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The antibacterial activity and silk dyeing of the crude pigment extract from J4 actinobacteria strain

Sittichai Urtgam[1,2], Tawatchai Sumpradit[3] and Naruemol Thurnkul[4]*

[1]Biology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University
[2]Center of Excellence for Biodiversity, Naresuan University
[3]Department of Microbiology and Parasitology, Faculty of Medical Sciences, Naresuan University
[4]Microbiology Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University

*Corresponding author; E-mail naruemol.t@psru.ac.th

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Abstract

Actinobacteria represent a vast variety of prokaryotic microbes that produce a diverse group of secondary metabolites used in medical, agricultural, and industrial applications. The objectives in this study were to investigate the antibacterial activities and potential for silk dyeing of the crude extract from the actinobacterial strain J4. Initially, the antibacterial activities of the crude pigment extracts obtained from the actinobacterial strain J4 isolated from the wasp's nest were determined. To extract the water-insoluble pigments used for antibacterial activity testing via the agar well diffusion method, ethyl acetate was applied during the pigment extraction. These extracts showed an inhibitory effect against *Salmonella* Typhimurium TISTR1472, *Bacillus subtilis* PSRU-01, and *Enterococcus faecalis* TISTR459. Secondly, evaluation of silk dyeing was carried out. We used the extractant as 70 % (v/v) ethanol in the alternative extraction process when the strain J4 was cultivated by solid-state fermentation with broken-milled rice to prepare actinobacterial dye for silk dyeing. The pigment extract obtained was tested for color stability based on washing methods, and the sensitivity to light was determined according to ISO105-C06:2010(E) and ISO 105-B02:2014(E), respectively. The color stability and sensitivity of the pigment extract to washing showed scores between 3-5, and the sensitivity to light demonstrated scores between 3-4. Taxonomically, the strain J4 was dark-violet colonies, and its water-soluble pigment diffused on sodium caseinate agar (SCA). To identify the strain J4, the phylogenetic identification based on partial sequencing of 16S rDNA was applied. The identification result indicated that the strain J4 was closely related to *Streptomyces coacervatus* (99.8% sequence similarity). In future, antibacterial activities of the crude extracts obtained from actinobacteria strain J4 should be tested with other food-borne pathogens. The development of Streptomyces dyes should also focus on the stability of the dyes during the silk dyeing process. For dyeing other fibers, we plan to work with both synthetic and natural fibers.

Keywords**:** *Antibacterial activity; Silk dyeing; Actinobacterial pigment*

1. Introduction

In the developing countries, multidrug-resistant strains of pathogenic bacteria pose significant health problems, causing illness and death to thousands. In order to solve these problems, the discoveries of the new drugs are important. Actinobacteria constitute a significant group, producing 45% of bioactive compounds synthesized by secondary metabolic pathways, compared to other groups of microorganisms like fungi (38%) and bacteria (17%) (Jose, and Jebakumar, 2013; Smaoui et al., 2012).

Actinobacteria are classified as gram-positive bacteria with free-living and saprophytic

lifestyles. They are predominantly found in soil, with thousands of actinobacterial strains producing bioactive compounds used in medicine and other applications. These metabolites are novel compounds exhibiting antibacterial, antifungal, anticancer, and antimycobacterial properties, as well as antioxidant, antienzymatic, and insecticidal activities. Additionally, the pigments extracted have been reported to be useful in the dyeing industry (Conlon et al., 2004; 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; Locci, 1989). Furthermore, crude pigment extracts of actinobacteria have been used as dyes for silk, cotton, and wool (Janković et al., 2023; Kramar, & Kostic, 2022; Chen et al., 2021; Urtgam, & Thurnkul, 2021; Kulkarni et al., 2014; Chakraborty et al., 2015). Actinobacterial dyes used in the textile industry are advantageous due to their safety and eco-friendliness, given that these dyes are naturally occurring in living organisms, especially actinobacteria, making them an appealing alternative to dyes derived from plants, animals, minerals, and fungi (Suomi, 2001; Velmurugan et al., 2010).

In this study, we isolated, and selected the actinobacterial strain J4 from a wasp's nest. The colony is dark-violet in color and produces water-soluble pigments when cultivated on media supplemented with broken-milled rice. The results suggest that these pigments could be developed into new bioactive compounds for application in the biopharmaceutical and textile industries.

2. Objectives

To investigate the antibacterial activities of the crude extract from the actinobacterial strain J4 and evaluate its potential for silk dyeing.

3. Materials and methods

3.1 Actinobacterial cultivation, and preparation of crude dye extract

Firstly, the actinobacterial strain J4 isolated from the wasp's nest was cultivated on sodium caseinate agar (SCA) and incubated at 30 ^{o}C for 5-7 days. Secondly, two protocols for preparation of actinobacterial crude dye extracts for antibacterial activity testing were carried out as mentioned below.

3.1.1 Cultivation of actinobacteria on media

The method was modified from the protocol of Radhakrishnan et al., (2016). Firstly, the actinobacterial colony grown on SCA previously mentioned above was transferred to the new SCA plate and incubated at 30 ^{o}C for 7 days. Secondly, the SCA covered by actinobacterial growth, and showed pigmentation were cut as small square pieces, and transferred to 500 mL Erlenmeyer flask soaked with 100 mL of ethyl acetate on rotary shaker at 120 rpm for 24 h. Finally, the filtrate was prepared with Whatman paper No. 1, and then rotary evaporation was applied to remove ethyl acetate from crude dye extract.

3.1.2 Cultivation of actinobacteria on broken-milled rice

The method was modified from the protocol of Abraham, & Chauhan (2018). Firstly, 50 grams of the broken-milled rice used as actinobacterial substrate for cultivation was put into 250 mL Erlenmeyer flask soaked with tap water for 30 minutes. Secondly, the soaked water was rinsed, and the broken-milled rice obtained in this process was packed and sterilized by autoclaving. The sterilized substrate was added with 10 mL sterile water and used for actinobacterial inoculation. Thirdly, the strain J4 was used for inoculum preparation by dissolved with the sterile distilled water equivalent as McFarland No.0.5 (1.5×10^8 CFU/ml) that measured and had score between 0.08-0.1 by spectrophotometer at λ 625 nm and inoculated on the substrate prepared as mentioned above (Kermeoglu et al., 2018). 1 mL of the prepared J4 inoculum was added in the sterilized broken-milled rice and incubated at 30 ^{o}C for 7 days. Finally, the pigment extraction was done by addition of 100 mL of ethyl acetate shaking on rotary shaker at 120 rpm for 24 h. The filtrate was obtained by filtering with Whatman paper No.1 via separating funnel. Then the mixture solution of methanol, and hexane (3:5) was added to the filtrate. Finally, rotary evaporation was applied to remove ethyl acetate from crude dye extract.

3.1.3 Antibacterial Activity Testing

The antibacterial activities of crude dye extract obtained from the J4 actinobacterial strain were determined by agar well diffusion method as described by Abraham, & Chauhan (2018) and Kermeoglu et al., (2018). The protocol was described as follows:

The microbial testers are *Escherichia coli* PSRU-01, *Pseudomonas aeruginosa* PSRU-01, *Salmonella* Typhimurium TISTR1472, *Staphylococcus aureus* PSRU-01, *Bacillus subtilis* PSRU-01, and *Enterococcus faecalis* TISTR459. The cultures were grown on NA plates and incubated at 37 ºC for 24 h. The single colony of each bacterial tester was transferred into NB, and incubated on rotary shaker at 120 rpm, 37 ºC for 24 h. The inoculant was determined, and equivalent as McFarland No.0.5 (1.5 x 10^8 CFU/ml) that measured and had score between 0.08-0.1 by spectro-photometer at λ 625 nm and inoculated on the substrate prepared as mentioned above.

To test the antibacterial activities of the crude dye extract, the solution prepared mentioned above was swab on Muller Hinton Agar (MHA) and applied the cork borer with 5 mm. in order to prepare the hole before the prepared crude dye extract was fill into the hole at the final concentration as 50 µg/mL that was diluted with 20 µL of Dimethyl sulfoxide (DMSO). The positive control was 50 µg/mL of chloramphenicol, and the negative control was DMSO. All experimental sets were incubated at 37 ºC for 24-48 h, and the clear zone diameter was evaluated around the tested hole filled with the crude dye extract, and the control sets.

3.1.4 Data analysis

Data analysis was done triplicate by analysis of variance (ANOVA). The means were compared by Duncan' new multiple range test (DMRT) at 95 % of confidence with SPSS version 23 for statistical analysis.

3.2 Silk dyeing

Silk dyeing was carried out with the crude dye extract previously prepared from the actinobacterial strain J4. The details are as follows.

3.2.1 The actinobacterial dye preparation

The actinobacterial strain J4 was cultivated on the sterilized broken-milled rice soaked with tap water for 30 min and removed the tap water before autoclaving. The broken-milled rice covered by the actinobacterial J4 growth was used for preparation of actinobacterial spore suspension at the initial concentration as 10^6 spores per mL. 1 mL of spore suspension was pipetted into the sterilized broken-milled rice soaked, incubated at 30 ºC for 3-5 days. The sterilized broken-milled rice covered with the actinobacterial growth was dried at 100 ºC for 30 min before milling and kept at room temperature. The pigment dye was extracted by 70% ethanol (v/v) as described by Urtgam, and Thurnkul (2021).

3.2.2 The stability of dyeing

The stability of dyeing was evaluated by the protocol as follows: 0.3 g of silk thread was soaked into 30 mL of the actinobacterial dye solution, incubated on rotary shaker at 120 rpm, room temperature for 4 h. Then, the silk thread obtained was dried at room temperature for 24 h, and the second dyeing was done by the method mentioned before and dried at room temperature for 24 h. The excess dye was removed by washing with tap water and dried at room temperature for 24 h. The silk thread obtained was measured of stability of actinobacterial color after dyeing by CIELAB system. The stability of silk thread after dyeing was determined by washing, and light sensitivity test followed by the ISO105-C06:2010(E) and ISO 105-B02:2014(E), respectively.

3.3 Actinobacterial identification

Polyphasic identification was applied for species delineation of the strain J4. The actinobacterial DNA was extracted with BioFact™Genomic DNA prep Kit (Biofactory, Korea), and the purification of DNA was done. The 16S rDNA of the J4 actinobacterial strain was amplified by the method described by Williams (1989) and Mullis et al., (1986). The primers used in this study are universal bacterial forward primer 27F (5'-GAG TTT GAT CCT GGC TCA G-3'), and universal bacterial reversed primer 1492R (5'-GTT ACC TTG TTA CGA CTT-3') as described by Lane (1991). The PCR products were polymerized by the activities of BioFact™ *Taq* DNA Polymerase (Biofactory, Korea), and purified by BioFact™ Gel & PCR Purification System (Biofactory, Korea). The 16S rDNA sequencing was determined by the standard sequencing protocols described by the Bionics company (Korea). The blast homology search was used for determination of 16S rDNA sequence similarities between the 16S rDNA sequence of strain J4, and the same gene of the phylogenetic relatives. The phylogenetic tree was constructed by the Neighbor-joining method in the MegaX (Kumar et al., 2018). Phenotypic characteristics were also determined, such as morphological, and cultural characteristics.

4. Results

4.1 Antibacterial Activity Testing

Table 1 shows the antibacterial activity of the crude dye extract of the actinobacterial strain J4. The crude dye extract of the actinobacterial strain J4 was shown in Figure 1

.

Table 1 Antibacterial activity of the crude dye extract of the actinobacterial strain J4

Bacteria / Testing	Clear Zone Diameter (mean ± SD) (mm)					
	Gram-negative bacteria			Gram-positive bacteria		
	***Escherichia coli* PSRU-01**	***Pseudomonas aeruginosa* PSRU-01**	***Salmonella* Typhimurium TISTR1472**	***Staphylococcus aureus* PSRU-01**	***Bacillus subtilis* PSRU-01**	***Enterococcus faecalis* TISTR459**
Positive control	15.67±1.15	8.33±0.58	25.32±0.44[a]	30.79±1.14	24.40±0.01[a]	10.52±0.45[b]
Negative control	-	-	-	-	-	-
J4 (A)	-	-	11.12±1.00[c]	-	11.07±0.70[c]	9.42±0.00[c]
J4 (B)	-	-	14.92±1.11[b]	-	15.00±0.00[b]	15.42±0.24[a]

Notes: Positive control = 50 µg/ml Chloramphenicol/Negative control = DMSO (A) cultivation of J4 on SCA (B) cultivation of J4 on the broken-milled rice, [a, b, c] Statistical analysis value ($p<0.05$)

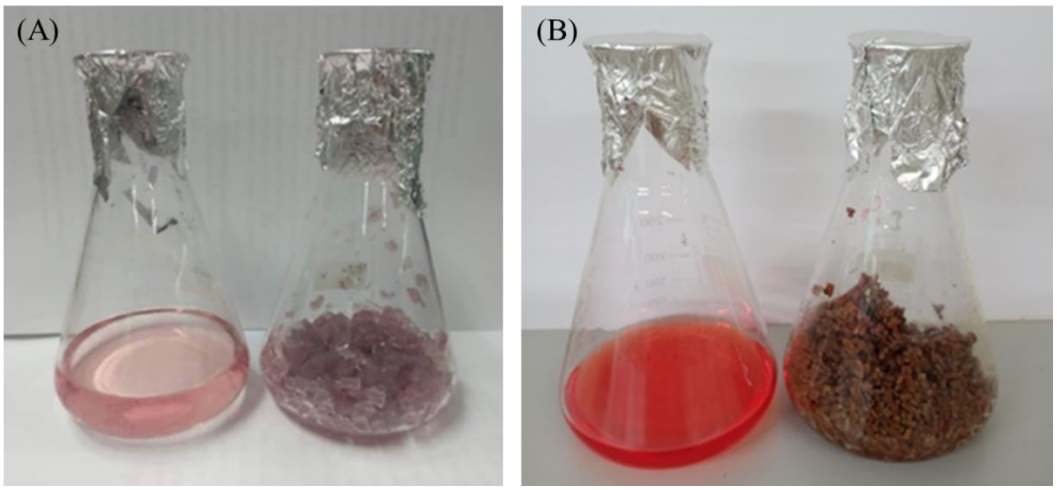

Figure 1 (A) The crude dye extract of the actinobacterial strain J4 cultivated on SCA, and (B) on the broken-milled rice.

Figure 2 (A) The dark-violet dye powder extracted from the actinobacterial strain J4, and (B) the silk thread after dyeing

4.2 Silk dyeing and the stability of dyeing

The crude dye extract from the actinobacterial strain J4 had dark-violet color presented as the pigments when cultivated the J4 on SCA, and the broken-milled rice at 30 ºC for 5 days (Figure 2 and Table 2). The crude dye extract obtained from 70% (v/v) ethanol extraction was applied for silk dyeing and was subsequently used to produce a color powder. This powder was tested for stability and sensitivity to light, specifically after the dyed silk thread was subjected to washing and light exposure.

The results showed that the silk thread presented the resistant characteristics to washing at the score 3-5 evaluated by the standard of ISO105-C06:2010(E) and ISO 105-B02:2014(E), respectively. For the test of sensitivity to light, the silk thread had the score between 3-4 on the basis of two standards mentioned above (Table 3).

Table 2 The actinobacterial dye powder color, color of silk thread after dyeing, and color score based on CIELAB system used as standard.

Color	Silk thread color	Score compared with CIELAB system				
		L	**a***	**b***	**C**	**h**
Dark violet	Violet	52.80	+13.50	-	14.60	22.20

Note: L refers to lightness, with values ranging from 0 (black) to 100 (white); a* and b* are considered chromatic coordinates: a* for red (+), and green (−), and b* for yellow (+), and blue (−). C* represents chroma, and h is the hue angle. The value of chroma C* is the distance from the lightness axis (L*) and starts at 0 in the center. Hue angle starts at the +a* axis and is expressed in degrees (0° is +a*, or red, and 90° is +b, or yellow).*

Table 3 Stability test of silk thread after dyeing with the actinobacterial dye on washing, and light.

Stability Test	Standard used	Color stability	Value Level
Washing	ISO105-C06:2010(E)	Change	3
		Color staining - cotton	4-5
		- silk	4-5
Light	ISO 105-B02:2014(E)	Change	3-4

Note: Color fastness to light; rated from number 1 (very low color fastness) to 8 (very high color fastness)
Color fastness to washing; rated from number 1 (excessive color change), 2 (considerable color change), 3 (noticeable color change), 4 (slightly color change), and 5 (no color change)

4.3 Actinobacterial Identification

The actinobacterial strain J4 was morphologically examined. The results indicated that it was gram-positive, produced substrate and aerial mycelia with retinaculiaperti (Goodfellow et al, 2012). On the basis of the phylogenetic identification by the partially compared 16S rDNA sequence analysis, the actinobacterial strain J4 was phylogenetically identified as *Streptomyces coacervatus* with 99.8 % sequence similarity.

Additionally, the strain J4 showed the phylogenetic related to *Streptomyces rameus* (98.8 % sequence similarity) *Streptomyces deccanensis* (98.4 % sequence similarity) *Streptomyces griseoruber* (98.4 % % sequence similarity), and *Streptomyces olivochromogenes* (98.4 % sequence similarity) Phenotypically, the actinobacterial strain J4 shared morphological, and cultural characteristics similar to those of *Streptomyces coacervatus* (Figure 3-4).

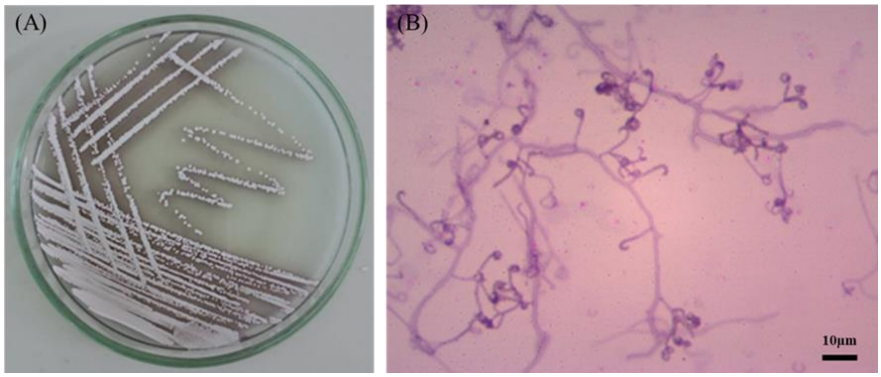

Figure 3 Morphological, and cultural characteristics of the actinobacterial strain J4 (A) Cultural characteristics of J4 on SCA (B) Morphological characteristics of J4, 1000X

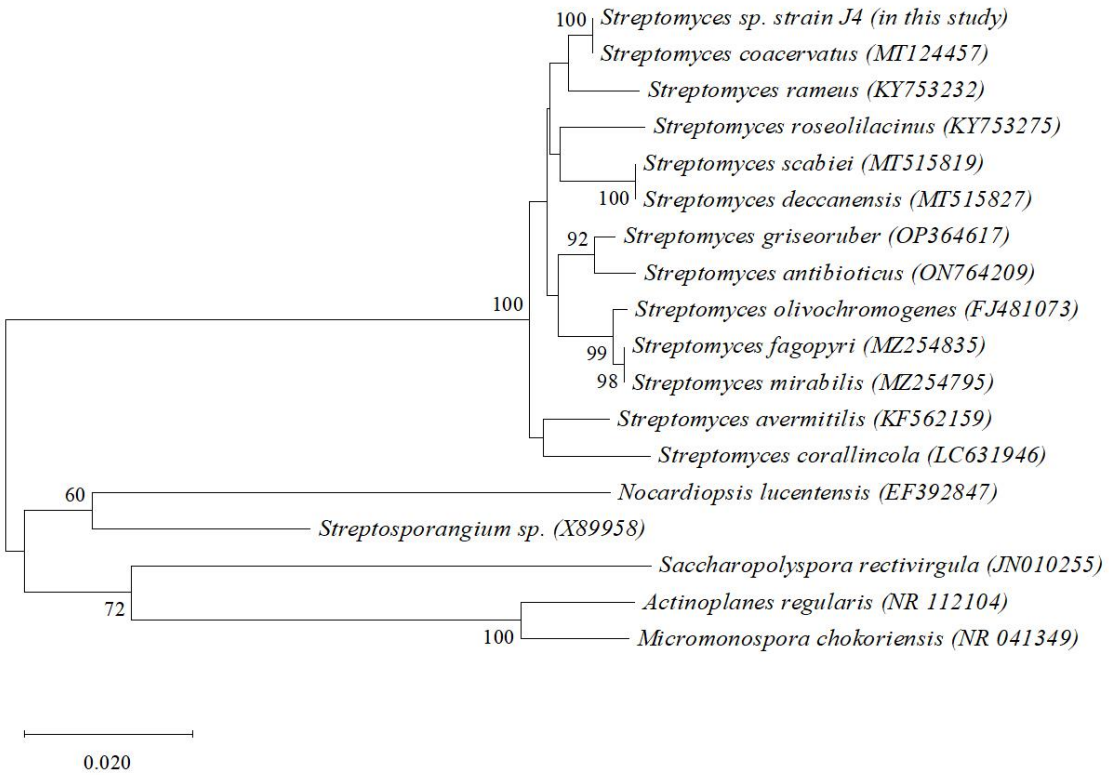

Figure 4 Phylogenetic relationship of the actinobacterial strain J4, and the closely related species belong to *Streptomyces* based on the partially compared 16S rDNA analysis constructed with MEGA X software.

5. Discussion

The crude dye extract of strain J4 cultivated on SCA, and the broken-milled rice showed the antibacterial activities inhibited growth of *Salmonella* Typhimurium TISTR1472, *Bacillus subtilis* PSRU-01, and *Enterococcus faecalis* TISTR459. However, the crude extract obtained from the actinobacterial strain J4 presented higher bacterial inhibition than one obtained from the strain J4 cultivated on SCA. We thought that the broken-milled rice contains nutrients and growth factors that supported growth and pigment production of actinobacterial strain J4. The cultivation media used as actinobacterial substrate, and solvents used for pigment extraction are the factors effecting on efficiency of antibacterial activities of strain J4 confirmed by inhibition of *E. faecalis* TISTR459 growth that was compared with the positive control. These results obtained were supported by Abraham, & Chauhan (2018) and Radhakrishnan et al., (2016). Abraham, & Chauhan (2018) found that the crude extract of Streptomyces strain JAR6 cultivated on wheat bran with solid state fermentation had antibacterial activity. The results indicated that the crude dye extract inhibited growth of *Staphylococcus aureus, Shigella, Pseudomonas aeruginosa, Enterococcus, Escherichia coli, Bacillus subtilis, Proteus mirabilis, Klebsiella pneumonia*, and *Salmonella*. Similarly, Radhakrishnan et al., (2016) found that the crude dye extract of *Streptomyces* D25 cultivated on yeast extract malt extract agar had antibacterial activity. The results indicated that the crude dye extract inhibited growth of *Staphylococcus, Lactobacillus, Alcaligenes, Bacillus, Micrococcus, Pseudomonas*, and *Kurthia.* To test the stability of J4 actinobacterial dye on the silk thread after washing, the score of stability confirmed that the crude dye extract could be developed for use in textile industry.

The phylogenetic result indicated that the strain J4 closely related to *Streptomyces coacervatus* based on 99.86 % partial 16S rDNA sequence similarity. Additionally, the strain J4 showed the partial 16S rDNA sequence similarities to certain species of *Streptomyces*, including *S. rameus* (98.8%), *S. deccanensis* (98.4%), *S. griseoruber* (98.4%), and *S. olivochromogenes* (98.4%). All species of *Streptomyces* previously described were reported as non-pathogenic

Streptomyces, and the biosynthesis of secondary metabolites accumulated in the cells was also proposed. Their secondary metabolites were applied in medicine, agriculture, and industry. However, no scientific papers were published the utilization of *Streptomyces* pigments on textile industry (Gu et al., 2020; Dohra et al., 2017; Balachandran et al., 2016; Phankhajon et al., 2016; Li et al., 2012; Wang et al., 2012; Praveen, & Tripathi, 2009). *S. coacervatus*, and their closest species were studied on antimicrobial properties as described below. Gu et al., (2022) indicated the antifungal potential of the filtrate extracted from *Streptomyces deccanensis* QY-3 onto growth of the rubber anthracnose fungus *Colletotrichum gloeosporioides. S. coacervatus* isolated from the intestinal tract of *Armadillidium vulgare* was reported the antifungal activities on *Aspergillus niger* IFM 5368, and *Candida albicans* ATCC 90028 (Shibazaki et al., 2011). Meena et al., (2022) found the antifungal potential of *S. rameus* GgS 48 against mungbean root rot caused by colonization of *Rhizoctonia bataticola.* Al_husnan, and Alkahtani (2016) found the antibacterial, and antifungal potentials of *S. griseoruber* onto bacterial and fungal pathogens, including *Staphylococcus aureus*, *Bacillus cereus*, and *Candida albicans*. In this study, we found the antibacterial potential of the pigment crude extract of the strain J4 onto *Salmonella* Typhimurium TISTR1472, *Bacillus subtilis* PSRU-01, and *Enterococcus faecalis* TISTR459. The results previously mentioned showed that *S. coacervatus*, and the closest species based on the 16S rDNA sequence similarities had antimicrobial properties, including inhibition of bacterial, and fungal pathogens used as the testers.

Our publication is the first report on application of the pigment extracts of *Streptomyces* strain phylogenetically related *S. coacervatus*, and the closest species on the textile industry. Actually, the next research in development of Streptomyces dyes should be concentrated in the stability of the dyes in dyeing process with silk. It is essential to apply the Streptomyces dye in the textile industry. For other fiber dyeing, we have plan to carry out of synthetic fibers (satin, chiffon, and polyester fibers), and natural fibers (wool, cotton, and jute fibers) dyeing (Nair et al., 2017; Chakraborty et al., 2015; Kramar et al., 2014; Kulkarni et al., 2014).

6. Conclusion

Regarding the antibacterial activities of the pigment extracts from actinobacterial strain J4, these extracts demonstrated an inhibitory effect on *Salmonella* Typhimurium TISTR1472, *Bacillus subtilis* PSRU-01, and *Enterococcus faecalis* TISTR459. Additionally, these extracts have shown good color stability and moderate sensitivity to washing (score 3-5 value), while exhibiting moderate sensitivity to light (score of 3-4). Phylogenetic identification based on partial sequencing of 16S rDNA has revealed that strain J4 is closely related to *Streptomyces coacervatus*, exhibiting a high sequence similarity of 99.8%. Moving forward, further investigations should focus on evaluating the antibacterial activities of crude extracts from strain J4 against other food-borne pathogens. Additionally, the development of Streptomyces dyes should be also focus on the stability of the dyes during the silk dyeing process. These future studies will contribute to a more comprehensive understanding of the antibacterial properties and dyeing capabilities of strain J4, paving the way for potential applications in various industries.

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