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Exploring The Potential Of Bacterial Pigments As Antioxidants

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Abstract:
Background*: Antioxidants can be obtained from various sources, including bacterial pigments, which are natural compounds with great potential in various applications. Therefore, this study aimed to explore microbial pigments as antioxidants from various sources, to identify isolates macroscopically, microscopically, and molecularly, as well as to conduct bacterial antioxidant tests followed by molecular identification.*
Materials and Methods*: Materials and chemicals used in this study were nutrient broth, bactoagar, KIT gram staining alcohol 70%, aquadest, DPPH, (2,2-diphenyl-1-picrylhydrazyl), methanol, ethanol, isolation KIT DNA Favorgen, 1X TAE buffer, PBS, gel agarose, GelREd, Master Mix, universal primer 27F 907R and ddH2O. The antioxidant activity of each isolate was determined using a DPPH (2,2-diphenyl-1-picrylhydrazyl) test, and the molecular identification of each isolate was based on the analysis of its 16S rRNA gene using the BioEdit and MEGA X programs.*
Results*: The results of the antioxidant test showed that the bacterial isolates M.1, AS-12, and U.5 had strong antioxidant potential, while R.1 had moderate potential. These isolates were identified using classical and molecular methods. The 16S rRNA gene region analysis showed that M.1, AS-12, and U.5 were identified as Cytobacillus kochii, Bacillus aryabhattai, and Sagittula marina, respectively*.
Conclusion: *This study yielded the molecular identification of the pigmented bacterial isolates, namely M.1 as Cytobacillus kochii, AS-12 as Bacillus aryabhattai, and U.5 as Sagittula marina. Antioxidant testing showed that isolate M.1 exhibited a very strong antioxidant category, suggesting its potential to produce pigment extracts with high antioxidant properties. The antioxidant potential of the isolates was categorized into very strong for M.1, very strong for AS-12, and moderate for U.5.*
Key Word: *microbial pigment, antioxidant, 16S rRNA, macroscopically, microscopically, molecularly*

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I. Introduction

Antioxidants are compounds that can prevent the oxidation process of free radicals, and their activity can be determined using a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The DPPH (α,α-diphenyl-β-picrylhydrazyl) free radical capture method is an approach to evaluate the antioxidant potential of a compound, extract, or other biological source. In this assay, the compound or extract is mixed with a DPPH solution, and the colour change is measured over time [1].

Antioxidants can be obtained from various sources, one of which is bacterial pigments. Bacteria that produce pigments are referred to as chromogenic bacteria. The pigments produced by these bacteria are a result of their normal metabolic processes, and the spectrum of colours produced includes black, white, brown, gold, silver, fluorescent green, yellow and blue. The specific colour of the pigment is indicative of the bacteria. Notably, pigmented bacterial cultures can exhibit a multitude of colours. The significance of pigments extends beyond mere aesthetic applications in the cosmetic and food industries; they have also been documented to possess antioxidant properties. Bacteria are a significant source of pigments and can serve as an alternative for pigment synthesis due to their enhanced biodegradability and favourable compatibility with the environment [2].

The pigments derived from plants possess several drawbacks as compared to pigments from bacteria e.g., instability against adverse pH, heat or light, low solubility in water, and are often consistently not available. Certain properties make microbial pigments an emerging field of research and of great interest for various

applications [3]. Furthermore, Micro-organisms produce pigments with lower residue and higher fermentation yield as compared to animals and plants [4].

Microorganisms can thrive in a variety of environments, each of which possesses distinct and unique characteristics. Moreover, the rapid rate of propagation exhibited by these organisms can be advantageous in the context of antioxidant production [5] and [6]. According to an investigation, Kochuria sp bacteria are capable of producing pigments with antioxidant properties. Bacteria can produce extracellular or intracellular pigments as a defense mechanism against environmental stressors, such as protection from UV light [7].

Certain bacterial strains are capable of synthesizing secondary metabolites in the form of pigments, which exhibit a wide spectrum of colours. Previous studies reported that bacteria produce various pigments, including carotenoids and their derivatives (e.g. β-carotene, astaxanthin, canthaxanthin, zeaxanthin), prodigiosin, pyocyanin, melanin, and violacein. This study was conducted to explore microbial pigments as antioxidants from various sources, and to identify isolates macroscopically, microscopically, and molecularly. During the experimental phase, molecular identification was carried out using the 16S rRNA gene with its corresponding sequence. The DNA sequence was deposited in GenBank, a database maintained by the National Centre for Biotechnology Information (NCBI), with the accession number.

II. Material And Methods

Materials

Materials and chemicals used in this study were nutrient broth, bactoagar, KIT gram staining alcohol 70%, aquadest, DPPH, (2,2-diphenyl-1-picrylhydrazyl), methanol, ethanol, isolation KIT DNA Favorgen, 1X TAE buffer, PBS, gel agarose, GelREd, Master Mix, universal primer 27F 907R and ddH2O.

Bacterial Isolation and Selection

The sample obtained was weighed precisely to 1 gram and subsequently crushed using a porcelain cup. Subsequently, dilutions were executed to about 10^{-3}, 10^{-4}, and 10^{-5} concentrations. Each dilution series was dispensed with 0.1 mL using a micropipette and cultured by the spread plate method. The cultures were then subjected to an incubation period of 24 hours at 30°C, and coloured colonies were purified and stored for further study.

Subculture of Pigment Producing Bacteria

Bacterial colonies that appeared red were meticulously selected and subcultured on nutrient agar medium by quadrant streak method. The cultures were then incubated at room temperature for 72 hours. Pure and isolated colonies from previously streaked plates were subsequently inoculated on nutrient agar slant and in nutrient broth and then grown at optimal conditions.

Macroscopic observations were carried out by growing isolates on NA medium using the streak plate method. Then the shape, color, edge, texture, and surface of the colony was observed. Microscopic observations were done by cleaning the slide with alcohol and dried it on the spritus lamp. Then 1 ose of bacterial suspension was dropped aseptically on the slide, 1-2 drops of violet crystal was added for 1-2 minutes and washed with distilled water and then dried it in the air. Next, as much as 2-3 drops of lugol was added and waited for 1 minute, washed with distilled water and then air dried. The preparation slide was dropped with 96% alcohol drop by drop until it were clean and washed with distilled water then air dried. Next, he preparations were dropped with safranin as much as 1-2 drops, left for 1-2 minutes then washed with running water and air dried. The cell shape was observed using a microscope with a magnification of 1000x.

Biopigment Production

Pre-cultures were grown in 5 mL of NB medium and then incubated at room temperature for a time that was determined to be overnight. Subsequent pre-cultures were inoculated into 1 mL of NB production medium. Pigment production was then undertaken in 50 mL of NB in a 100 mL Erlenmeyer flask. The flasks were then placed in a shaker incubator at room temperature, with a shaking speed of 120 rpm, for seven days.

Pigment Extraction

After seven days of incubation in an incubator shaker, alterations in colour were observed. Pigments were extracted using a centrifuge at 6000 rpm for 15 min at 12 °C. The pigment-containing pellet and its supernatant (the liquid separated from the pellet by centrifugation) were mixed with 10 mL of 95% methanol for extraction. The resulting mixture was then filtered using Whatman No. 1 filter paper. The filtrate was evaporated at 50°C for 30 minutes to yield a dry pigment extract free of solvent. The resultant pigments were then stored at 4°C until further use.

Pigment Antioxidant Activity of Pigment Coarse Extract

Antioxidant testing of bacterial pigments was carried out using the DPPH method as described by [8]. The stock solution of crude enzyme extract was prepared at a concentration of 1000 ppm, and from this, standard solutions of 20, 40, 60, 80, and 100 ppm were then created and then left in darkness at room temperature for 30 minutes. The DPPH solution was measured at a wavelength of 400-800 nm to obtain the maximum absorption and wavelength. The percentage of inhibition was calculated for the standard solution using the following formula.

$$\%inhibition = \left(\frac{Ab - As}{Ab}\right) x\ 100\%$$

Note:
Ab : Blank absorbance = DPPH absorbance value
As : Sample absorbance = sample absorbance value

Molecular Identification of Bacterial Isolates Based on 16S rRNA Gene

The selection of isolates was based on the 16S rRNA encoding gene. The 16S rRNA gene was amplified using primers 27F and 907R, and the bacterial culture genome was isolated using Favorgen, following the manufacturer's instructions. The purity and concentration of the DNA were measured using a Nanodrop Spectrophotometer. The 16S rRNA gene was then amplified using universal primers 27F (forward: 5'-AGAGAGTTTGATCMTGGCTCAG-3') and 907R (reverse: 5'-CTTGTGCGGGCCCGTCAATTC-3'). The predenaturation stage was conducted at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds, an elongation stage at 72°C for 1 minute and 30 seconds, and a post-elongation stage at 72°C for 7 minutes. The post-PCR process was conducted at 4°C for 5 minutes.

The subsequent stage of the procedure involved the visualization of the PCR products using electrophoresis on a 1% agarose gel in 1x TAE buffer. The voltage applied was 70 V for 25 minutes, after which the DNA bands were stained with fluorosafe and visualized using a UV illuminator. After this, the PCR products were subjected to sequencing at the 1st BASE DNA Sequencing Service, Malaysia. The resulting data were then analyzed using the MEGA X program, by aligning 16S rRNA sequences at NCBI using BLAST-N. Phylogenetic tree construction was carried out using MEGA X software with the Neighbor-Joining method with a bootstrap value of 1000 times.

III. Result

Bacterial Isolation and Selection

A total of 28 pigment-producing bacterial isolates were obtained from a variety of samples, including three isolates from rhizosphere samples, nine isolates from river water samples, four marine bacterial isolates, one estuary bacterial isolate, and 12 rhizosphere bacterial isolates. A total of four isolates from different environments were characterized macroscopically and microscopically, after which the pigment antioxidant test was conducted. The macroscopic and microscopic characteristics of the isolates are presented in Table 1 below.

Table 1. Macroscopic and microscopic characteristics of bacterial isolates from different environments.

Isolate Code	Source	Characteristics					
		Colony Morphology				Cell Morphology	
U.5	Macroalga	Yellow	Round	Convex	Flat	-	Coccus
M.1	Estuary Water	Yellow	Round	Convex	Flat	-	Bacil
AS-12	River Water	Yellow	Round	Flat	Not flat	-	Bacil
R.1	Rhizosphere	Cream	Round	Convex	Flat	-	Bacil

Macroscopic characteristics of the 4 isolates are all round, the elevation shape of 3 isolates is convex and 1 is flat. From the edge characteristics there are 3 isolates with flat edges and 1 isolate is uneven. In terms of the color of the isolates there is 1 isolate in cream color, and 3 isolates are yellow. The results of gram staining as microscopic characteristics show that all four isolates are gram-negative bacteria. Isolate U.5 is in the form of a bacillus, while isolates M.1, AS-12, and R.1 are in the form of cocci. [9] and [10] isolated gram-negative and coccus-shaped marine pigment bacteria. [11] obtained isolates of pigment bacteria from soil with coccus-shaped and gram-negative cell characters.

Pigment Extraction

The extraction of pigments using methanol solvent in all isolates, according to [1] The utilization of methanol in the extraction of pigments has been demonstrated to yield colours that are distinguished by their high degree of saturation. Researchers have delineated pigment extractions from four isolates as exhibiting characteristic yellow and cream colours. Bacterial pigments have potential for industrial applications, [12] explain

that using bacteria as a pigment source offers several advantages. It is inexpensive and easy to produce, requires minimal space for cultivation, and does not raise ethical concerns. Their research about identification of bacteria producing red pigments and their application in the textile industry.

Antioxidant Testing

Antioxidant tests on crude pigment extracts were grouped based on their ability to degrade DPPH by 50%. The IC50 values are presented in Table 2.

Table 2. Antioxidant activity values of crude extracts of bacterial pigment samples.

Pigment Coarse Extract	DPPH Antioxidant Activity IC_{50} (µg/mL)	Antioxidant Activity Category
Vitamin C	4,716	Very strong
U.5	138,139	Moderate
M.1	15,924	Very strong
AS-12	86.032	Strong
R.1	61,444	Strong

Pigments represent a class of metabolites that are produced by a variety of bacterial species. As a comparator, vitamin C, which has well-documented antioxidant capacity, was utilized. The isolates obtained in this study exhibited antioxidant activity levels that ranged from moderate to very strong. The antioxidant activity of bacterial pigment isolate U.5 was classified as moderate with a value of 138.139 µg/mL, while M.1 and R.1 were categorized as strong, with IC_{50} values of 86.032 µg/mL and 61.4444 µg/mL, respectively. Furthermore, three isolates exhibited a strong level of antioxidant activity with an IC_{50} value of less than 50 µg/mL. Isolate M.1 exhibited particularly strong antioxidant activity, with an IC_{50} value of 15.942 µg/mL, suggesting its potential for use in relevant biological contexts. A study conducted on bacterial isolates from the rhizosphere of the sea identified the presence of orange and yellow pigments obtaining the same colony morphological characteristics.

The extraction process using methanol solvents has been shown to cause the disintegration of cell walls, although the efficiency is relatively low and the process is time-consuming [13]. The application of thermal extraction treatment, utilizing a temperature of 50°C, has been shown to enhance the extraction rate. This extraction stage consists of the processes of mixing the substrate and solvent, and subsequently, phase separation. This method has been shown to bind compounds (pigments) without causing damage to the target compounds, and the solvent is capable of binding the pigments contained in the supernatant. The solvent is then subjected to evaporation at temperatures ranging from 40°C to 50°C, resulting in the extraction of the crude pigment extract. The antioxidant activity is categorized into five distinct classes based on the IC50 values, namely very strong (IC50 < 50 µg/mL), strong (50 - 100 µg/mL), moderate (101 - 250 µg/mL), weak (250 - 500 µg/mL), and inactive (>500 µg/mL [14]. M.1 has the highest IC50 value, which is 15.924 µg/mL, while isolates U.5, AS-12, and R.1 have strong antioxidant activity. As marine bacteria, isolate M.1 and U.5 could become a fine alternative as source of natural pigment from marine and not damaging marine ecosystem. Another advanted is they easy to be cultured in laboratory. At least, there are 7 kinds of pigment that can be found from marine bacteria. There are prodiginines, carotenoids, violacein, phenazine compound, quinones, melanins [2]. Among those pigments, carotenoids have attractive research object for researcher due to their biological activity that useful.

As marine bacteria, M.1 and U.5 may develop into a fine substitute for other sources of natural pigment from the ocean without endangering the marine ecology. They also have the advantage of being simple to cultivate in a lab. Marine bacteria can produce at least seven different types of color. [15] Prodiginines, carotenoids, violacein, phenazine compounds, quinones, and melanins are among them. Because of their beneficial biological action, carotenoids are an appealing research object among those pigments. Similar results for antioxidant from marine bacteria were observed by several researchers such as [16] dan [17] that show marine microbial pigment potential as antioxidant. Meanwhile, [18] reported *Rhodococcus* spp. has an antioxidant activity isolated from soil.

Molecular Identification of Bacterial Isolates Based on 16S rRNA Gene

The bacterial isolates identified were found to possess antioxidant abilities that were moderate, strong, and very strong. Three isolates were selected as gram-negative bacteria, namely U.5, M.1, and AS-12. The 16S rRNA gene amplification results were visualized on electrophoresis, which showed the length of the DNA fragment obtained was 800-900 bp (Figure 1). Subsequent analysis of the consensus sequences was conducted using BioEdit software, with the forward and reverse sequences being analyzed separately. The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (http://blast.ncbi.nim.nih.gov).

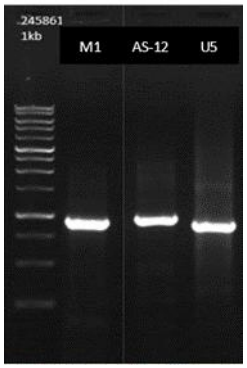

Figure 1. Electrophoresis of isolates M.1, AS-12, and U.5.

Table 3. BLAST Nucleotides of Isolate M.1

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
***Cytobacillus kochii* strain H 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	OQ195933.1
***Bacillus kochii* strain DDWB 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	MK537364.1
***Bacillus* sp. SV14 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	GU143796.1
***Cytobacillus kochii* strain BL-YJ0 5-22 ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	MZ674048
***Bacillus kochii* V25 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	MK229091.1
Bacterium W15C5a 16S ribosomal RNA gene, partial sequence	1509	1509	100%	0.0	99.64%	JQ043044.1
***Cytobacillus solani* strain DOS-CAP-2 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	MF076230.1
***Bacillus* sp. Strain IAE16 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	MK414919.1
***Cytobacillus kochii* strain EpV25 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	MZ452524.1
***Bacillus pocheonensis* strain BPSCM 16S ribosomal RNA gene, partial sequence**	1509	1509	100%	0.0	99.64%	KX161844.1

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Table 4. BLAST Nucleotides of Isolate AS-12.

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
***Bacillus aryabhattai* strain Pg120-1 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	MK519189.1
***Priestia aryabhattai* strain CT07 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	PP838165.1
***Bacterium* strain NS1432 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	MK824620.1
***Bacillus aryabhattai* strain NGB-SF105 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	MK318226.1
***Bacillus* sp. Strain HM.12 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	OR.131092.1
***Priestia aryabhattai* strain SP2 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	PQ278296.1
***Bacillus aryabhattai* strain ZS3-5 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	OL304880.1
***Bacillus aryabhattai* strain WC12-1 16S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	PQ510638.1
***Bacillus aryabhattai* strain SX1016S ribosomal RNA gene, partial sequence**	1476	1476	100%	0.0	98.78%	MT378548.1

Table 5. BLAST Nucleotides of Isolate U.5.

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
***Sagittula marina* strain XY-148 16S ribosomal RNA gene, partial sequence**	1358	1358	99%	0.0	99.46%	OR262791.1
***Sagitulla* sp. Strain T-29 16S ribosomal RNA gene, partial sequence**	1267	1267	99%	0.0	97.20%	MH650987.1
***Sagitulla* sp. Strain CfWS2d 16S ribosomal RNA gene, partial sequence**	1267	1267	99%	0.0	97.20%	MN099588.1

***Sagitulla* sp. RKEM 724B 16S ribosomal RNA gene, partial sequence**	1267	1267	99%	0.0	99.57%	KU198756.1
***Sagitulla marina* strain F028-2 16S ribosomal RNA, partial sequence**	1267	1267	99%	0.0	97.20%	NR 109096.1
***Sagitulla* sp. Strain MA-2 16S ribosomal RNA gene, partial sequence**	1249	1249	99%	0.0	96.79%	OQ842247.1
***Sagitulla* sp. F1 16S ribosomal RNA gene, partial sequence**	1247	1247	99%	0.0	96.79%	EU697073.1
***Sagitulla stellata* strain P11 16S ribosomal RNA gene, partial sequence**	1247	1247	99%	0.0	96.79%	MZ276329.1
***Sagitulla stellata* strain CAU 1629 16S RNA gene, partial sequence**	1243	1243	99%	0.0	96.66%	MW020256.1

A comparison was made of the 16S rRNA gene sequences of isolates M.1, AS-12, U.5 and R.1 with reference sequences in GenBank, with Query Cover values > 97% indicating homology. Based on the results of the BLAST analysis, it was determined that isolate M.1 has 100% homology to the bacterium *Cytobacillus kochii*, while isolate AS-12 has 100% homology to *Bacillus aryabhattai* bacteria. [19] According to a study, *Cytobacillus kochii* has antioxidant, antimicrobial, and catalytic potential. [20] It was reported that a strain of *Bacillus aryabhattai*, isolated from wastewater, produced a peroxidase enzyme capable of reducing phenol. In this study, the isolate from river water was identified as *Bacillus aryabhattai*.

Isolate U.5 has 99% homology to the bacterium *Sagittula marina* [21] The isolated *Sagittula marina* samples obtained from the marine waters of Pohang, South Korea, were characterized as rod-shaped cells with a diameter of 0.5 mm and a length of 1.0 mm. Following a three-day incubation period at 25 °C, colonies formed on MA exhibited a circular, convex, intact, and smooth morphology, with a diameter ranging from 1.0 to 3.0 mm. Growth occurred at temperatures ranging from 4-30 °C (optimal at 25 °C), at pH values between 6.5-9.0 (optimal between pH 7.0 and pH 8.0), and with 1-7% (w/v) NaCl (optimal at 3.0%).

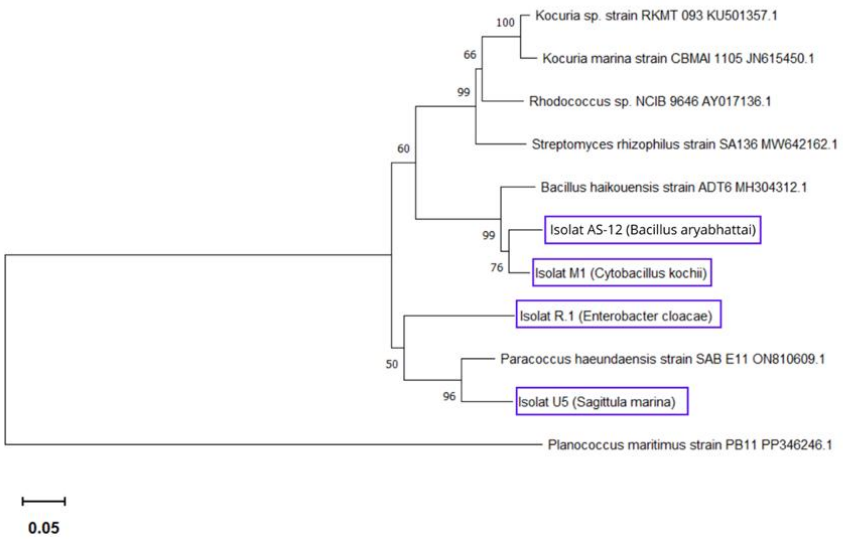

Figure 2. Phylogenetic tree of bacterial cultures

The constructed phylogenetic tree demonstrates the evolutionary relationship between the bacterial isolates and several antioxidant-capable bacteria, as determined by DNA sequence analysis. Isolate M.1 (*Cytobacillus kochii*) exhibited a significant phylogenetic relationship with *Bacillus haikouensis*, with a bootstrap value of 76%. Isolate AS-12 (*Bacillus aryabhattai*) exhibited a highly significant relationship with *Bacillus haikouensis*, as indicated by a bootstrap value of 99%, suggesting substantial sequence similarity. Isolate R.1 (*Enterobacter cloacae*) formed a distinct branch with a greater evolutionary distance, suggesting a more distant relationship compared to the other isolates. [22] reporting the antioxidant activity produced by *Enterobacter cloacae* with sulfate modification to enhance antioxidant activity.

Conversely, Isolate U.5 (*Sagittula marina*) exhibited a close phylogenetic relationship with *Paracoccus haeundaensis*, as shown by a bootstrap value of 96%. [21] explain phylogenetic analysis based on 16S rRNA gene sequences showed that strain F028-2T formed a lineage within the family Rhodobacteraceae of the class

Alphaproteobacteria, and was closely related to members of the genera *Sagittula* and *Antarctobacter* with 96.3–96.4% sequence similarities. In contrast, reference bacteria such as *Kocuria marina* and *Kocuria* sp. formed a clade with a bootstrap value of 100%, signifying a very close relationship within the same genus. *Rhodococcus* sp. and *Streptomyces rhizophilus* appear to be in a more distant clade from the isolates. In general, the isolates show strong phylogenetic affinities with species of the same genus or closely related in the corresponding phylum.

IV. Conclusion

This study yielded the molecular identification of the pigmented bacterial isolates, namely M.1 as *Cytobacillus kochii*, AS-12 as *Bacillus aryabhattai*, and U.5 as *Sagittula marina*. Antioxidant testing showed that isolate M.1 exhibited a very strong antioxidant category, suggesting its potential to produce pigment extracts with high antioxidant properties. The antioxidant potential of the isolates was categorized into very strong for M.1, very strong for AS-12, and moderate for U.5.

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