonas putida* strain OS-18, HTB2 to *Pantoea* sp., and HTD2 to *Bacillus subtilis* strain G-13. The production of the yellow pigment in this experiment resulted from the solvent extraction process (please specify the solvent used). Characterization of the extracted pigment using UV-Vis revealed a maximum absorbance at 452 nm, which is characteristic of carotenoid pigments. The FTIR analysis further supported this identification.

Keywords: Isolation, Characterization, Endosymbiotic Bacteria, Pigment, Dye-Yielding Plant

Received: December 14, 2025 | Revised: April 21, 2026 | Accepted: April 30, 2026

## Introduction

Endophytes are those microorganisms that inhabit inside the plants, especially leaves, stems, and roots, and show no apparent harm to the host (Moi et al., 2018a). Almost all classes of vascular plants and grasses examined to date are found to host endophytic organisms (Abidin et al., 2018). Different groups of organisms, such as fungi, bacteria, actinomycetes, and mycoplasma, are reported as endophytes of plants. Endophytes are presumably ubiquitous in plants, which depend on the host species and location, and they have been recognized as a valuable source of novel bioactive metabolites (Khanam et al., 2015). Utilizing microorganisms (especially the fungi) to produce some chemicals and bioactive compounds by fermentation is highly promising because it could be further enhanced by optimizing the fermentation techniques or improving the seed of microorganisms. The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine which including carotenoids, flavonoids, anthocyanins, and some tetrapyrroles (Daramola & Awojobi, 2025).

In recent years, interest in synthetic pigments has declined due to their toxic, carcinogenic, and teratogenic risks; consequently, microbial sources have gained atten-

tion as safer alternatives (Kumar et al., 2022). Several species of algae, fungi, and bacteria have been exploited commercially for the production of pigments (Chaturwedi et al., 2023). Microbial pigments are of industrial interest because they are often more stable and soluble than those from plant or animal sources (Pallath et al., 2023). Microorganisms can grow rapidly, which can lead to high productivity and can produce a product throughout the year (Abidin et al., 2018). Over the past decade, numerous secondary metabolites from these microorganisms have been evaluated for their potential applications as medicinal and agrochemical agents (Venil et al., 2020). Nowadays, the utilization of natural pigments in food and feedstuff has increased due to the marketing advantages of employing natural ingredients. Bacterial groups are significant natural-pigment sources because bacterial pigments could easily be produced in high quantities via fermentation technology and the potential application of metabolic engineering tools. Many of the endophytic bacterial strains have attracted special attention because they have the capability of producing different colouring pigments with high chemical stability (Agarwal et al., 2023; Muhammad et al., 2024)

Bacterial pigments, which are remarkable natural compounds, have garnered the interest of industry in recent times (Das & Singh, 2024). Bacterial pigments are produced as secondary metabolites under specific growth media and conditions. The pigments do not have a proven purpose in the growth of the organisms, but they do aid in shielding the cell from the detrimental effects of ultraviolet light. Pigment-producing bacteria are found everywhere, although they are more common in environments that are stressed due to physical, chemical, and biological factors (Bashir et al., 2022). Pigmented microorganisms (chromogenic microorganisms) have been discovered in

\* Corresponding Author:

Maria Yasinta Moi

Department of Biologi Education, Faculty of Teacher Training and Education, Timor University

Phone: 082330116380

E-mail: mariayasinta@unimor.ac.id

many environmental and geographic conditions, such as marine, aerial, and terrestrial environments. The pigment-producing organisms identified are bacteria, microalgae, and archaea, namely Haloarchaea (Daramola & Awojobi, 2025). Carotenoids are the most prevalent and possess a more intricate molecular composition compared to other microbial pigments, indicating their presence in a diverse range of organisms. A wide range of microorganisms, such as *Deinococcus sp.*, *Myxococcus sp.*, *Rhodobacter sp.*, *Erythrobacter sp.*, *Planococcus sp.*, *Rhodococcus sp.*, *Corynebacterium sp.*, *Micrococcus sp.*, *Anabaena sp.*, and *Cryptococcus sp.* have been reported to produce about 1200 distinct carotenoids (Muhammad et al., 2024). Owing to their significant potential as cost-effective, safe, and environmentally friendly substitutes, bacterial pigments have been utilized as innovative colourant's, fortifiers for food, and vitamins (Daramola & Awojobi, 2025). The study of bacterial pigment production has become a burgeoning research area, highlighting its versatility across multiple sectors. Beyond their traditional role as natural colorants in the food and cosmetic industries, these pigments are increasingly recognized for their significant therapeutic potential, including antimicrobial, antioxidant, antiviral, and anticancer properties (Kumar et al., 2022). This study aims to isolate and identify bacteria that have the ability to produce pigments from natural habitats such as plants used for natural dyes. The pigments from these isolates are processed for production, extraction, and characterization of the types of pigments produced.

## Methods

### Sample Collection

The plant material was collected from various places in the North Central Timor Regency. For isolation of endophytes, healthy and mature plants of Ta'um (*Indigofera tinctoria* L.), Koto (*Phaseolus lunatus* L.), Bauk Ulu (*Morinda citrifolia* L.), Meko (*Phyllanthus reticulatus* Poir) were carefully chosen and collected during August 2025. Leaves, roots, seeds, and stems of selected plants were carefully cut out and packed in sterile zip-lock plastic bags. Collected samples were brought to the laboratory.

### Surface Sterilization, Isolation and Culture of the Endosymbiotic Bacteria

Plant organs in the form of roots, stems, and leaves from the Ta'um, Koto, Bauk ulu, and Meko plants were cleaned under running tap water to remove attached dirt. Samples were surface-sterilized successively with 70% ethanol for 5 min, rinsed with sterile water, and then treated with 7-8 drops of Tween20 for 10 min for gentle reduction in surface tension and to allow the sterilizing agent to reach into niches and grooves beyond the epidermal cells (Moi et al., 2018). This was followed by sequential immersion of each plant part in 2% bavistin solution for 10 min, then in 0.1% HgCl<sub>2</sub> for 10 min under a Biological Safety Cabinet. To remove the disinfectants, explants were rinsed three to four times with sterilized distilled water, then blotted into autoclaved tissue paper (Kandasamy & Kathirvel, 2024). The surface-sterilized explants were cut into approximately 0.5 x 1x 0.5 cm

pieces using a sterile blade and placed on nutrient agar petri-dishes. The plates with explants were sealed using paraffin and incubated at 37± °C in order to recover the maximum possible endophytes. The observation is recorded after six to seven days. After a few days, the bacterial cultures were repeatedly streaked to achieve pure endophytic isolates. The isolates were preserved on nutrient agar (NA) slants and stored at 4 °C.

### Morphological Identification of the Endosymbiotic Bacteria

For observing the general colonial morphology, the strain was cultured on NA plates at 37±1 °C for 24 hrs. Macroscopic characteristics of the colony such as the shape, size, color, surface texture, morphological elevation, form, and margin, were recorded by using the criteria described by Harley and Prescott (2007).

### Confirmatory test for carotenoids

Bacterial colonies were inoculated into LB broth, incubated at 37 °C at 120 rpm for 3 days. After the desired incubation, the broth was centrifuged at 4 °C at 8000 rpm for 10 min. The pellet was washed repeatedly using distilled water and centrifuged. and mixed with methanol and incubated at 60 °C for 15 min. The supernatant was filtered through Whatman No.1 filter paper and collect the orange-pigmented precipitate was mixed with Sulfuric acid and water in a ratio of 1:9. The appearance of blue color is the confirmatory test of carotenoid (Pallath et al., 2023).

### Extraction of pigment and spectrophotometric analysis

For the extraction of pigment, the bacterial cells were first grown for 72 hours in nutrient broth, followed by centrifugation at 3000rpm for 20 minutes. The pigment-containing layer (pellet/supernatant) was taken, mixed with 10 ml 95% (v/v) methanol for extraction (Pallath et al., 2023). The colored methanolic supernatant extract was collected and filtered through Whatman No. 1 filter paper. The filtrate was poured in an evaporating dish and incubated at 50 °C hot air oven for 30 min to obtain a dry pigment extract free of solvent. The mass was stored at 4 °C until further use (Pallath et al., 2023). The pigment extracted with methanol was characterized using an FTIR spectrophotometer (SHIMADZU 1730 model, JAPAN) in diffuse reflectance mode operating at a resolution of 0.2 cm<sup>-1</sup>.

### Molecular Identification of Potential Endosymbiotic Bacterial Isolates

Endosymbiotic bacterial isolates then potentially subjected to molecular identification. The DNA extract obtained was amplified using a Biorad PCR machine in 30 µl of a solution consisting of 15 µl of Nexpro PCR Master Mix, 1.5 µl of DNA template sample (100 ng/µl), 11.5 µl of water, and 3 µl of primer (5 pmol each forward and reverse primer). The primers used were 16S 27F and 16S 1492R'. Amplification was carried out with the following temperature settings: pre-denaturation at 95°C for 2 minutes, then continued with 20 cycles consisting of denaturation at 95 °C for 2 minutes, annealing at 55 °C for

30 seconds, and extension at 72 °C for 1 minutes. Next, the post-extension process was carried out at a temperature of 72 °C for 5 minutes. The PCR results were then electrophoresed on 1% agarose (Moi et al., 2018). The PCR results were then sequenced using Widya Life Science services, Indonesia. The sequencing results in the form of DNA base sequences were aligned using MEGA 11 software. The alignment results were further analyzed using BLAST on the NCBI website (<http://www.ncbi.nlm.nih.gov>) to determine the species of potential endosymbiotic bacterial isolates.

## Results and Discussion

The process of isolating endosymbiotic bacteria uses Nutrient Agar and Starch Casein Agar media. Sterilized plant sample pieces are placed on the surface of the solid media and incubated at 37°C. Endosymbiotic bacteria from natural dye plants begin to show growth around the plant sample pieces with an incubation time of 48 hours

for Koto (*Phaseolus lunatus* L.) and Ta'um (*Indigofera tinctoria* L.) plants, whereas for Bauk ulu (*Morinda citrifolia* L.) and Meko (*Phyllanthus reticulatus* Poir) plants, the incubation time is 72 hours. Endosymbiont bacteria isolated from Hoto plants were given the codes HTB, HTD, and HTA. Bacterial isolates from Ta'um plants were given the codes TRB, TRD, and TRA. Bacterial isolates from Bauk ulu plants were given the code BUB. Meanwhile, bacterial isolates from Meko plants were given the code MTB. Endosymbiotic bacterial isolates showed diversity in terms of colony morphology, which is presented in Table 1 below.

Observation of isolates in Table 1 was carried out macroscopically based on the shape, color, and edges of different colonies representing different isolates. Each colony with a different morphology was separated into one pure isolate, which was inoculated on NA medium by spotting in Figure 1.

### Biochemical Tests and Molecular Identification

Table 1. Colony Morphology of Endosymbiotic Bacteria

Isolate Code	Morphology Characterization					
	Shape	Colour	Margin	Teksture	Surface	Elevation
HTB1	Circular	Dark yellow	Entire	Butyrous	Shiny	Convex
HTB2	Circular	light yellow	Entire	Dry	Shiny	Convex
HTB3	Circular	brownish yellow	Undulate	Dry	Rough	Rata
HTD1	Irregular	milky white	Undulate	Viscid	Smooth	Flat
HTD2	Circular	brownish yellow	Entire	Dry	Smooth	Flat
HTA1	Circular	transparent white	Entire	Dry	Shiny	Convex
HTA2	Circular	milky white	Undulate	Viscid	Smooth	Convex
TRD1	Circular	Creamy	Entire	Dry	Smooth	flat
TRD2	Irregular	Dark yellow	Lobate	Butyrous	Rough	flat
TRD3	Circular	milky white	Entire	Dry	Smooth	convex
TRA	Irregular	transparent white	Undulate	Viscid	Shiny	convex
TRB	Irregular	milky white	Undulate	Dry	Wrinkled	flat
BUB1	Circular	milky white	Entire	Butyrous	Shiny	convex
BUB	Irregular	light yellow	Lobate	Butyrous	Shiny	flat
MTB	Circular	milky white	Entire	Viscid	Smooth	convex

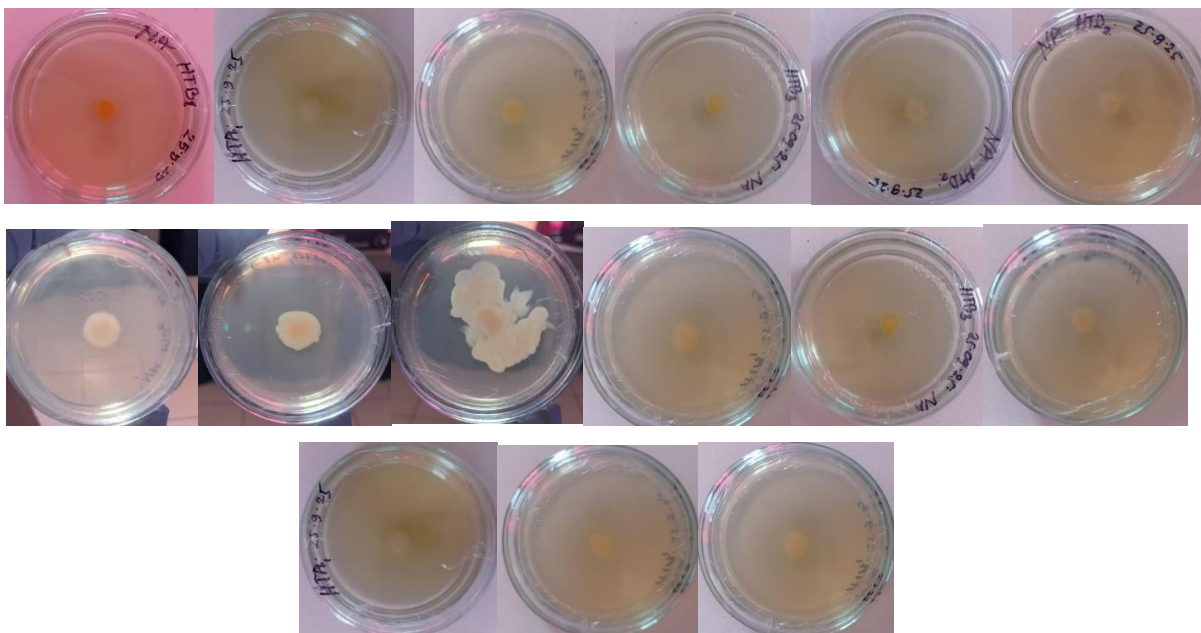


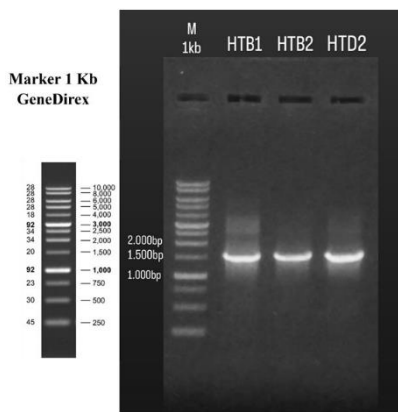
Fig. 1. Colony morphology of Endosymbiotic Bacteria Isolates on NA medium

Potential endosymbiotic bacterial isolates were then subjected to biochemical activity tests and molecular identification to determine the endosymbiotic bacteria. The results of morphological observations and

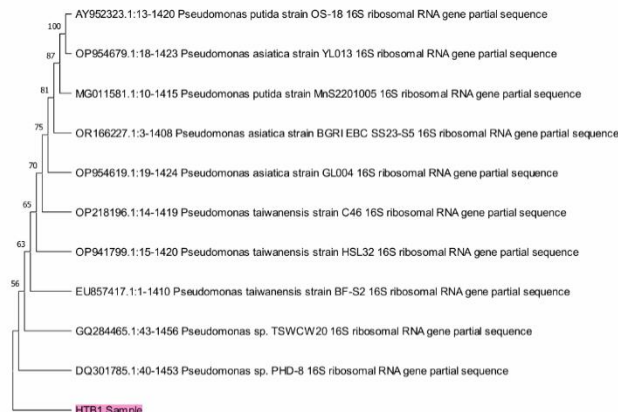
biochemical tests of potential endosymbiotic bacterial isolates are presented in Table 2.

**Table 2.** Results of morphological observations and biochemical tests of potential isolates

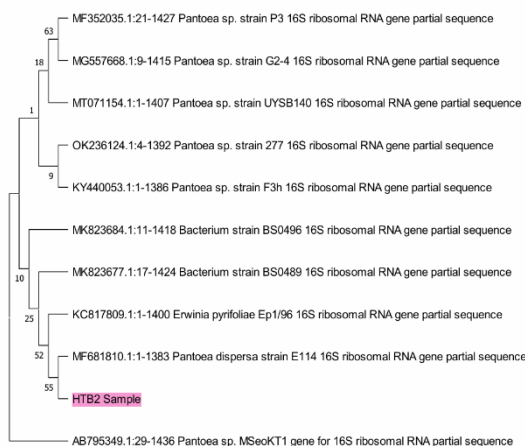
Isolate Code	Indicator Test						Cell Shape
	Catalase	Sitrate	H <sub>2</sub> S	Oxidase	Koagulase	Gram	
HTB1	+	+	+	+	+	-	Bacilli
HTB2	-	+	-	-	-	-	Bacilli
HTB3	-	+	+	+	-	-	Bacillus
HTD1	+	-	+	+	+	+	Monococcus
HTD2	+	-	+	-	+	+	Bacillus
HTA1	+	+	-	-	+	-	Diplobacilli
HTA2	+	+	+	+	+	-	Bacilli
TRD1	-	-	-	-	+	+	Diplobasil
TRD2	-	-	-	+	-	+	Bacillus
TRD3	-	+	-	+	+	-	Monococci
TRA	+	-	+	-	-	-	Bacillus
TRB	+	+	+	+	+	+	Monococci
BUB1	-	+	-	-	+	+	Diplobacilli
BUB	+	+	-	-	-	+	Monococci
MTB	+	-	+	-	+	+	Bacilli



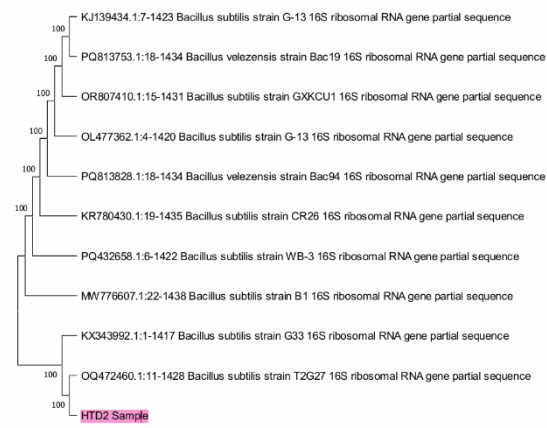
(a)



(b)



(c)



(d)

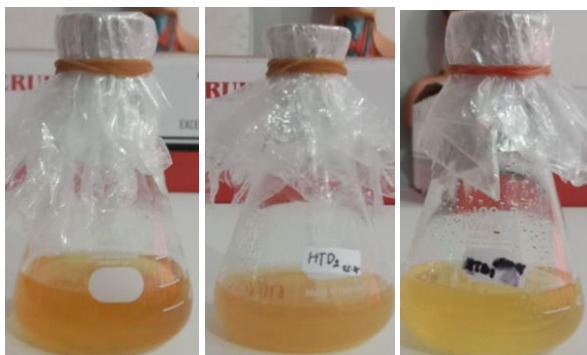
**Fig. 3.** (a) PCR amplification of single 1.5 kb of 16 S rDNA amplicon 16S rDNA gene; (b,c,d) Phylogenetic tree of endosymbion bacteria of isolates HTB1, HTB2, and HTD2 based on 16S rDNA sequences

From the 15 isolates obtained, three isolates with the potential to produce yellow pigment were selected, namely isolates HTB1, HTB2, and HTD2. These three isolates come from the Hoto plant. The preliminary identification of the bacterial isolate was revealed by Bergey's Manual Determinative Bacteriology (Table 2) and Molecular Identification. The genomic DNA of

HTB1, HTB2, and HTD2 was isolated, quality was checked using Agarose gel electrophoresis (Fig.3a), and was captured under UV light using the Gel Documentation System (Bio-rad). The DNA visualization results showed that all samples successfully demonstrated amplification of the 16S rRNA gene, approximately 1500 bp in length. The sequencing reaction was done in PCR

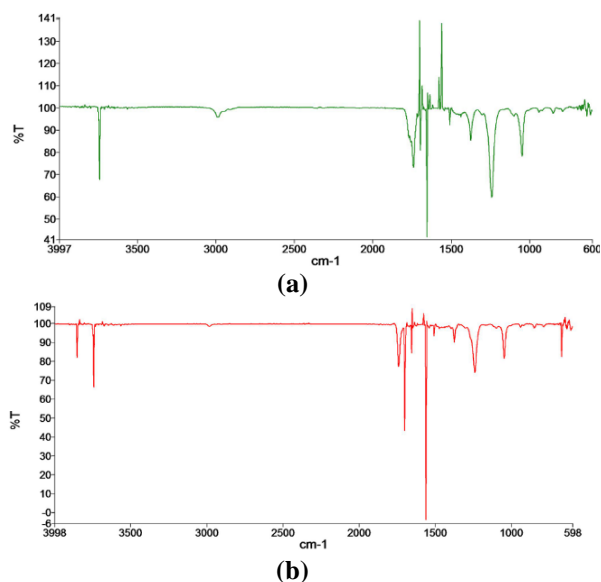
thermal cycle and sequence quality was checked using sequence scanner software v1 (Applied Biosystem). Sequences were BLAST in NCBI, showing that HTB1 was 89,66% similarity with *Pseudomonas putida* strain OS-18 accession number ID: AY952323.1; HTB2 isolate was 99,06% similarity with *Pantoea* sp. strain 277 Sequence ID: OK236124.1, and the HTD2 isolate was similar to *Bacillus subtilis* strain G-13 Sequence ID: KJ139434.1. Evolutionary analyses were conducted in MEGA11. The tree was constructed with a maximum likelihood tree, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and 100 repeated numbers used with the traditional rectangular method (Fig.3b-d).

Pigment production was quantified the optimum conditions like, pH7, incubation time 72hours by using the nutrient broth medium. The extracted pigment in methanol was quantified 2.064 g /L (Fig.4). In UV-VIS spectroscopy, maximum absorbance of yellow colored pigment was obtained at 452 nm. It was confirmed the presence of carotenoid pigment. Devi et al. (2024) stated that maximum absorption at a wave length of 400 – 500 nm using an acetone solvent will absorb several color spectra/pigments. Specifically, at a wavelength of 448 nm ( $\alpha$ -carotenoid), 480 nm (astaxanthin), and 452 nm ( $\beta$ -carotenoid and zeaxanthin).



**Fig. 4.** The optimization process of yellow pigments quantification by using nutrient broth medium (optimum pH7 at 72h)

The FTIR spectrum report was analyzed and interpreted corresponding to the standard peak values  $3461.69\text{ cm}^{-1}$  Symmetrical stretching of Hydroxyl group ( $-\text{OH}$ ),  $1450.26 - 1544.65\text{ cm}^{-1}$  stretching vibration presence of aromatics ( $\text{C} = \text{C}$ ),  $1239.76\text{ cm}^{-1}$  stretching vibrations presence of fenolik compounds ( $\text{C}-\text{O}$ ),  $1085.62$  and  $1653.40-1695.88\text{ cm}^{-1}$  stretching vibration presence of flavones (Fig.5a). Similar studies reported the FTIR bands at  $1653-1661\text{ cm}^{-1}$  indicates the presence of chlorophyll  $1424-1426\text{ cm}^{-1}$  as  $-\text{C}-\text{H}(\text{CH}_2)$  bending vibration from methylene of carotenoids or lycopene,  $1366-1367\text{ cm}^{-1}$  band as the  $\beta$ -ionone of  $\beta$ -carotene due to the  $\text{C}-\text{H}$ , ( $-\text{CH}_3$ ) symmetrical bending (Trivedi et al., 2017). FTIR spectrum of extracted pigment from bacteria *Bacillus subtilis* PD5, showed similarity to  $\beta$ -carotene in fingerprinting patterns of peaks. It indicates that the extracted pigment is a  $\beta$ -carotene or carotenoid derivative (Trivedi et al., 2017). Analysis of pigment highlighted the stretching of different functional groups with peaks  $1661$ ,  $1653$ ,  $1654$ ,  $1655\text{ cm}^{-1}$ , respectively (Devi et al., 2024; Stannius et al., 2024).



**Fig. 5.** Characterizations of identified pigment producing bacteria (a) FTIR spectrum of carotenoid pigment from bacterial species HTB1; (b) FTIR spectrum of carotenoid pigment from bacterial species HTD2

Based on previous studies, *Pantoea* sp. is known to produce various bioactive compounds. For example, lycopene can be cyclized to  $\beta$ -carotene by lycopene  $\beta$ -cyclase (CrtY) and subsequently hydroxylated to zeaxanthin by  $\beta$ -carotene 3,3'-hydroxylase (CrtZ), compounds that are also found in higher plants (Pandya et al., 2023). Carotenoids offer crucial functions beyond merely creating red, pink, and yellow hues. These compounds are highly valuable across the medical, pharmaceutical, and food sectors, where they act as a vital source of vitamin A, powerful anti-cancer agents, effective antioxidants, and as natural dyes for textiles, food, and beverages (Daramola & Awojobi, 2025; Kumar et al., 2022; Pringgenies & Riniatsih, 2014). Crucially, obtaining natural pigments from bacteria provides significant logistical benefits over sourcing them from animals or plants, chiefly by eliminating the requirement for extensive land use and lengthy harvesting periods. Consequently, pigments can be generated quickly when bacteria are cultivated in a bioreactor (Khanam et al., 2015). Ultimately, bacterial pigments hold the potential to become a sustainable and primary resource for various industries, satisfying the demand for natural coloring agents. Bacterial pigment production represents a superior alternative to traditional plant-based extraction due to its rapid growth kinetics and independence from seasonal or climatic constraints (Daramola & Awojobi, 2025). While botanical sources are limited by geographical variability and extensive land requirements, microbial pigments are produced in controlled bioreactors, ensuring standardized quality and high batch-to-batch consistency (Muhammad et al., 2024). Furthermore, the scalability of bacterial fermentation is enhanced by the ability of microorganisms to utilize agro-industrial waste as a cost-effective substrate, significantly reducing the environmental footprint compared to large-scale plantations (Kandasamy & Kathirvel, 2024). This shift away from synthetics would significantly mitigate the health concerns currently associated with synthetic pigment consumption. Recommendation: As the safety profile of this strain and its derived

products has not yet been established, further studies are required to verify that the strain is non-toxic and non-pathogenic before its application as a food or textile colorant.

## Conclusion

In this study, 15 Endosymbiotic bacteria have been isolated from various dye-yielding plants. Isolates HTB1, HTB2, and HTD2 from Hoto (*Phaseolus lunatus* L.) plant were selected as potential best pigment producers based on the confirmation test results of carotenoid pigment content. This selection was supported by the results of morphological identification, biochemical tests, and 16S rDNA gene molecular analysis, isolate HTB1 was 89,66% similarity with *Pseudomonas putida* strain OS-18; isolate HTB2 was 99,06% similarity with *Pantoea* sp. strain 277, and isolate HTD2 was similar to *Bacillus subtilis* strain G-13.

## Acknowledgment

We would like to thank the DPPM Kemendikisaintek for providing the author with the opportunity in the form of research grant funds for the 2025 fiscal year, which will enable us to carry out this research effectively. Thank you to the Biology Education Laboratory, Timor University, and the NTT Provincial Health Laboratory UPTD for providing facilities during the implementation of the study.

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