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Antibacterial activity of bioactive brown pigment produced from Streptomyces SPP. Against pathogenic bacteria

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Abstract

Streptomyces spp. are considered the richest sources of various bioactive secondary metabolites important for antibiotic production. Here, we screened Streptomyces spp. for bioactive metabolite production, especially the brown pigments. This study aims to exploit the bioactive metabolites from Streptomyces spp. isolates against highly resistant Gram-Negative bacteria. Streptomyces spp. Strains with strong brown pigment were isolated from the rhizosphere using conventional methods, passaged in starch-casein broth, and then the sensitivity of the supernatant to extended-spectrum β-lactamase was tested. (ESBL)-producing Klebsiella pneumonia, E. coli, proteus, and Pseudomonas aeruginosa and exhibited colistin and carbapenem resistance using the well diffusion method. Out of 16 Streptomyces isolates, only 3 isolates were associated with brown pigment production. Only one Brown pigment-derived isolate demonstrated a pronounced and valuable zone of inhibition against tested resistant pathogens. A brown pigment derived from Streptomyces spp. is a promising and effective product against highly resistant bacteria.

Keywords: Secondary metabolite, Beta-Lactam, Antibiotic, Extraction, Inhibition zone

Introduction

The emergence of Multi-Drug Resistant (MDR) bacteria (1) including the formidable KPEP pathogens *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E coli*, and *Proteus* spp.), poses a critical global health threat. These bacteria can cause severe and often untreatable infections, resulting in prolonged illness and increased risk of death. ESBL-producing organisms are primarily *Klebsiella* and *E. coli*, which can be partially inhibited by certain extended-spectrum cephalosporins and β-lactamase inhibitors like clavulanic acid (2). These bacteria often exhibit multidrug resistance due to plasmids carrying genes for resistance to aminoglycosides, chloramphenicol, sulfonamides, trimethoprim, and tetracycline. These mobile genetic elements facilitate rapid resistance spread among Gram-negative bacteria, limiting effective antibiotic options (3). there is an urgent need for alternative treatments. This study explored the potential of *Streptomyces* spp. bioactive brown pigment therapeutic option against KPEP isolates (4). Soil is a rich reservoir of microbial diversity, harboring a multitude of ecological niches that support the production of various bioactive compounds. A staggering (70-75%) of known secondary metabolites, including those with antibiotic, anti-cancer, antioxidant, antimicrobial, and immunosuppressant properties, originate from microbial sources. Actinomycetes, particularly the genus *Streptomyces*, predominate in soil ecosystems (5). These Gram-positive, filamentous bacteria possess large genomes with high GC content and are renowned for their prolific secondary metabolite biosynthesis. *Streptomyces* is a genus of bacteria comprising over 500 species, renowned as a prolific source of antibiotics for human medicine. This bacterium produces more than 70% of antibiotics, including black, red, and blue pigments, which are considered antibacterial agents according to previous studies. Notably, each *Streptomyces* strain has the potential to synthesize more than 100 secondary metabolites, underscoring the vast untapped potential of this genus for drug discovery (6).

Materials and Methods

Tested microorganisms used for antimicrobial activities.

Gram-negative bacteria, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus* spp., served as test organisms to evaluate the antimicrobial potential of *Streptomyces* isolates. These pathogens were collected from the

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Biotechnology College, Al-Nahrain University, and the Biology Department, University of Baghdad. Microorganisms were activated by culturing in Nutrient Broth at 37°C for 24 hours before testing (7). Detection of ESBLs-producing bacteria by *a* double diffusion sensitivity test (DDST) is positive when an enlarged inhibition zone is observed around any antibiotic disc compared to the clavulanic acid disc (Figure 5B), or a faint inhibition zone appears between the central disc and another antibiotic disc as shown in Table (1). Also, Tabel (2), showed the antibiotic discs employed in this study to identify Carbapenem) and Colistin-resistant bacteria.

Table (1): Presents the antibiotic discs employed in this study for detecting ESBL-producing bacteria using the Double Disc Synergy Test (DDST)

Antibiotics	Discs symbols	Concentration(µg)	Company	Origin
Ceftriaxone	CRO	10		
Cefotaxime	CTX	10	Bioanalyse	Turkey
Ceftazidime	CAZ	10		
Amoxycillin/Clavulanic acid	AMC	10		

Table (2): The antibiotic discs employed in this study to identify Carbapenem and Colistin-resistant bacteria

Antibiotics	Disc symbols	Concentration (µg)	Company	Origin
Colistin	CT	10		
Trovafloxacin	TRV	10	Bioanalyse	Turkey
Imipenem	IPM	10		
Meropenem	MEM	10		

Isolation and identification of Streptomyces SPP

One gram of dried rhizosphere soil was suspended in 10 mL of sterile distilled water to create a stock suspension. This mixture was shaken at 150 rpm for 15 minutes at room temperature. Serial dilutions ranging from 10^-1 to 10^-5 were prepared from the stock suspension and allowed to stand for 10 minutes. Subsequently, 0.1 mL of 10^3 dilution was pipetted onto supplemented starch casein agar (SCA) plates containing tetracycline (50 mg/L) and nystatin (50 mg/L). The suspensions were evenly distributed across the media surface using a sterile swab. The inoculated plates were incubated at 28°C for 7 to 10 days (8).

Cultural and biochemical characterization of Streptomyces isolates

Bacterial isolates were cultivated on Starch casein agar and mannitol soya bean agar media. Subsequent morphological characterization, including colony appearance and Gram staining, was performed. Physiological and biochemical tests were conducted following the International *Streptomyces* Project (ISP) guidelines to identify Streptomyces spp. isolates. These tests are crucial for the accurate characterization of *Streptomyces* spp. (9).

Preparation of Streptomyces inoculum

Streptomyces sp. spore inoculum was generated by cultivating *Streptomyces* spp. on Starch Casein (SC) agar for one week (7-10 days) at 30°C. A fresh culture, derived from a stock SC slant, was used for inoculation. After incubation, 5 ml of sterile distilled water was added to the culture. Spores were gently dislodged by scraping with a sterile loop, and the resulting suspension was transferred to a sterile tube. To purify and concentrate the spores, the suspension was centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded, and the pelleted spores

were re-suspended in 1 ml of sterile distilled water. The spore concentration was then determined using a hemocytometer (10,29).

Cultivation conditions.

Streptomyces spp. spores were inoculated into a Starch casein broth medium for pigment production. The culture was incubated at 30°C, pH 7.2, with a spore concentration of (1×10^9 spores/mL), and agitated at 200 rpm on a rotary shaker. Pigment production was monitored, and after 10 days, the culture was centrifuged at 8,000 rpm for 15 minutes to separate the biomass. The extracellular brown pigment supernatant was collected for further analysis (11).

Primary screening for tested brown pigment

Nutrient agar plates were inoculated with a *Streptomyces* isolate by streaking a single line of inoculum down the center. Following a 7-10 days' incubation period at 30°C, fully developed *Streptomyces* colonies were established. Subsequently, the tested bacterial pathogens were streaked perpendicularly to the *Streptomyces* growth (a single line at a 90° angle). Plates were then re-incubated at 37°C for 24 hours. Antimicrobial activity, indicated by inhibition zones around the *Streptomyces*, was visually apparent as the reference strains failed to grow near the *Streptomyces* line (12).

Optimization condition

To optimize Streptomyces growth and pigment production, a comprehensive evaluation of culture conditions was conducted. Isolates were cultured in three different media including starch casein broth, mannitol soya bean broth, and malt yeast extract broth (Table 3) across a range of temperatures (20, 25, 30, and 35°C), pH levels (4,5,6,7,7.5,8,9), and sodium chloride concentrations (0.5,1,2,2.5,3,3.5%) (13).

Table 3: Media used in the study for *Streptomyces* characterization and pigment production

No.	Media name	Composition	Amount	Ref
1	Starch casein inorganic salt agar	Soluble starch	10 gm	-13
		Casein	0.3 gm	
		K2HP04	2 gm	
		KNO3	2 gm	
		MgS04.7HzO	0.05 gm	
		NaCl	2 g	
		FeSo4.7H2O	0.01 gm	
		CaC03	0.02 gm	
		Agar	20 gm	
		Distilled water	1000 ml	
		pH 7.5		
2	Mannitol Soya Flour (MS or SFM) agar	Mannitol	20 gm	-14
		Soya flour	20 gm	
		agar	20gm	
		Distilled water	1000 ml	
		PH 7.5		
3	Malt Yeast Agar	Malt extract	10 gm	-15
		Yeast extract	4 gm	
		Dextrose	4 gm	
		Distilled water		
		PH 7.5	1000 ml	

Secondary screening for brown pigment

Streptomyces isolates exhibiting prominent bioactive brown pigment production during primary screening underwent secondary screening for antimicrobial activity. The potent isolate was selected for further antimicrobial pigment characterization. The supernatant, collected from the isolate's culture after centrifugation at 8,000 rpm for 15 minutes to remove crude precipitate, was subjected to antimicrobial testing. Following the solidification of 20 ml sterile Muller-Hinton agar, 200 microliters of activated pathogenic bacteria were spread evenly. Wells (6 mm diameter) were created in the inoculated agar and filled with 75 microliters of filtered supernatant (extracellular brown pigment) (0.45µm.). Plates were incubated at 37 °C for 24 hours, and the zone of inhibition was measured to assess antimicrobial activity (16).

Results and Discussion

Nineteen soil samples were collected from various rhizosphere locations and screened for *Streptomyces* strains producing antibacterial brown pigment. After culturing diluted soil samples (10^-1 to 10^-5) on starch casein salt agar for 7-10 days, *Streptomyces* colonies were observed with pigment production among mixed microbial colonies (17) (Figure 1) depicts the initial culture containing white and colorful, chalky, powdery colonies indicative of potential *streptomyces*, alongside other microorganisms such as bacteria and fungi (18). (Figure 2) shows an isolated *Streptomyces* colony. The presence of non-*streptomyces* colonies in the culture might be attributed to resistant spores in the soil or insufficient heat treatment. Suspected *Streptomyces* colonies were further cultivated on SC agar and selected based on their gray, creamy, or white coloration, with colony diameters ranging from (1.5 to 12 mm) (19). Initially smooth, the colony morphology evolved into a powdery, soft, granular appearance due to aerial mycelium formation. Of the 19 rhizosphere soil samples collected, 17 were presumptively positive for *Streptomyces*. Subsequent isolation yielded four distinct Streptomyces isolates (89.4%) exhibiting diverse morphological traits. Suspected *Streptomyces* colonies were meticulously sub-cultured on mannitol soya flour agar to obtain pure cultures. These isolates displayed varied coloration in aerial and substrate mycelia and presented as dry, rough, or smooth colonies with irregular or regular margins. The colony morphology was generally convex, and most isolates produced the earthy odor characteristic of Streptomyces as described by Williams and Cross (20).

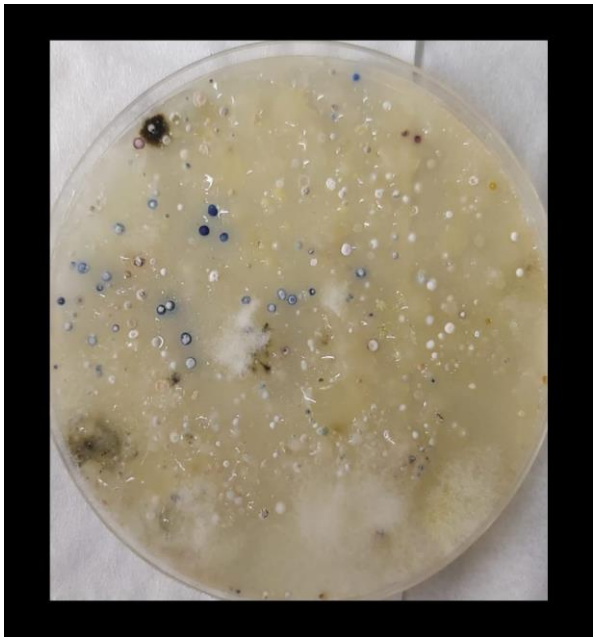

Figure 1. The colorful chalky/dusty/powdery appearance *Streptomyces* on starch casein salt agar (single colony), incubated at 30C for (7-10) days with different pigment production

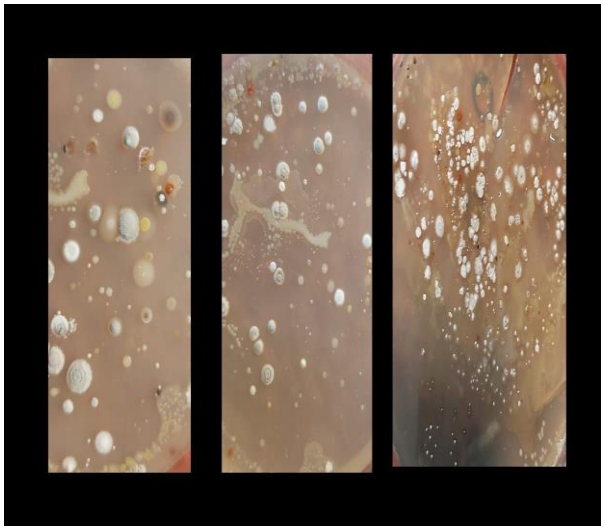

Figure 2. Streptomyces spp. were initially screened by plating serial dilutions (10^-3) of soil samples onto starch salt casein agar plates. These plates were incubated at 30°C for (7-10) days. A single *Streptomyces* spp. colony was identified among the diverse microbial growth

Pure cultures of pigment-producing Streptomyces SPP

Isolate was purified by repeated streaking on fresh SCA plates using sterile inoculating loops. Pure cultures of pigment-producing *Streptomyces* spp. were subsequently maintained on the SCA plate at 4°C for long-term storage (21).

Identification and characterization of Streptomyces SPP

Morphological characterization

The isolate was identified based on its colony morphology and microscopic characteristics, including aerial and substrate mycelium, soluble pigment production, and spore chain arrangement. This isolate exhibited diffusible pigments in the surrounding media that corresponded to the color of its aerial mycelium (22). Additionally, soluble pigments were observed within the isolate's cellular structures. Figure 3 showcases the isolate displaying distinct brown pigmentation, consistent with the classification scheme outlined in Bergey's Manual of Determinative Bacteriology by Buchanan, and Gibbons.

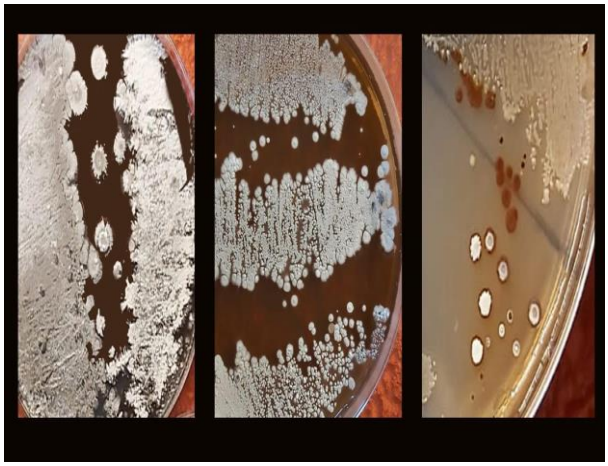

Figure 3. Pure cultures of brown pigment-producing Streptomyces ssp. on starch casein salt agar incubated at 30 C for 7-10 days

Microscopic examination of the isolate was conducted after 7-10 days of incubation to visualize hyphae, as depicted in Figure 4. Spore chain morphology observed following 10 days of incubation, demonstrated variations in spore arrangement characteristic of different *Streptomyces* species (23).

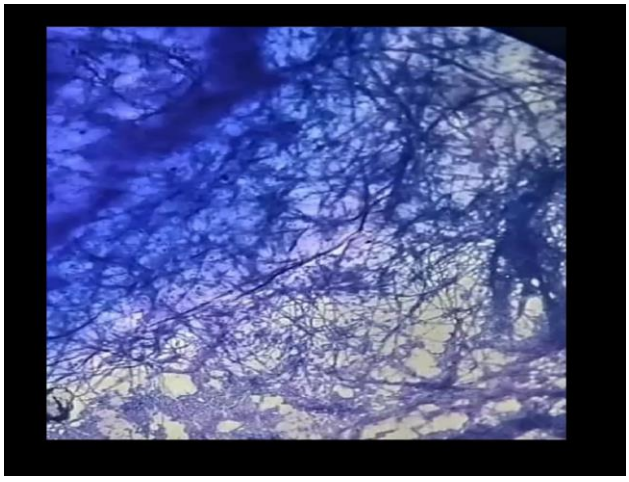

Figure 4. Slide of a *Streptomyces* spp. hyphae, grown on SC agar. Branching filaments, abundant aerial mycelia, and long chains of small spores are visible, all are characteristic of *Streptomyces* spp. 100X

Physiological and biochemical test

Biochemical characterization of *Streptomyces* spp. is summarized in Table 1. These strains exhibited positive results for catalase, gelatinase, urease, and citrate utilization tests. Indole production was negative. Additionally, the ability to utilize mannitol, melanin, nacl, and optimum ph. optimum temperature, starch, or yeast as a carbon source was assessed through growth on respective media (24).

Table 4. Physiological and biochemical properties of brown pigment-producing Streptomyces *species*

NO	Test	Reaction	Result
1	Melanin	Brown	+++
2	Urease	Pink	++
3	Sugar utilization	growth	+++
4	Catalase	Bubbles	+++
5	Gelatinase	Zone	+++
6	Indole test	No color	Negative
7	Citrate utilization	Blue color	++
8	Optimum temperature	Growth	30C
9	Optimum pH	Growth	7.5
10	Growth in the presence of NaCl	Growth	2-10%
11	Shape and growth	Growth	Filamentous aerial growth

Detection ESBLs producing bacteria

A DDST is positive when an enlarged inhibition zone is observed around any antibiotic disc compared to the clavulanic acid disc (Figure 5B), or a faint inhibition zone appears between the central disc and another antibiotic disc. This indicates the presence of ESBLs, which are inhibited by clavulanic acid. The enzyme's activity is blocked near the central disc, resulting in growth inhibition only towards the clavulanic acid disc. A negative test excludes ESBL-mediated cephalosporin resistance (25).

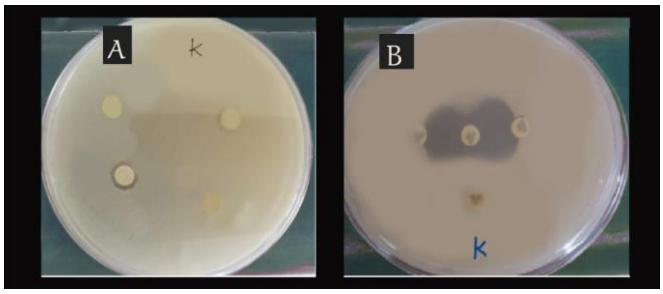

Figure (5): Detection of ESBLs producing *K. pneumoniae* (B) and resistance to Carbapenem and Colistin (A)

Figure (5A), an example of tested bacteria illustrates that approximately 1% (5 isolates) of our *K. pneumoniae* samples exhibited resistance to both carbapenem and colistin. This finding is notably lower than the 33% co-resistance reported by Attalla *et al*., (26). This discrepancy may be attributed to the smaller sample size in our study. The emergence of carbapenem-resistant *K. pneumoniae* strains is a critical concern as colistin is often the last-line treatment option. Consequently, the development of alternative therapeutic strategies is urgently needed.

Brown pigment-producing Streptomyces strains were primarily screened for antibacterial properties

A brown pigment-producing *Streptomyces* isolate was recovered from rhizosphere soil samples in the region. This isolate was evaluated for its antibacterial potential against the resistant pathogens *Klebsiella pneumoniae, E. coli, Pseudomonas aeruginosa, and Proteus* spp. using the cross-streaking method. Figure (6), summarizes the antibacterial activity of the bioactive brown *Streptomyces* pigment. Positive results, indicating the ability of *Streptomyces* metabolites to inhibit the growth of these pathogenic bacteria, identified promising strains for further antibacterial screening as high-potential producers. The active dye demonstrated moderate to high efficacy overall. It exhibited moderate activity against Gram-negative bacteria such as *Klebsiella pneumoniae, Proteus, and E. coli,* but proved highly effective against Gram-positive bacteria. This differential activity likely stems from inherent differences between the two bacterial groups, with Gram-negative bacteria possessing protective mechanisms, including specific enzymes, that impede the dye's action. Chaturwedi *et al.,* (27) demonstrated similar antibacterial efficacy against pathogenic bacteria.

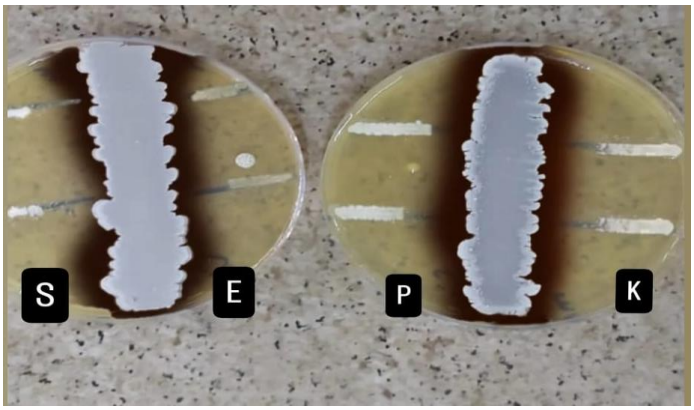

Figure (6): Primary screening of bioactive brown pigment-producing *Streptomyces* spp. against resistant *K. pneumoniae (K), Proteus* spp. *(P), E coli (E), P.* aeruginosa

Selection of fermentation medium for brown pigment production

To determine the optimal medium for brown pigment production, three different broth media were evaluated: Yeast Malt Extract broth (YME), starch casein broth (SCB), and mannitol soya bean broth (MS). Pigment production was assessed by measuring the absorbance at the previously determined lambda max of 450 nm. SCB medium exhibited the highest pigment production (0.65 absorbance) among the tested media (Figure 9). It was therefore selected for subsequent submerged fermentation. MS broth did not support pigment production, while YME broth showed lower yields than SCB. Parmar *et al*. (28) found that starch casein broth was optimal for pigment production by *Streptomyces sp*. The type and concentration of carbon, nitrogen sources, and minerals significantly influenced pigment yield.

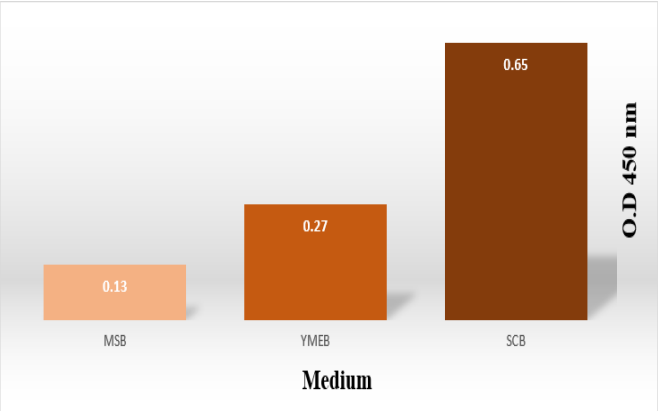

Figure 9. Best fermentation medium selection for brown pigment production by *Streptomyces sp* At 30 C for 10 days measured at 450 nm spectrophotometer

Selection of temperature for brown pigment production

Temperature significantly influences the growth, pigment production, and activity of Streptomyces sp. To determine the optimal temperature for these processes, a selected isolate was cultured in starch casein broth at various temperatures (20, 25, 30, and 35°C) while shaking at 200 rpm. Results indicated that 30°C was the ideal temperature for both growth and the the efficiency of cultural growth production of a brown pigment Figure (10). Deviations from this optimal temperature adversely affected the efficiency of cultural growth.

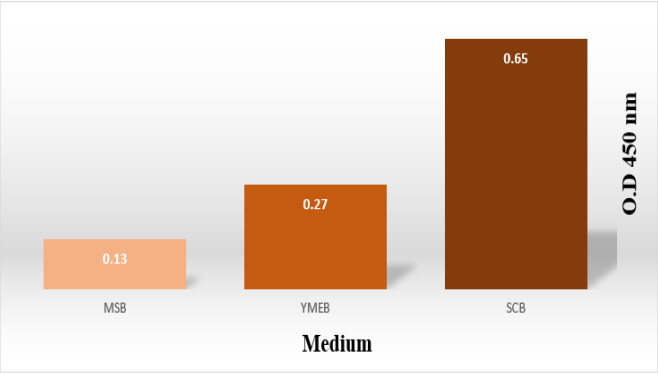

Figure 10. Best temperature selection for brown pigment production by *Streptomyces SP* at 30 C for 10 days measured at 450 nm spectrophotometer

Selection of PH for brown pigment production

Optimization of *Streptomyces sp.* revealed that pigment production is optimal within a pH range of 4-9, with the maximum yield occurring at pH 7.5. However, pigment production declined at both acidic (pH 4) and alkaline (pH 9) extremes. While pH 5 yielded minimal pigment, pH 8 demonstrated a gradual increase in pigment production over 120 hours of incubation. pH 7 was also found to be favorable, but not as optimal as pH 7.5. Therefore, it can be concluded that a pH range of 7-7.5 represents the ideal medium conditions for pigment production by this *Streptomyces* isolate Figure (11)

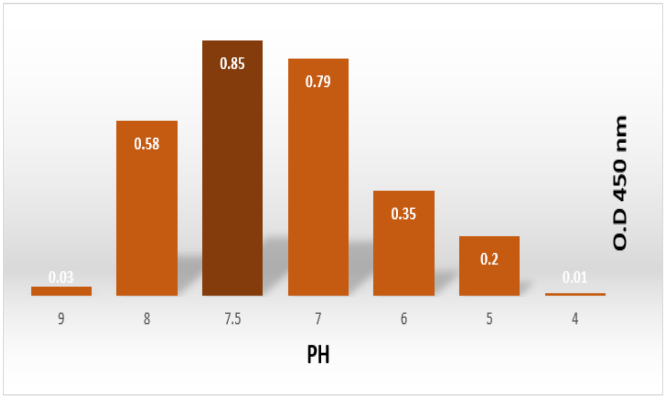

Figure 11. Best PH selection for brown pigment production by Streptomyces sp At 30 C for 10 days measured at 450 nm spectrophotometer

Best sodium chloride (NACl) concentration for brown pigment production

SC broth supplemented with various concentrations of NaCl, ranging from 0.5% to 3.5% (w/v), was incubated for 10 days at 30°C. The optical density (OD) at 450 nm was measured to determine pigment production. Figure (12) demonstrates that the isolate exhibited optimal pigment production at a NaCl concentration of 2.5%, with good growth. While the isolate tolerated NaCl concentrations up to 3.5%, pigment production was low at this level. Minimal pigment production was observed at NaCl concentrations of 0.5%, 1%, 2%, and 3%. Therefore, it can be concluded that a concentration of 2.5% NaCl represents the optimum concentration for pigment production by *Streptomyces sp.* The importance of salt in bacterial growth and pigment production can be attributed to its role in maintaining osmotic pressure or stress conditions. This facilitates the movement of molecules across the bacterial cell. However, high salt concentrations can also be toxic to the cell due to its elevated sodium ion concentration.

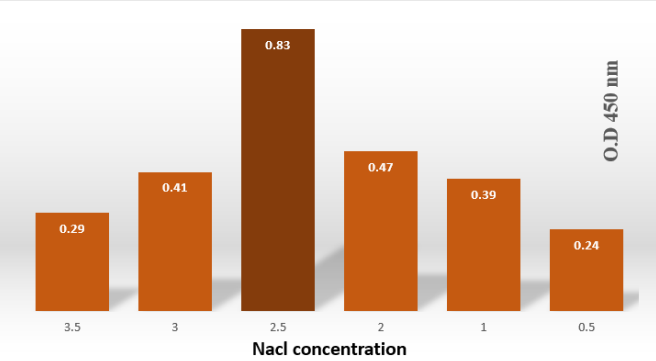

Figure 12. Best salt concentration selection for brown pigment production by *Streptomyces* spp. at 30 C for 10 days measured at 450 nm spectrophotometer

Secondary screening of *Streptomyces* isolates for bioactive brown pigment production

The *Streptomyces* isolate from the primary screen was cultivated in SC broth for 10 days at 30°C in a shaking incubator at 200 rpm Figure (7). The resulting fermentation broth(supernatant) extracellular was subjected to a plate well assay to assess the activity of the pigment, and the crude precipitate was discarded. The supernatant, filtered through a 0.45 µm filter, containing the bioactive brown pigment produced by the *Streptomyces* isolate, was evaluated for its antimicrobial activity against the Gram-negative *bacteria Klebsiella pneumoniae, E. coli, and Proteus sp.*, as well as the Gram-positive Bacterium *S. aureus* figure (8). The bioactive pigment (supernatant) exhibited moderate antibacterial activity against the Gram-negative bacteria *Klebsiella pneumoniae, E. coli, and Proteus sp.* However, its potent pronounced antimicrobial effect was observed against the Gram-positive Bacterium *S. aureus.* Chaturwedi et al., (26), reported that the pigment extracts from pigmented bacteria might have beneficial antibacterial roles against pathogenic bacteria.

Figure 7. Brown pigment production was assessed through shake flask fermentation. *Streptomyces* isolates were cultured in 30 mL of SCb production medium under aerobic conditions at 200 rpm and 30°C for 10 days.

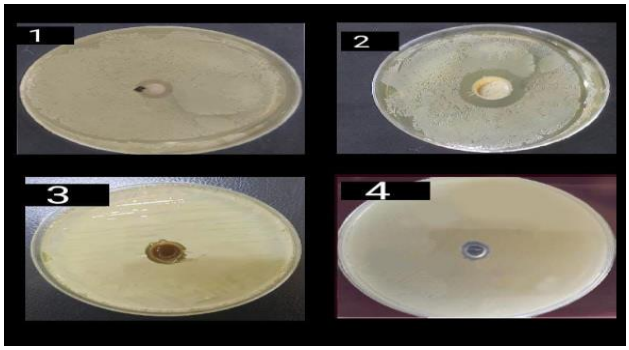

Figure 8. Antibacterial activity of (supernatant) bioactive brown pigment-producing Streptomyces against *(S. aureus* 2*), (E. coli* 1*), (Proteus* spp. 3*), (K. pneumoniae* 4*)*

Conclusion

This study demonstrates that the isolate of *Streptomyces* spp. possesses the potential to produce a brown pigment with antagonistic activity against pathogenic *E. coli, Proteus sp., K. pneumoniae, and S. aureus.* Optimizing the conditions for pigment production could have significant ecological implications. Moreover, the pigment derived from *Streptomyces* could serve as a valuable and cost-effective source for medicinal and cosmetic applications.

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