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Enhancing the production of secreted melanin by *Streptomyces* spp. using common tyrosine-containing media

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ABSTRACT: Soil-isolated bacterial strains producing black pigment, identified as *Streptomyces* spp. strains AQ2 and CQ2 through 16S rRNA gene analysis, exhibited distinct morphological characteristics. Among the tested culture media, potato dextrose agar (PDA), which contains a high concentration of the tyrosine precursor, induced the highest production of black pigment compared to malt extract agar (MEA), yeast malt extract agar (YMA), oatmeal agar (ISP3), and glycerol asparagine agar (ISP5). FTIR analysis confirmed that the black pigment closely resembles melanin, with its production significantly increasing in response to higher tyrosine concentrations. Additionally, melanin biosynthesis was investigated through the induction of secondary metabolite gene expression using chloramphenicol-supplemented media at subinhibitory concentrations (0–40 µg/ml). The results indicated that antibiotic-supplemented ISP1 (tryptone yeast extract agar), ISP6 (peptone yeast extract iron agar), and ISP7 (tyrosine agar; L-tyrosine 0.5 g/l) were less effective at promoting black pigment production than PDA. Furthermore, amplification of the tyrosinase gene *melC1* confirmed its presence in both strains, with the expected product size obtained from CQ2, while AQ2 yielded a larger amplicon. These findings suggest that *Streptomyces* spp. strains AQ2 and CQ2 utilize the tyrosinase pathway for melanin biosynthesis, which is significantly enhanced in the presence of tyrosine. Although preliminary tests demonstrated fungicidal activity of the melanin-producing bacteria against selected fungal strains, it remains unclear whether melanin alone is responsible for this activity. Further studies are required to confirm the antifungal properties of the extracted melanin. In summary, this study highlights the potential of actinomycete-derived metabolites for future antifungal applications.

KEYWORDS: melanin, tyrosine, tyrosinase, *melC*, *Streptomyces* spp., antifungal activity

INTRODUCTION

Melanin is a multifunctional biopolymer which is diversely produced among animals, plants, and microorganisms [1, 2]. Melanin primarily provides protection against environmental stresses such as ultraviolet (UV) light, oxidizing agents, and ionizing radiation that harm host cells [3]. The unique characteristic of melanin is its dark colors ranging from brown-black to red-yellow corresponding to major structure of eumelanin and pheomelanin, respectively. The difference of structural melanin depends on substrates involved in the complex biosynthesis. Microbial melanin is primarily synthesized through 2 pathways: the L-3,4-dihydroxyphenylalanine (DOPA) pathway and the 1,8-dihydroxynaphthalene (DHN) pathway. In the DOPA-pathway, tyrosine is converted to L-DOPA, which is then oxidized to dopaquinone by tyrosinase and laccase enzymes and further converted into dopachrome and then to eumelanin [4]. In addition, pheomelanin is produced through the cysteinylation of dopaquinone, generating a sulfur-containing polymer that differs from eumelanin [1, 5, 6]. The DHN pathway particularly found in fungi, in contrast, utilizes malonyl-coenzyme A as a precursor, produced via polyketide synthases that catalyze sequential decarboxylative condensations, leading to the formation of 1,3,6,8-tetrahydroxynaphthalene. Microorganisms that use the DOPA pathway are generally preferred for efficient, high-yield melanin production because harsh extraction processes are not required for the extracellular melanin they produce [4].

Most melanin is currently sourced from the cuttlefish *Sepia officinalis*. However, this method is costly, unsustainable, and heavily reliant on the availability of these natural resources [7]. Microbial melanin production has recently gained interest as a cost-effective and eco-friendly alternative to chemical synthesis [8]. Although fungal strains are good choice for melanin production, they require a long fermentation period and challenging extraction and purification steps upon the physical properties of the isolated melanin. Recently, it has been reported on the bacterial melanin production studied in the genera *Rhizobium*, *Marinomonas*, *Vibrio*, *Bacillus*, *Streptomyces*, *Serratia*, and *Pseudomonas* [1, 9]. These bacteria utilize different enzymatic pathways to synthesize melanin, which serves various ecological and industrial purposes.

Actinomycetes are Gram-positive, filamentous bacteria found in various natural environments, where they play a significant role as producers of secondary metabolites, including antimicrobial and anticancer agents, immunosuppressants, enzymes, and pigments [10]. The genus *Streptomyces* is a major source of novel secondary metabolites, contributing to drug discovery and other diverse applications. The lichen-derived *Streptomyces parvulus* strain Lp03, which produces Actinomycin D, has demonstrated both antimicrobial and antitumor activities [11]. Additionally, a new compound, pudicin, which exhibits slight antioxidative activity, was identified in *Streptomyces mimosae* 3MP-14, isolated from the root of *Mimosa pudica* [12].

Streptomyces kathirae SC-1 and *Streptomyces lusitanus* DMZ-3 produced melanin in the presence of tyrosine [13, 14]. *Streptomyces djakartensis* NSS-3 and *Streptomyces nashvillensis* DSM 40314 produced a dark brown melanin pigment extracellularly [15, 16]. *Streptomyces cavourensis* SV 21 isolated from the sea cucumber was cultured for melanin production in marine broth containing various mineral ingredients [17]. Research has been conducted on various fermentation strategies to increase melanin production, including the optimization of growth conditions such as pH, temperature, nutrients, and salts [9]. L-tyrosine, an essential precursor for melanin synthesis via the DOPA pathway, has been the focus of recent studies exploring alternative, cost-effective supplementation strategies. These studies have demonstrated that incorporating alternative sources of L-tyrosine, such as hydrolysate residues of cyanobacteria (*Arthrospira* (*Spirulina*) *platensis*) and fava bean seed peels, can enhance melanin production in *Streptomyces antibioticus* NRRL B-1701 and *Streptomyces cyaneus*, respectively [18, 19].

Imai et al [20] found that the actinomycete *Streptomyces coelicolor* A3(2) produced higher amounts of the pigmented antibiotic metabolite actinorhodin (ACT) when stimulated with subinhibitory concentration of the ribosome-targeting antibiotics such as lincomycin, chloramphenicol, and tetracycline. They found that 1/10 MIC (minimum inhibitory concentration) of lincomycin was effective for inducing the expression of genes involved in secondary metabolism in *Streptomyces* strains.

This study aimed to identify the influencing cultivation parameter for melanin production of soil isolated *Streptomyces* spp. We approached common culture media such as potato dextrose agar (PDA), yeast malt extract agar (YMA), malt extract agar (MEA), and International *Streptomyces* Project (ISP) media in the presence and absence of chloramphenicol (at subinhibitory concentrations of 0–40 µg/ml). Additionally, we have verified that melanin production is mediated through the tyrosinase pathway.

MATERIALS AND METHODS

Isolation of actinomycete bacteria

Actinomycete bacteria were isolated from soil samples collected from sunlit areas in Bangkok, Thailand, leveraging the melanin properties that enhance UV radiation resistance [13]. We aimed to induce the expression of genes involved in the secondary metabolite biosynthesis of *Streptomyces* spp. and to inhibit other well-grown bacteria. Thus, a standard 10-fold dilution plate method was performed on PDA supplemented with 50 µg/ml Chloramphenicol (Sigma-Aldrich, USA) (below the MIC of 80 µg/ml) [21]. The dilution plates were incubated at 28 °C, and 2 distinct colonies, designated AQ2 and CQ2, were selected based on differences in morphology and color among the slow-growing actinomycete colonies. The isolated strains were identified as Gram-positive bacteria with filamentous growth and long spore chains resembling fungal structures [22].

Each isolate was subsequently purified and cultured on solid YMA incubated at 28 °C for 14 days to allow sufficient growth and preserved as a spore suspension in 20% (v/v) glycerol at −20 °C for further analysis. Additionally, strains AQ2 and CQ2 were sent to the Thailand Bioresource Research Center (TBRC) in Bangkok, Thailand, for 16S rRNA gene sequence analysis.

Extracellular melanin on culture plates

The *Streptomyces* spp. strains AQ2 and CQ2 were examined on various International *Streptomyces* Project (ISP) media (HIMEDIA, India), including ISP1 (tryptone yeast extract agar, pH 7.0), ISP2 (yeast malt extract agar or YMA, pH 6.4), ISP3 (oatmeal agar, pH 7.2), ISP5 (glycerol-asparagine agar, pH 7.4), ISP6 (peptone yeast extract iron agar, pH 6.7), and ISP7 (tyrosine agar, pH 7.3) media compared to PDA medium (HIMEDIA), pH 5.7. The growth capability and pigment production were recorded every day after incubation at 28 °C for 12 days. The macroscopic morphology of their colonies was visualized by stereomicroscope.

DNA extraction and tyrosinase gene amplification

Genomic DNA was isolated by the method modified from Ravi et al [23]. For genomic DNA preparation, the *Streptomyces* spp. strains were obtained from YMA plates using 0.01% Tween80 and inoculated into 50 ml of potato dextrose broth (PDB; HIMEDIA) for cultivation at 28 °C, 200 rpm for 7 days. A liquid PDB culture of each strain (AQ2 and CQ2) was centrifuged and homogenized with 0.1 mm glass beads using TissueLyser (QIAGEN, Germany) at 30 Hz for 5 min. The homogenized samples were incubated in the 600 µl of 0.5% SDS containing TEN buffer (1 M Tris-HCl pH 7.5, 0.7 M NaCl, and 0.5 M EDTA) at 65 °C for 20 min. The genomic DNA was extracted using phenol:chloroform:isoamyl alcohol, pre-

cipitated, and dissolved in elution buffer. In addition, according to the manufacturer's instruction, the GeneAll Exgene Cell SV kit (GeneAll Biotechnology, South Korea) was the alternative method to obtain the genomic DNA. The amplification of tyrosinase genes were carried out using the specific primer sets of *melC1* gene (*melC1*F; 5′-ATGCCGGAACTCACCCGCCG-3′ and *melC1*R; 5′-CAGTTGGCGGGGAAGGGGAG-3′) and the specific primer sets of *melC2* gene (*melC2*F; 5′-ATGACCGTACGCAAGAACCAG-3′ and *melC2*R; 5′-TCAGACGTCGAACGTGTAGAAC-3′) [8].

Testing black pigment production under antibiotic stress

The *Streptomyces* spp. strains AQ2 and CQ2 were cultured in media supplemented with the antibiotic chloramphenicol, which targets bacterial ribosomes. The antibiotic was added at concentrations below its MIC, specifically at 0, 0.5, 5, 10, 20, and 40 µg/ml, to assess its effect on melanin production. The culture media used for the experiment included ISP1, ISP6, and ISP7. The goal was to identify the antibiotic concentration that most effectively stimulated melanin production [20].

Testing the antifungal activity of melanin-producing strains

The antifungal properties of the secreted black pigment by *Streptomyces* spp. strains AQ2 and CQ2 were rapidly tested on culture plates against the growth of 4 different fungi including *Aspergillus flavus* TISTR 3041, *Aspergillus niger* TISTR 3061, *Candida albicans* TISTR 5554, and *Talaromyces marneffei* ATCC200051. The method was adapted from the bioassay agar disc method [24]. The dual-culture inhibition assay was performed to assess the interaction between fungi and actinomycete bacteria on the same test plate. The actinomycete strain was streaked as a 1-inch line at the center of the test media (PDA, ISP6, and ISP7) and incubated at 28 °C for 14 days. During incubation, the bacterium secreted a black pigment, resulting in darkening of the culture medium. Following the incubation period, 4 distinct fungal isolates were inoculated onto the culture medium by spotting 5 µl of each spore suspension (6 mm in diameter) at 4 equidistant positions from the actinomycete streak. Fungal growth was monitored daily for 3 days and qualitatively compared with control plates containing single fungal cultures incubated at 28 °C. The dual-culture inhibition assay was conducted using 3 plates per set and repeated in 2 independent experiments.

RESULTS AND DISCUSSION

Isolation of melanin-producing actinomycete bacteria

Among the isolates capable of producing black pigments on culture plates, 2 strains, designated AQ2 and CQ2, were selected for further investigation due to their pronounced black pigment production and distinct morphological characteristics. The AQ2 strain exhibits an orangish color in the mature central area of the colony, whereas CQ2 forms an entirely whitish colony on YMA after 6 days of incubation at 28 °C, as shown in Fig. 1.

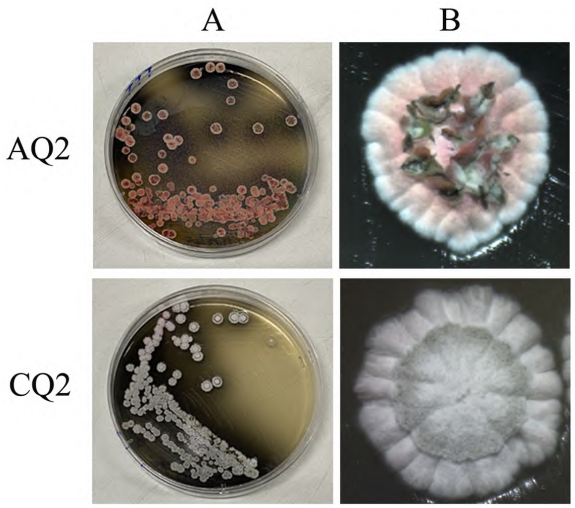

Fig. 1 (A) Pure culture of *Streptomyces* spp. strains AQ2 and CQ2 grown on YMA, showing black pigment production. (B) The close-up colony morphology using a stereomicroscope.

Comparative analysis of the 16S rRNA gene sequences revealed that AQ2 shares the highest similarity (99.30%) with *Streptomyces spectabilis*, while CQ2 exhibits an even higher similarity (99.79%) to the same species (Table S1, Table S2, and Fig. S1). However, due to the observed variation in the 16S rRNA gene sequence of strain AQ2, further verification at the species level via whole-genome sequencing is required. Given that both strains were classified within the genus *Streptomyces*, they are referred to in this study as *Streptomyces* spp. strains AQ2 and CQ2.

Extracellular melanin on different culture plates

The *Streptomyces* spp. strains AQ2 and CQ2 exhibited normal growth and black pigment production on various media, including YMA, ISP3, ISP5, PDA, and MEA. The amount of black pigment produced varied across the media, with the highest production observed on PDA, followed by YMA, MEA, ISP3, and ISP5, respectively (Fig. 2). All media types contain different kinds and amount of carbon sources which impact on melanin production of AQ2 and CQ2 strains. In this study, PDA medium was the most suitable culture medium for extracellular melanin production of the *Streptomyces* spp. strains AQ2 and CQ2. The PDA medium contains a high amount of potato infusion

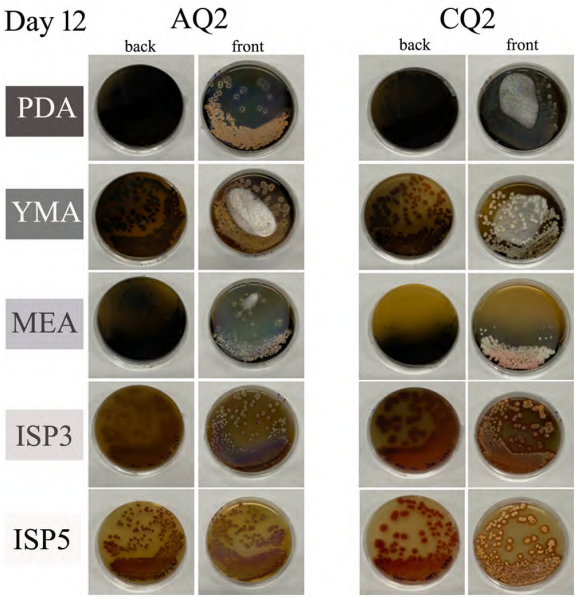

Fig. 2 Bacterial colonies of *Streptomyces* spp. strains AQ2 and CQ2, producing varying amounts of black pigment on PDA, YMA, MEA, ISP3, and ISP5 media, shown on both the front and back sides of the Day 12-culture plates and arranged in descending order of pigment production.

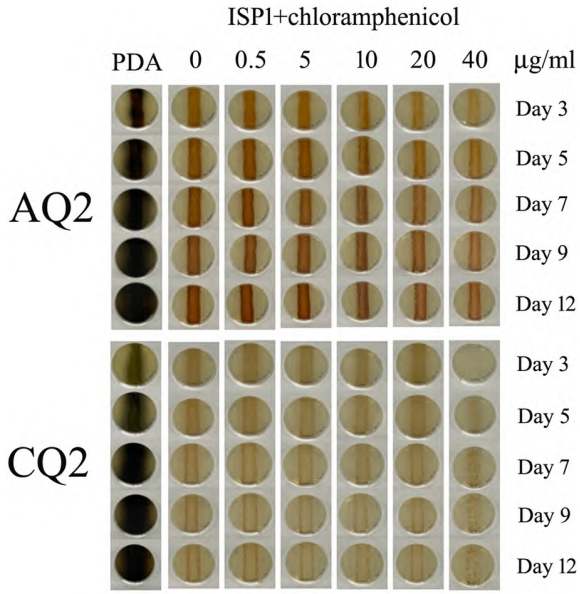

Fig. 3 The effect of subinhibitory concentrations of chloramphenicol on black pigment production by *Streptomyces* spp. strains AQ2 and CQ2. Chloramphenicol at concentrations of 0–40 µg/ml was supplemented into ISP1 medium and compared to PDA medium over a period of 12 days.

(20%) and dextrose (2%). Potatoes contain a relatively high amount of tyrosine [25], which is also a precursor in melanin biosynthesis. Additionally, MEA and YMA media, which include 3% and 0.3% malt extract, respectively, seem to produce black pigment in proportion to the amount of the tyrosine precursor. Black pigment was less present in ISP3 (containing 2% oatmeal and 0.1 mg% trace minerals) and ISP5 (containing 1% glycerol and 0.1 mg% trace minerals). Therefore, the type of culture medium suitable for black pigment or melanin production, as observed in the experiment, correlates with the importance of tyrosine as a precursor.

Antibiotic stress having no impact on melanin production

Recently, lincomycin at 1/10 of its MIC has been shown to effectively induce the expression of genes associated with secondary metabolism in *S. coelicolor* A3(2) [20]. Therefore, to test the efficiency of antibiotics in stimulating black pigment production in the *Streptomyces* spp. strains AQ2 and CQ2, chloramphenicol was selected. This antibiotic targets bacterial ribosomes and was tested at concentrations lower than the MIC (0–40 µg/ml) in 3 different culture media: ISP1 (Fig. 3), a medium that promotes the growth of *Streptomyces* spp.; ISP6 (Fig. 4), a medium known to stimulate melanin production in *Streptomyces glaucescens* strain NEAE-H [5]. Additionally, ISP7 medium (Fig. 5), supplemented with 0.5 g/l L-tyrosine, is recognized as a standard tyrosine-containing medium. Previous

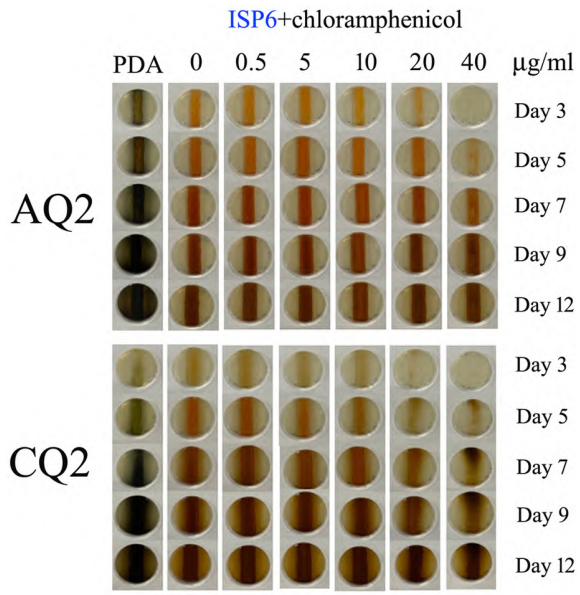

Fig. 4 The effect of subinhibitory concentrations of chloramphenicol on black pigment production by *Streptomyces* spp. strains AQ2 and CQ2. Chloramphenicol at concentrations of 0–40 µg/ml was supplemented into ISP6 medium and compared to PDA medium over a period of 12 days.

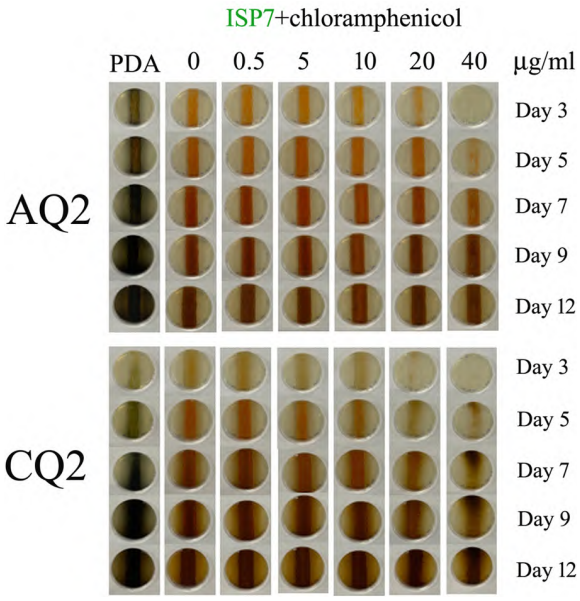

Fig. 5 The effect of subinhibitory concentrations of chloramphenicol on black pigment production by *Streptomyces* spp. strains AQ2 and CQ2. Chloramphenicol at concentrations of 0–40 µg/ml was supplemented into ISP7 medium and compared to PDA medium over a period of 12 days.

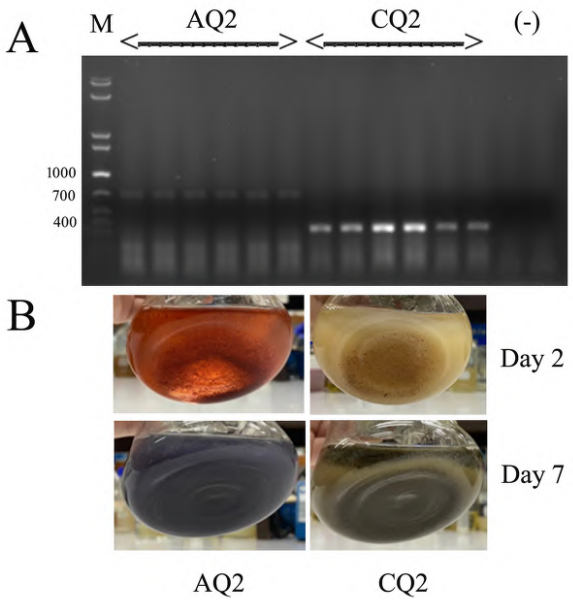

Fig. 6 (A) PCR amplification of the *melC1* gene from *Streptomyces* spp. strains AQ2 and CQ2, as indicated. (–) represents the negative control with no DNA template. The expected product size is 375 bp. (B) Extracellular melanin production darkening the potato dextrose broth after 7 days of cultivation.

studies have reported that a concentration of 2 g/l tyrosine facilitates melanin production in *Pseudomonas stutzeri* BTCZ10 [26]. Moreover, the synergistic effects of 2 g/l tyrosine, a temperature of 50 °C, pH 8.5, and 0.5 g/l beef extract have been shown to enhance melanin production in *S. lusitanus* DMZ-3 [14].

This experiment aimed to evaluate the effectiveness of subinhibitory concentration of chloramphenicol in stimulating black pigment production of the *Streptomyces* spp. strains AQ2 and CQ2. However, the results indicated that chloramphenicol at a concentration of 0–40 µg/ml failed to stimulate black pigment production of both strains in ISP1 medium, whereas the highest concentration (40 µg/ml) showed the inhibitory effect on bacterial growth more pronounced than lower concentration. ISP6 and ISP7 supplemented with chloramphenicol showed similar results to ISP1, although the bacterial colonies darkened over the cultivation time. Surprisingly, the ISP7 media with or without chloramphenicol were able to stimulate the black pigment production of the CQ2 strain but showed no effect in the AQ2 strain. Among the 3 different media, the results showed that subinhibitory concentration of chloramphenicol has no stimulatory effect on black pigment production of the *Streptomyces* spp. strains AQ2 and CQ2 when compared to PDA. In summary, culture media with high tyrosine content derived from potatoes play an essential role as a critical precursor for melanin production, surpassing the stimulatory effects of antibiotic stress.

Although ISP6 and ISP7 media have been utilized for melanin detection in *Streptomyces* species [27], the *Streptomyces* strains AQ2 and CQ2 exhibited less pronounced melanin production. The black pigment secreted in PDA cultures of these strains was analyzed using FTIR spectroscopy and compared to standard melanin (Fig. S2). The FTIR spectra of melanin exhibit characteristic absorption bands in the ranges of 3600–2800 cm^{-1}, 1650–1620 cm^{-1}, and 1500–1400 cm^{-1} [5]. The results suggest that the black pigment closely resembles melanin.

Tyrosinase gene amplification

The DOPA (3,4-dihydroxyphenylalanine) pathway is a metabolic route utilized by *Streptomyces*, where tyrosine serves as a precursor to L-DOPA. This conversion is catalyzed by tyrosinase, and L-DOPA is subsequently oxidized and polymerized to form melanin. Tyrosinase is encoded by the *melC1* and *melC2* genes in *S. kathirae* SC-1 [8]. These 2 genes were amplified by PCR from the genomic DNA of the AQ2 and CQ2 strains using the primers *melC1*F/*melC1*R and *melC2*F/*melC2*R, respectively.

The results showed that only the expected 375 bp fragment of the *melC1* gene was amplified in the CQ2 isolate, while a fragment of double the size was present in the AQ2 isolate (Fig. 6A). In contrast to *melC1*,

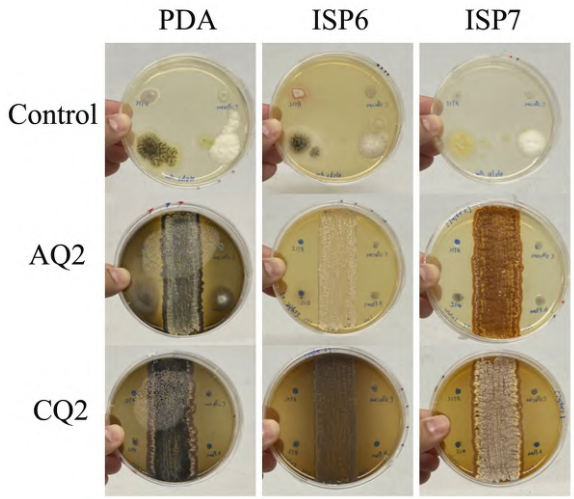

Fig. 7 The antifungal assay of black pigment secreted in culture plates of strains AQ2 and CQ2 grown for 14 days on PDA, ISP6, and ISP7 media, tested against the fungi *A. flavus* TISTR 3041, *A. niger* TISTR 3061, *C. albicans* TISTR 5554, and *T. marneffei* ATCC200051.

no amplification product of the 822 bp *melC2* gene was obtained from either isolate (data not shown). These amplification results suggest that the CQ2 isolate contains a nucleotide sequence for *melC1* that is similar to the *S. kathirae* SC-1 database sequence (GenBank no. KJ868795). However, the AQ2 isolate may have nucleotide substitutions at the primer-binding sites or a larger *melC1* gene fragment.

The inability to amplify the *melC2* gene (GenBank no. KJ868796) from both strains suggests significant differences in the nucleotide sequences of tyrosinase genes among actinomycete species. Both strains were cultivated in PDB, and extracellular melanin production darkened the liquid culture after 7 days of incubation (Fig. 6B). Since the AQ2 isolate has an orangish color and CQ2 has a whitish color, their appearance after combining with melanin production differs accordingly. Therefore, the sequences of the tyrosinase genes in both strains need to be further verified.

Antifungal activity of melanin-producing strains

The melanin production by *Streptomyces* spp. strains AQ2 and CQ2 was confirmed to utilize tyrosine and be mediated through the tyrosinase pathway. We subsequently conducted preliminary tests to evaluate the properties of the secreted melanin from these strains. The results showed that the strains could inhibit the growth of yeast and molds, including *A. flavus* TISTR 3041 [22], *A. niger* TISTR 3061, *C. albicans* TISTR 5554, and *T. marneffei* ATCC200051 [28], in dual culture assays compared to the control fungal plates. However, the data suggests that the inhibitory effect may not be solely attributed to the melanin product, as bacterial growth on ISP6 and ISP7 media also inhibited fungal growth even in the absence of black pigment on the plates (Fig. 7).

CONCLUSION

The *Streptomyces* spp. strains AQ2 and CQ2 secreted a black pigment resembling melanin into the cultivation medium with production levels positively correlated with the concentration of available tyrosine. The highest enhancement of melanin production was observed in PDA containing 20% potato infusion. Subinhibitory concentrations of antibiotics such as chloramphenicol were ineffective in stimulating the tyrosinase-mediated L-DOPA pathway. One of the *melC*-encoding genes, *melC1*, was detected in both *Streptomyces* spp. strains, indicating a high degree of similarity to the *melC1* homolog of *S. kathirae* SC-1, while *melC2* exhibited significant divergence. These melanin-producing strains demonstrated promising fungicidal activity against 4 selected yeast and mold species. Further studies on melanin extraction and verification of its biological properties are essential to confirm its potential applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.038.

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Appendix A. Supplementary data

Table S1 Comparison of 16S rRNA gene sequences of strain AQ2 with reference strain(s).

Rank	Name	Strain	Accession no.	Pairwise similarity (%)	Diff/total nt
1	*Streptomyces spectabilis*	NBRC 13424	AB184393	99.30	10/1433
2	*Streptomyces lasiicapitis*	3H-HV17(2)	KX777589	99.03	14/1439
3	*Streptomyces indiaensis*	NBRC 13964	AB184553	98.68	19/1435
4	*Streptomyces coeruleorubidus*	ISP 5145	AJ306622	98.57	20/1402
5	*Streptomyces alboflavus*	NRRL B-2373	JNXT01000131	98.54	21/1438
6	*Streptomyces flavofungini*	NBRC 13371	AB184359	98.54	21/1434
7	*Streptomyces venetus*	CMU-AB225	LC073310	98.53	21/1432
8	*Streptomyces deserti*	C63T	HE577172	98.44	22/1409
9	*Streptomyces coerulescens*	ISP 5146	AY999720	98.44	22/1408
10	*Streptomyces coeruleofuscus*	NBRC 12757	AB184840	98.40	23/1435

Table S2 Comparison of 16S rRNA gene sequences of strain CQ2 with reference strain(s).

Rank	Name	Strain	Accession no.	Pairwise similarity (%)	Diff/total nt
1	*Streptomyces spectabilis*	NBRC 13424	AB184393	99.79	3/1442
2	*Streptomyces lasiicapitis*	3H-HV17(2)	KX777589	98.69	19/1446
3	*Streptomyces deserti*	C63T	HE577172	98.66	19/1418
4	*Streptomyces flavofungini*	NBRC 13371	AB184359	98.55	21/1444
5	*Streptomyces indiaensis*	NBRC 13964	AB184553	98.48	22/1445
6	*Streptomyces alboflavus*	NRRL B-2373	JNXT01000131	98.13	27/1446
7	*Streptomyces venetus*	CMU-AB225	LC073310	98.13	27/1446
8	*Streptomyces coeruleofuscus*	NBRC 12757	AB184840	98.00	29/1448
9	*Streptomyces coerulescens*	ISP 5146	AY999720	97.97	29/1427
10	*Streptomyces coeruleorubidus*	ISP 5145	AJ306622	97.55	35/1426

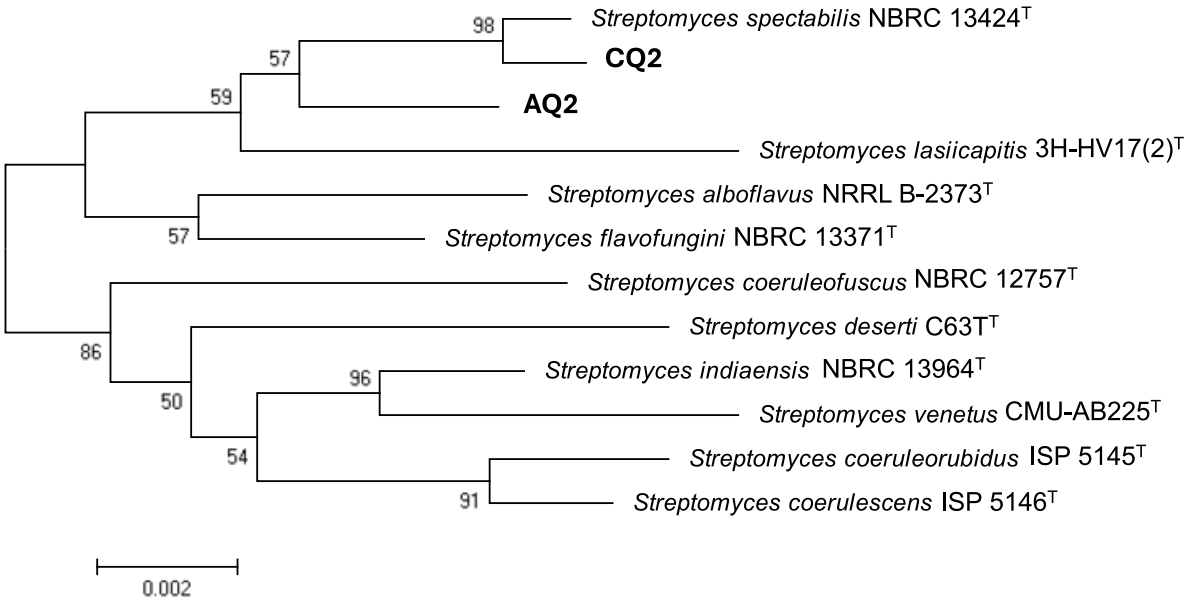

Fig. S1 Neighbour-joining concatenated phylogenetic tree of the 16S rRNA gene sequences of strain AQ2 and CQ2 and closely related type strains constructed by MEGA 6 software.

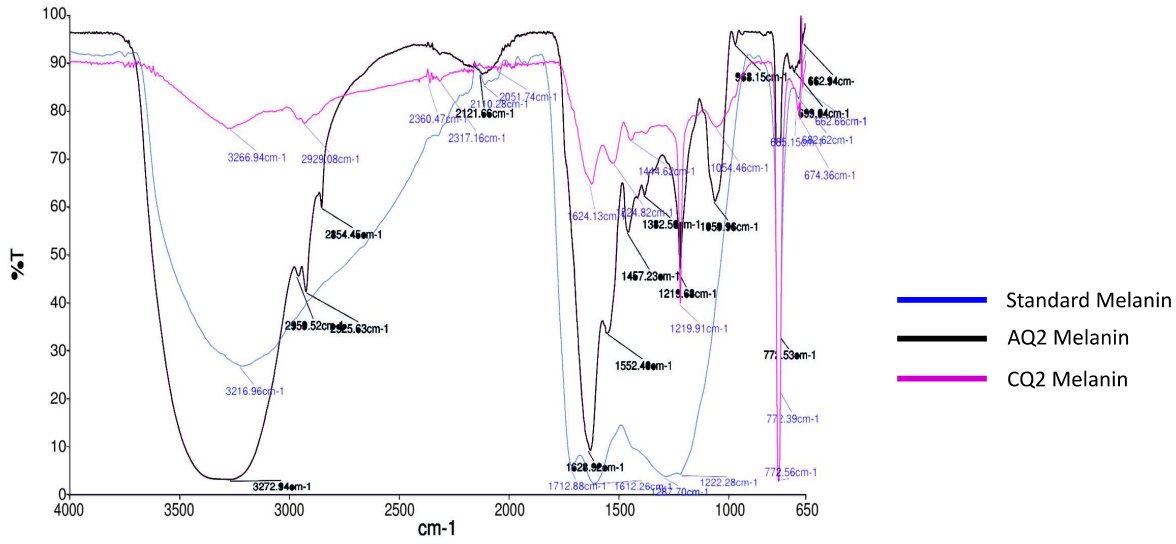

Fig. S2 The FTIR spectra of strain AQ2 and CQ2 melanin compared to standard melanin. The FTIR spectra of melanin include characteristic bands covering 3600–2800 cm^{-1}, 1650–1620 cm^{-1}, and 1500–1400 cm^{-1}.