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Biomedical exploration of Bacterial pigments extracted from *Staphylococcus* sp. and *Pseudomonas* sp.

Ramya Gunasekaran[1], Hemapriya Janarthanam[2] and Vijayanand Selvaraj[1*]

[1]Bioresource Technology Lab, Department of Biotechnology, Thiruvalluvar University, Vellore, Tamil Nadu- 632 115, India
[2]Department of Microbiology, DKM College for Women, Sainathapuram, Vellore, Tamil Nadu- 632 001, India
**Corresponding author*

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A B S T R A C T

Bio colorants from microbial resources are highly explored now days for their biotechnological applications. These bio colorants serve asa potential alternate for chemically synthesized coloring agents and also numerous biomedical applications. This study deals with the isolation and optimization of chromogenic bacteria from different soil samples. The bacterial isolates (BRT-CB1 and BRT-CB2) were further subjected to pigment extraction using methanol and chloroform as active solvents. Extracted pigments were evaluated for its antioxidant efficiency against DPPH as free radical. The extracted pigments scavenge free radical with increasing concentration. The antagonistic ability of the pigments were studied on selected bacterial pathogens such as *Bacillus subtilis, E. coli, Shigella* sp., *Klebsiella* sp. *and Streptococcus pyogenes* where the inhibitory activity was found to be directly proportional with the increasing concentration of pigment. Thus, the work concludes that bacterial pigments are prospective metabolite which can be applied in biotechnological and biomedical applications.

Introduction

Natural pigments acquired a key status in therapeutic and industrial applications due to its nontoxic, biocompatibility, safe and ecofriendly nature. In early periods, plants and algae were considered as the best source of natural colorants. Later, microbes were considered to be one of the effective resources of pigments. Algae, fungi and bacteria are few potential microbes which produce pigments with numerous biological applications (Tuli *et al.*, 2015).

Among various sources, chromogenic bacteria play a key role due its unique features such as utilization of low-cost growth supplements, shorter fermentation time, high yield and easy extraction procedure which makes them a better candidate in research and industrial uses (Venil *et al.*, 2014). Currently bacterial pigments are highly utilized in

pharmaceutical, food and textile industries for its biological activities such as antimicrobial, antioxidant, anti-inflammatory, antibiofilm formation and anticancerous properties. Bacteria were reported to produce various pigments such as Indigoidine, Carotenoids, Prodigiosin, Pyocyanin, Pyoverdine, Violacein and Melanin. Apart from industrial applications, bacterial pigments play an essential role in defense mechanism against photooxidation (Franceschelli *et al.,* 2014).

Free radicals generated in the microbial cells are responsible for oxidative stress and cellular damage. Carotenoids are group of yellow – orange pigments responsible for its antioxidant and anticancerous properties. These dietary substances scavenge the ROS molecules thereby decrease the adverse effects (Kodach *et al.,* 2006). Under oxidative stress, the carotenoid molecules act as antioxidant and enhances the cellular integrity and stability (Ungureanu and Ferdes 2012).

On the other hand, due to the discovery of numerous synthetic antibiotics the microbial flora has acquired an undesirable feature called Multi Drug Resistance (MDR). MDR bacteria creates a challenging situation for development of antibacterial drugs against them(Keith *et al.,* 2000). Natural pigments from bacteria possess a unique defense property which inhibits the growth of MDR bacterial species (Tuli *et al.,* 2013).

Pyoverdine, extracted from *Pseudomonas* sp, were reported for its antagonistic activity against several MDR bacteria. In the present study, carotenoid producing bacterial strain and Pyoverdine producing *Pseudomonas* sp. has been isolated and optimized for production of maximum pigment yield. Further the pigments from the strain were further studied for its antioxidant and antibacterial properties.

Materials and Methods

Sample collection and isolation of chromogenic bacteria

Soil samples from three different sites (A1, A2 and A3) were collected in a sterile polythene bag aseptically and designated accordingly. Samples were serially diluted in autoclaved water and plated over freshly prepared nutrient agar plate and incubated for 48 h at 37 ºC. Following incubation, the plates were observed for presence of chromogenic colonies. The selected strains were purified and designated as BRT- CB1 and BRT-CB2. The isolates were purified using purification techniques and stored at 4ºC for further uses. The isolates were subjected to morphological and biochemical characterization.

Optimization of growth condition for maximum pigment production

Effect of pH on pigment production

To determine the effect of pH on pigment yield, the isolates were inoculated separately in 100 ml of freshly prepared nutrient broth culture. The pH of the nutrient media was altered using sodium hydroxide and HCl to varying levels of pH from 2 to 9. The intensity of the pigment production was determined using spectrometric analysis at OD_{600}nm.

Effect of temperature on pigment production

To evaluate the optimum temperature required for the production of maximum yield, 100 ml of freshly prepared nutrient broth culture was inoculated with the isolated cultures (BRT-CB1 and BRT-CB2). The cultures were further incubated at varying temperature ranging from 20 ºC to 60 ºC. The effect of temperature on pigment production

was determined using spectrometric analysis at OD_{600} nm.

Effect of time on pigment production

To estimate the optimum incubation time required for the pigment production, 100 ml of freshly prepared nutrient broth were inoculated with the isolates (BRT-CB1 and BRT-CB2). The growth of the bacterial cultures was observed at regular time intervals (6 h). The effect of time on pigment production was determined using spectrometric analysis at OD_{600}nm.

Extraction of pigment

Yellow pigment extraction from BRT-CB1

100 ml of freshly prepared nutrient broth media was incubated with active culture of BRT-CB1 and incubated at 30 ºC for 48 h. Following incubation, the culture media was transferred to sterile centrifuge tube and centrifuged at 8000 rpm for 15 minutes. The cell pellet was subjected to washing using distilled water and recentrifuged. Further, the pellet was treated with methanol and kept undisturbed for 4 h. Later the solvent- pellet mixture was centrifuged and the pigment in the supernatant was collected in a fresh tube.

Green pigment extraction from BRT-CB2

100 ml of freshly prepared nutrient broth was inoculated with 24 h active culture of BRT-CB2 and incubated at 30 ºC for 48 h. The water-soluble green pigment was extracted using chloroform and HCl. The active culture was centrifuged at 8000 rpm for 10 min. The supernatant was collected and treated with chloroform (1:2). The upper layer was collected in a sterile glass tube and treated with HCl. The acidified layer was neutralized using Tri-base. The procedure was repeated for three times and the pigment was collected in a sterile collecting tube (Devnath *et al.*, 2017).

Antioxidant activity of the bacterial pigments

Free radical scavenging assay using DPPH

Radical scavenging ability of the pigments extracted from the isolates (BRT-CB1 and BRT-CB2) was studied using DPPH assay. 1ml of DPPH solution (0.1mM) was mixed with 3 ml of pigments extracted from BRT-CB1 and BRT-CB2 at varying concentrations ranging from (100 - 500 µg/ml). The mixture was mixed well and incubated for 1 h. The absorbance was recorded at 517 nm using UV-Vis spectrophotometry (Brand-Williams *et al.,* 1995). The percentage of inhibition was calculated using the equation

$$\textbf{DPPH scavenging effect (\%)} = A_0\text{-}A_1/A_0 \text{ X } 100$$

Where, A_0 represent control and A_1 represents the absorbance of test sample (pigment)

Antibacterial activity of the bacterial pigments

Pigments extracted from chromogenic bacterial isolates (BRT-CB1 and BRT-CB2) were investigated for its antibacterial activity against bacterial pathogens such as *Bacillus subtilis, E. coli, Shigella sp., Klebsiella sp.* and *Streptococcus pyogenes*. Freshly prepared Muller Hinton agar plates were used for determining the antagonistic activity using well diffusion method. Wells were cut using sterile well cutter and overnight broth cultures of the selected bacterial pathogens were swabbed over the agar. Varying concentrations (20 - 100 µg/ml) of pigments was loaded and incubated at 37ºC for 24 h. Following incubation, the plates were observed for the zone of inhibition (Saha *et al.,* 2008).

Results and Discussion

Isolation and identification of BRT-CB1 and BRT-CB2

Two distinct chromogenic bacterial strains were isolated from soil samples. The isolates were studied for its morphological and biochemical characterizations. The colony morphology of BRT-CB1 on nutrient agar plate was found to be circular, convex, and smooth with golden yellow pigmentation. On the other hand, the morphology of BRT-CB2 was observed to be irregular, smooth, and convex with bluish green pigmentation. According to the analysis the isolate BRT-CB1 was found to be *Staphylococcus* sp. The isolate was observed to be catalase positive and could efficiently grow on the mannitol salt agar plate utilizing the mannitol which is a characteristic feature of *Staphylococcus* sp. (Parija 2012). Based on the microscopic and biochemical analysis the isolate BRT-CB2 was found as *Pseudomonas* sp. belonging to Pseudomonadaceae family. *Pseudomonas* sp. being considered as a human pathogen was reported to produce medicinally important secondary metabolites such as pigments, enzymes and toxins (Rubilar *et al.,* 2008).

Optimization of growth conditions for maximum pigment production

Optimization of growth conditions such as pH, temperature, agitation and incubation time play a key role in enhancing the yield of the byproduct and also responsible for the production of bacterial biomass. The isolate BRT-CB1, was found to produced maximum pigment when incubated with pH 6 to 7. On the other hand, the intensity of the pigment production in isolate BRT-CB2 was found to be maximum when incubated at pH 8 (Fig 1). The isolate BRT-CB1 was observed to produce maximum pigment at 30 ºC whereas the isolate BRT-CB2 produced maximum pigmentation at 20 ºC (Fig 2). Incubation time is one of the essential factors for determination of percentage of secondary metabolite extracted. BRT-CB1 showed high pigmentation when incubated for 48 h, where as maximum pigmentation was observed at 36 h in BRT-CB2 (Fig 3).

Antioxidant activity of the isolates

Antioxidant ability of the extracted pigment was studied using DPPH assay. The antioxidant efficiency of the pigment was determined based on the percentage of inhibition. The pigment extracted from BRT-CB1 shows effective radical scavenging ability at 200 µg/ml when compared with the other pigment (Fig 4). Previous study states that, free radical scavenging ability of carotenoids are mediated by the conjugated double bonds present in the structure (Clauditz *et al.,* 2006). These bonds make the carotenoid pigment more stable and enhance the radical scavenging ability more effective (El-Agamey *et al.*, 2004).

Antibacterial activity of bacterial pigments

Antagonistic ability of the bacterial pigments was studied using well diffusion method against five different pathogenic bacterial strains. Pigment extracted from BRT-CB1 exhibited maximum zone of inhibition against *Bacillus subtilis* (16mm) and *Streptococcus pyogenes* (15mm), however average range of inhibition was noted against *Shigella* sp. and *Klebsiella* sp. Minimum inhibition was observed against *E.coli* (6 mm) (Fig 5A). Whereas, the antagonistic activity of pigment extracted from BRT-CB2 was more effective. The pigment exhibited maximum zone of inhibition against *Bacillus subtilis* (17mm), *Streptococcus pyogenes* (15mm) and *Klebsiella* sp. (15 mm). Minimum zone of inhibition was observed against *E.coli* (8 mm) and *Shigella* sp (7 mm) (Fig 5B). Similarly, antibacterial activity of staphyloxanthin extracted from *Staphylococcus gallinarum* was reported to be effective against *E. coli, Staphylococcus aureus* and *Candida albicans* (Barretto 2018).

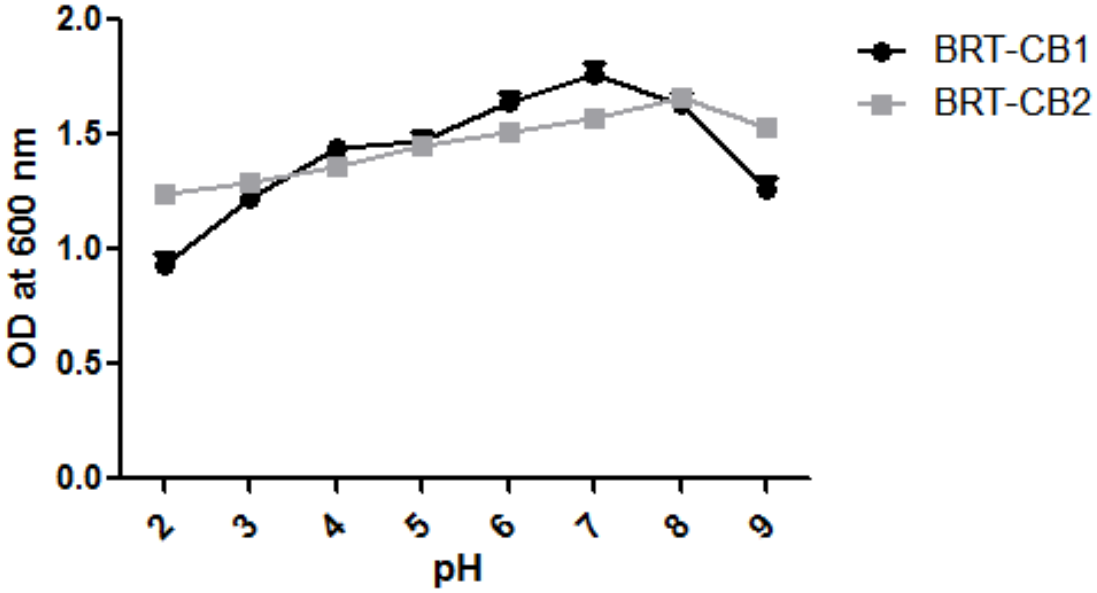

Figure.1 Effect of pH on pigment production of BRT-CB1 and BRT-CB2

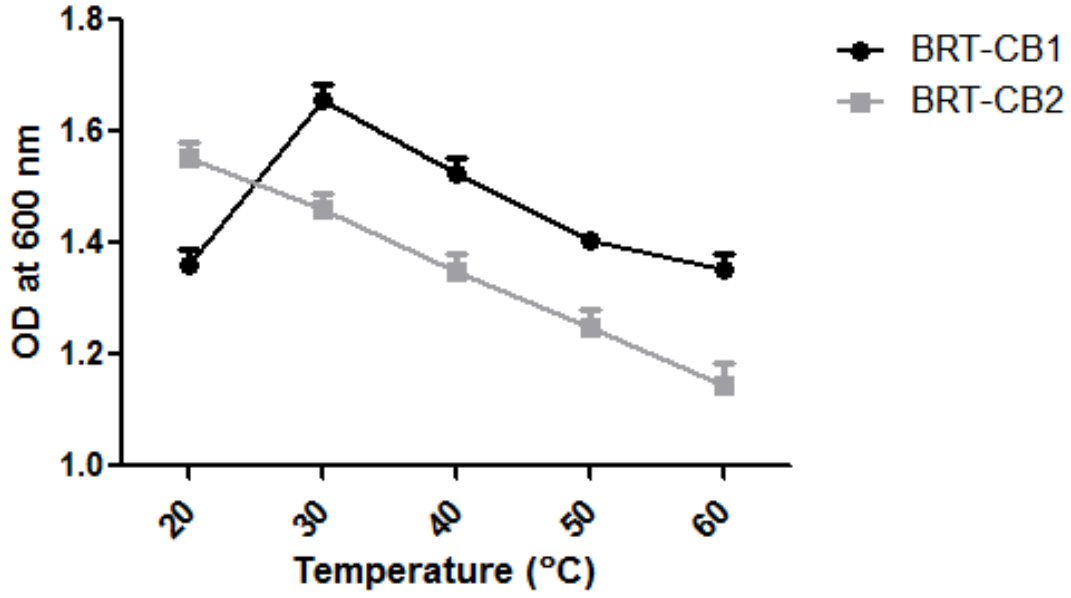

Figure.2 Effect of temperature on pigment production of BRT-CB1 and BRT-CB2

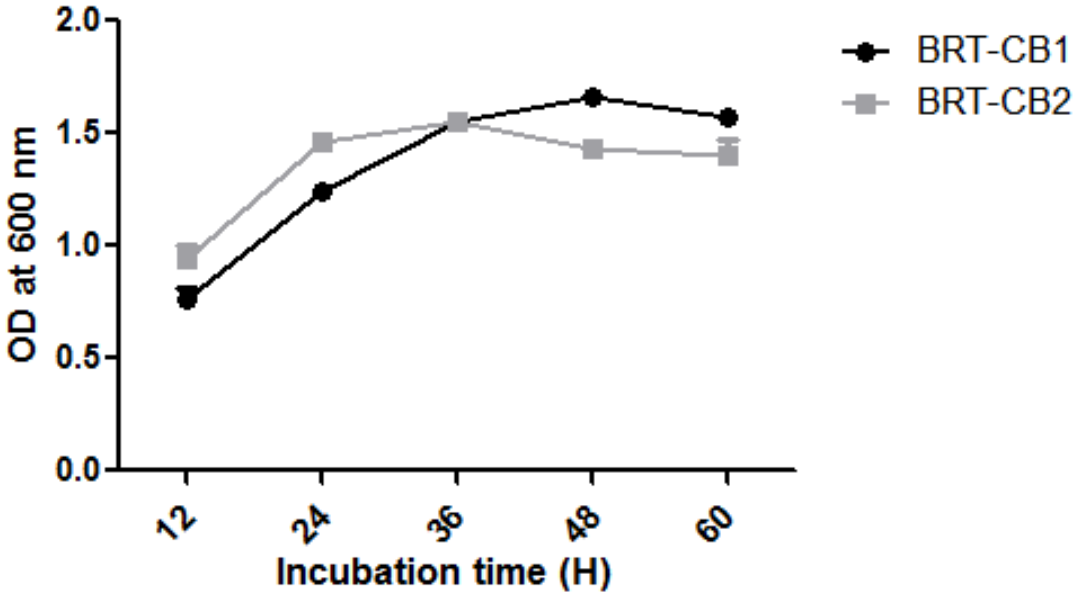

Figure.3 Effect of incubation time on pigment production of BRT-CB1 and BRT-CB2

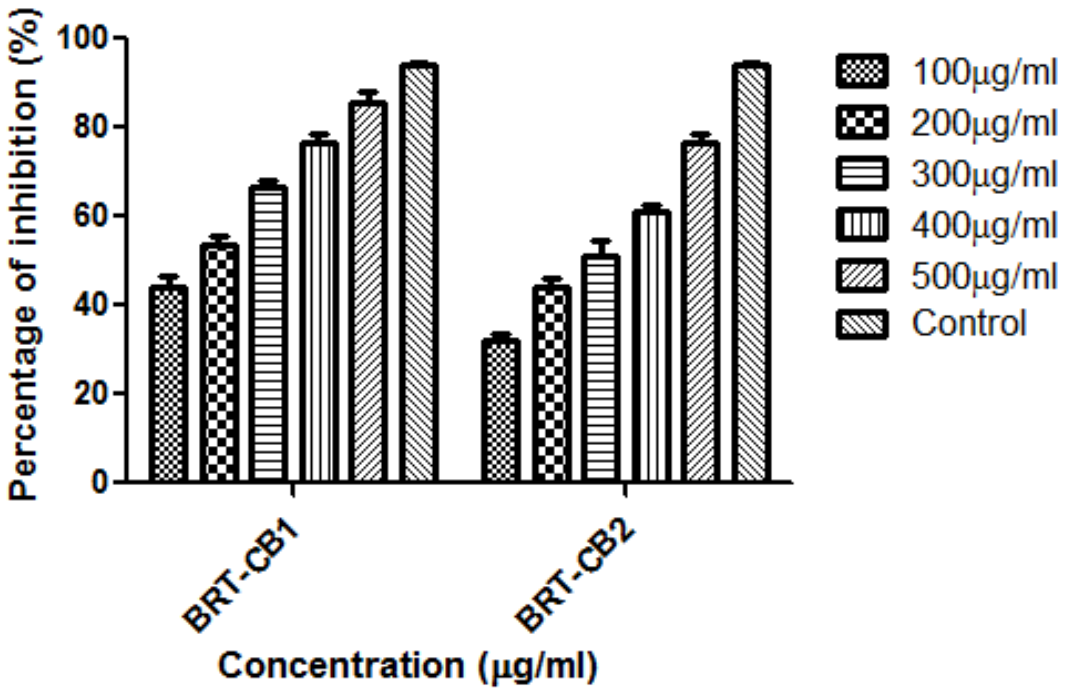

Figure.4 Antioxidant ability of pigment extracted from the isolates (BRT-CB1 and BRT-CB2)

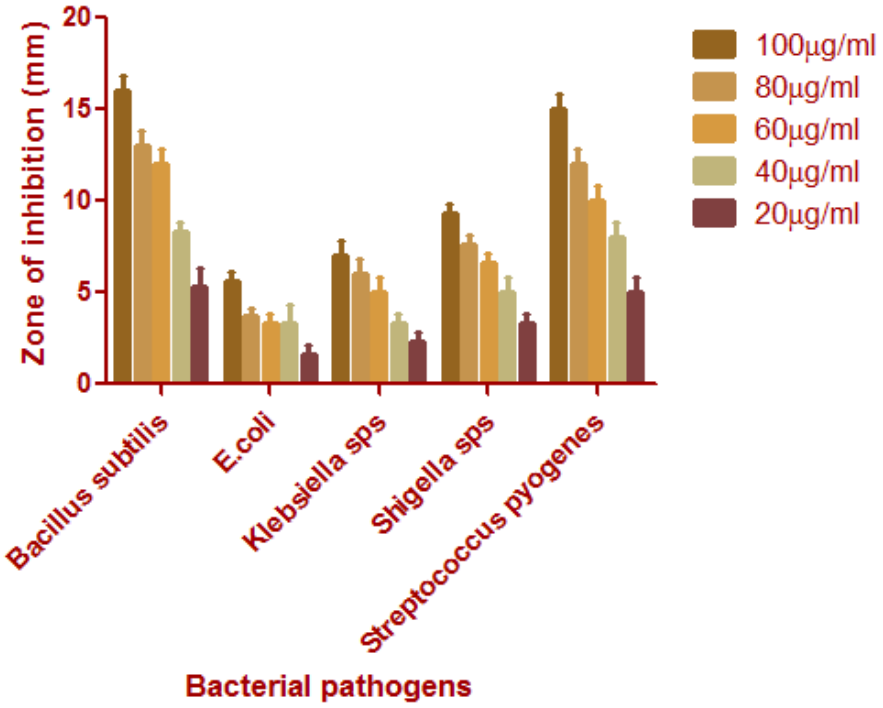

Figure.5 A. Antibacterial activity of pigment extracted from BRT-CB1

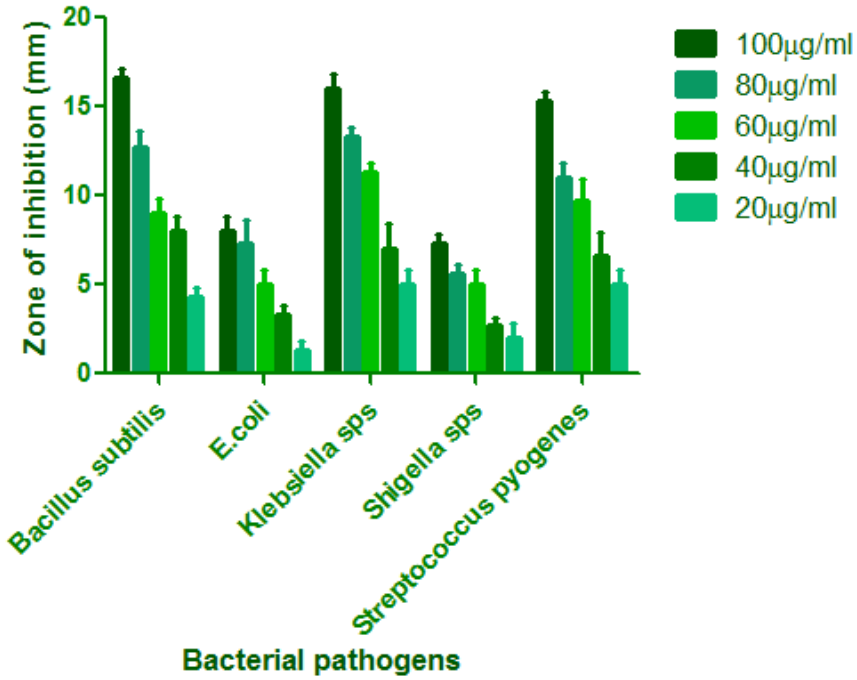

Figure.5 B. Antibacterial activity of bacterial pigment extracted from BRT-CB2

Pigments extracted from natural resources serve as potential substitute for chemically synthesized colorants. The present study demonstrated the biological properties of

bacterial pigments extracted from soil bacteria. It is been evident that bacterial pigments act as promising candidate in biotechnological applications. The radical scavenging against DPPH and antagonistic ability of chromogenic bacteria against pathogenic bacteria paves a way for exploiting these natural colorants in therapeutics and biomedical application.

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