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Evaluation of the antimicrobial activity of some bacteria-derived bio-active pigments

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Abstract

There is a growing interest and concern for both the safety of the human population and the protection of the environment which has spurred renewed interest in the search for alternative chemicals from microbial origins which are biodegradable and environmentally friendly. Production of stable bio-products has been reported to be strain-dependent. The aim of this study was to evaluate the antimicrobial activity of pigments extracted from some pigmented native bacteria on some tomato spoilage pathogenic bacteria and fungi. In total, four pigment-producing bacteria *spp.* were isolated from samples including underneath tomato roots, soils from vegetation sites, wastewater from potato processing sites, tree liters, and tree bark using the enrichment culture and pour plating technique. The isolates were morphologically and biochemically identified as *Serratia sp.*, *Salinococcus sp.*, *Exiguobacterium sp.*, and *Xanthomonas sp*. The crude bacteria pigments were tested for bio-activity (antimicrobial activity) against some tomato-derived spoilage bacteria (*Xanthomonas sp*. and *Clavibacter sp*.) and fungi pathogens (*Alternaria alternata and Phytophthora infections*) respectively. Among the isolates, pigments of Serratia *sp* had inhibition zones of 34.5mm and 32.1mm against *Xanthomonas sp*. and *Clavibacter sp*. bacteria and 27.8mm and 29.7mm against *Alternaria alternata and Phytophthora infectans* respectively. While *Salinococcus sp*. had 30.5mm and 34.2mm against the bacteria species and 27.9mm and 29.3mm against the fungi respectively. These are promising results. Therefore, further purification of the pigment should lead to discovering potent antimicrobial bio-active ingredients for drug development in the pharmaceutical industry.

Keywords: Antimicrobial; Pathogens; Bio-active; Bacteria; Fungi and Drugs

1. Introduction

From both medical and industrial points of view, natural products (NPs) still represent the richest source of novel molecular scaffolds and chemistry thereby standing as an outstanding source of drugs and drug-related products (Ciddi, 2012; Alejandro *et al*., 2022). Several microorganisms have been reported to produce arrays of pigments with antimicrobial activities against other microbes in varying degrees (Tanuka *et al*., 2019). *Pseudomonas*, *Streptomyces, Staphylococcus,* and *Serratia* are notable pigment-producing bacteria. They could be isolated from water, soil, and plant materials among other natural environments and grown on synthetic laboratory culture media containing different carbon sources (Panneerselvam *et al*., 2012). Bacteria have produced some medical and industrially important bioactive compounds including prodigiosin, a red-pigmented by some Serratia strains as a secondary metabolic product with unique chemical structure. Some of these microbial-derived bioactive compounds have been reported to have biocidal properties against pathogenic organisms by inhibiting proliferation without being toxic to the host cell (Nora *et al*., 2017). Production of the metabolites by these organisms has been reported to occur under various conditions such as temperatures, aeration, pH, strain variation, Nitrogen and oxygen concentrations, agitation rate, and incubation time. The large-scale production potentials of the crude and refined compounds from these pigments have also been assayed to depend on several other factors such as culture media with varying compositional ingredients such as proteins, carbohydrates, and minerals because the presence of these components in the right proportions have been reported to

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promote cell growth as well as increase pigment production (Ana *et al*., 2019). Some reports have indicated that pigment production such as prodigiosin was previously obtained by simple agitation in synthetic and complex culture media such as pure casein and industrial wastes from maize flour respectively. Whereas the proteins responsible for the biosynthesis of some metabolites are encoded within the bioactive biosynthesis gene cluster, the gene induction and range of biological activities are said to vary with environmental factors (Anki *et al*., 2017). Globally, the antimicrobial resistance threat is rising due to careless and unguided use of antibiotics in many cases (Francesca *et al*., 2015). Antibiotic resistance is the ability of the microbe to withstand the inhibitory effects caused by the particular antimicrobial agent at the given concentration. The ability of the microbe to withstand several antimicrobial agents, a multi-drug resistance phenomenon is a global health concern that has recently spurred researchers to seek alternative sources of modern antibiotics. The World Health Organization (WHO) has reported the incidence of these 'superbugs' (multidrug-resistant pathogens) as a global threat to human and animal existence. Microbes concerned are able to do this by adopting one or more strategies including genetic mutations, naturally occurring resistance ability, and the acquisition of resistance patterns from other species through conjugation. Aside from being environmentally friendly, the production of bioactive metabolites of medical importance could be the answer to the growing antibiotic resistance trend due to the amenable nature of the factors involved in bioactive compounds production from microbes. The molecular mechanism of action against bacteria by the bioactive substances which includes general mechanisms like DNA cleavage, pH disruption, phototoxicity, the generation of reactive oxygen species (ROS), and increased hydrophobic stress leading to plasma membrane disruption made the source relatively important. Also, some pigment-bioactive substances from bacteria have been found to inhibit the growth of plant pathogenic fungi such as *Pythium ultimum* and *Fusarium oxysporum using in vitro methods* (*Lingqing et al., 2014).* Therefore, the aim of this study was to isolate and screen some pigment-producing bacteria from the natural environment for antibacterial activity against both Gram-negative and Gram-positive bacteria and fungi.

2. Materials and Methods

2.1. Isolation and Identification of Bacteria

For the isolation of pigmented bacteria, the method of Alexis *et al.,* 2020 was used. Sample each was collected from underneath tomato root, soils from plantain vegetation sites, wastewater from potato processing sites, tree liters, and tree bark using the enrichment culture and pour plating technique. One milliliter of each sample was serially diluted up to 10^{-4} and 0.1ml aliquot of 10^{-2} and 10^{-4} dilutions were plated on nutrient agar and incubated at 37°C for 24 h. After the incubation period, colored (pigmented) colonies were selected and propagated on the same medium until pure cultures were obtained. Pigmentation of the colonies, Gram staining, and other standard biochemical characterization were carried out. The pure cultures of the identified isolates were maintained on nutrient agar until further needed.

2.2. Extraction of Pigment from Bacterial Isolates

Using the method of Narsing *et al.*, 2017, the pigment-producing bacteria isolates were grown in a Nutrient broth incu-shaker incubated for three days at 37°C. After three days, the respective cells were harvested by centrifugation at 4000 RPM for 10 min. The pellet of each culture was washed with sterile distilled water and centrifuged again after which the pellets were respectively suspended with 5 ml of methanol. It was then incubated in a water bath at 60^0 C for 15 min until all visible pigments were extracted and were then centrifuged again at 4000 RPM for 10 min. The colored supernatant was separated and filtered through Whatman no.1 filter paper. The colored extracts were evaporated by keeping them drying overnight. After the complete evaporation of the solvent, they were kept in a hot air oven set at 50^0C to remove trace moisture and ensure that dry powder was produced which was afterward kept in air-tight containers for further applications (Venil *et al*., 2013).

2.3. Validation of Microbial Strains and Inoculum Preparation

For the anti-bacterial evaluation, the method of Velmurugan, *et al.,* 2010 was adopted. Tomato spoilage bacteria; *Xanthomonas sp*. and *Clavibacter sp*. were previously isolated from rotten tomatoes and maintained on suitable media before use. Culture growth on nutrient agar was transferred to plates (Difco Laboratories, USA) and incubated at a temperature of 37°C for 24 hours.

The fungi evaluated, which are considered among the most economically significant spoilage pathogens in plants, were *Alternaria alternata and Phytophthora infectans.* In antifungal assays, all cultures were maintained on potato dextrose PDA and refrigerated at 4°C.

The bacteria inocula were prepared from one pure colony of each bacteria strain by transferring it into 5 ml of nutrient broth and incubated for 24 h at 37°C. The bacteria cultures were diluted with nutrient broth to adjust the concentration

to 0.5-1.0 McFarland standard of 1.3×10^8 Cfu/ml. In order to obtain the fungal inocula, spore suspension of each of the fungal cultures was prepared with 20 ml sterile 0.1 % (v/v) Tween 80 solutions with constant stirring by means of a magnetic stirrer for 10 min. The spore suspensions were adjusted to a concentration of 2×10^6 spores/ml by counting in the Neubauer chamber using a light microscope (Wolk *et al*., 2000).

2.4. Determination of Antimicrobial Activity Using Agar Well Dilution Method

Prior to assay for the antimicrobial activity, each pigment was dissolved in sterile distilled water and sterilized by filtration through a sterile syringe filter having a pore size of 0.02 micrometer.

The resultant sterile aqueous extract of pigment was tested for their antimicrobial activity which was carried out by agar well diffusion method. The lawn of each test micro-organism was made by spreading aliquot suspension of the culture on nutrient agar and PDA plates with the help of a sterile glass rod spreader. Afterward, wells of 6mm diameter were bored with the help of a sterile cork borer, and 100 microliters of each extract were added to the wells using a micropipette at 40mg/ml concentration. The plates were then incubated at 37^0C for 24 hours. After incubation plates were observed for a zone of inhibition (Mounyr *et al*., 2016).

2.5. Determination of Minimum Inhibitory Concentration (MIC)

To evaluate the MIC of the respective extract against the pathogens, the agar dilution method was used according to the method of Nayef (2016). In the procedure, the pigment extract at concentrations of 10, 20, 40, 60, 80, and 100 mg/mL was mixed with sterile MHA (for bacteria) and SDA (for fungi), followed by adding 0.1 mL of microbial suspension at 0.5 McFarland standard and was pour-plated. The plates were incubated at 37°C for 24 hours (for bacteria) and 27°C for 72 hours (for fungi), and then the MIC of the pigments was evaluated. The MIC was defined as the lowest concentration of the extract in a plate with no visible growth. A plate containing medium and without microorganisms, and a plate including medium and microorganism were considered as negative and positive control, respectively.

2.6. Determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

In this procedure, the agar dilution method was used. The bacteria and molds were sub-cultured from the plate containing MIC of the pigment to nutrient Agar and SDA modified with chloramphenicol, respectively. If the sub-cultured microorganisms cannot grow, the MIC MBC and/or MFC concentrations will be equal. However, in contrast to the situation, the grown bacteria were sub-cultured in MHA (for bacteria) and SDA (for fungi) containing the concentrations of the pigments (10, 20, 40, 60, 80, and 100 mg/mL) and the MBC considered as the concentration of the pigment that bacteria did not grow (Beata *et al*., 2021).

3. Results and Discussions

3.1. Isolation and Identification of Pigmented Bacteria

Considering that the various colors of the bacteria are visible, we attempted the isolation of the bacteria on various media. Distinct colony colors produced by the respective natural pigment that were predominant within the 24 hours of incubation were Redish, Orange, Yellow, and Yellow. Sequel to further identifications, the pigment-producing cultures were identified to be *Serratia sp., Salinococcus sp*., *Exiguobacterium sp*., and *Xanthomonas sp.* respectively, and were sub-cultured in sterile Nutrient agar and after the purification steps, the single-isolated colonies were selected for study (Figure 1 and Table2).

The pure cultures were subjected to biochemical tests and the results were compared with the standard, Bergeys Manual of Determinate Microbiology for cross-referencing and identification (Caroline, 2005). The results of the biochemical test are shown in Table 1.

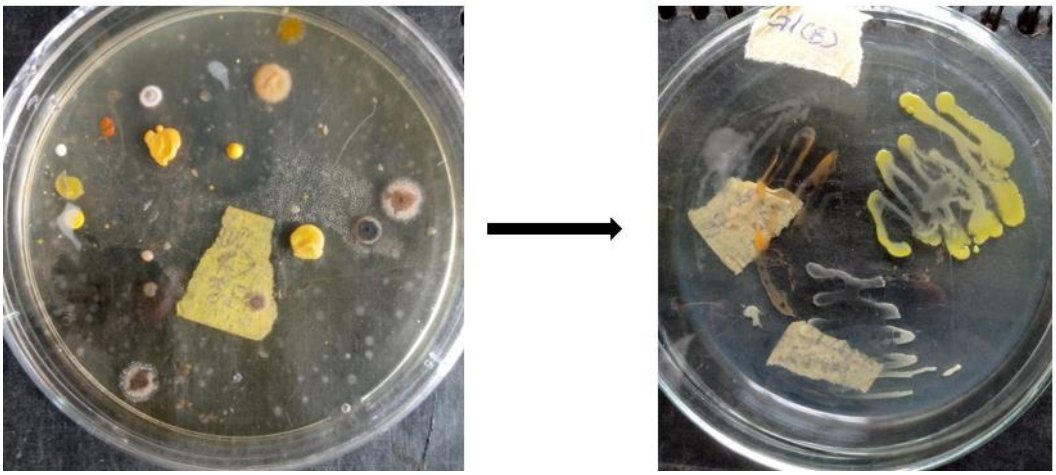

Figure 1 Subculturing and Purification of the Selected (Colored) Bacteria Strains**.**

Table 1 Biochemical Reactions of Pigment-producing Isolates

Bacteria Isolate	**Biochemical Test**							
	Indole	MR	VP	**Citrate**	**Oxidase**	**Catalase**	**Urease**	**Nitrate**
Serratia sp	-	+	+	+	+	+	-	-
Serratia sp	-	+	-	+	+	+	-	-
Exiguobacterium sp	-	+	+	-	+	+	-	+
Xanthomonas sp	+	+	-	+	-	+	-	-

+ = Positive; - = Negative

Sequel to further identifications, the pigment-producing cultures were identified to be *Serratia sp., Salinococcus sp*., *Exiguobacterium sp*., and *Xanthomonas sp.* The pigment and the mean percentage occurrence (%) are as shown in Table 2.

Table 2 The Occurrence of Pigmented Bacteria in the Native (Studied) Samples

Bacteria	**Color on Media**	**Occurrence (%)**
Serratia sp	Red	37.6
Salinococcus sp	Orange	29.4
Exiguobacterium sp	Yellow	12.8
Xanthomonas sp	Yellow	20.2

From the results, *Serratia sp*. has the highest occurrence according to the study (Table 2). It has red coloration because of the red pigment they produce under natural conditions. The study confirmed that their natural habitat included soil and vegetation. This agrees with the experiment of researchers such as Ines *et al*., 2021 who isolated some strains of Serratia from the environment including soil. Studies of the red-colored potato by Ehrenberg and the "bloody" bread earlier showed that *marcescens* were readily found in the environment (Monreal and Reese, 1969). In their study, it was concluded that water was one of the natural environments for several species, including *S. marcescens*, *S. fonticola*, *S. grimesii*, *S. liquefaciens*, *S. plymuthica*, *S. rubidaea*, and *S. ureilytica*. This corroborates our result in the study. A related study also found in river water, predominant species which included *S. marcescens*, followed by *S. liquefaciens*. *S. marcescens* subsp. *sakuensis* was also isolated from the suspended water of a wastewater treatment tank in Japan. The presence of the bacteria in the soil sample is in accord with other studies that perhaps the species are found in soil,

several are associated with plants including grass, tomatoes, green onions, and other vegetables (Steven, 2011). It is possible that in some cases soil is the source of organisms such as *S. marcescens* isolated from plants.

The soil sample screened revealed the presence of orange pigment bacteria which was later identified as *Salinococcus sp.* (Hizbullahi *et al*., 2018). The colonies of the isolate were round, convex, smooth, mucoid, and orange. The intensity of the orange pigment color increased on prolonged incubation from light to dark orange color on agar media. The physiological and biochemical characterization of bacterial isolates was done to confirm the genus. Based on the morphological and partial biochemical characteristics, isolates were found to be a motile, Gram-negative, rod-shaped bacterium with orange colonies in nutrient agar which gave a catalase-positive reaction (Table 1). This agrees with the study by Bhat *et al*., 2015.

The occurrence of Xanthomonas species is as given in Table 2. It is the third in occurrence and was isolated from a leaf litter sample. The study confirmed the presence of phytopathogenic bacteria such as Xanthomonas in leaf and soil samples from the studied sites. This was also identified in a similar environment in the study carried out by Akoua, *et al*., 2022. It also agrees with those of Kumar *et al*, 2017 which shows that the rhizospheric bacteria were a mixture of antagonists, neutral and mutualistic pathogens including Xanthomonas species. The presence of this organism also agrees with the research finding that there is a close connection between subsoil microbes and aboveground components of the plant ecosystem (Arnold *et al*., 2011).

Exiguobacterium sp. has 12.8% in terms of occurrence in the studied samples. They are Gram-positive, rod-shaped, non-spore-forming, and motile bacteria. The cell morphology ranges from ovoid, rods, double rods, or chain of cells which agrees with the work of Pietro et al., 2021. Colonies of the strain are yellow to orange pigmented, 1–1.5 mm in diameter, circular, and smooth. The species may be aerobic or anaerobic depending upon the growth conditions and oxygen availability.

3.2. Preparation of Test Organisms (Bacteria and Fungi)

Prior to the antimicrobial test, some tomato spoilage bacteria and fungi isolates were isolated and maintained in 10% glycerol (Figure II).

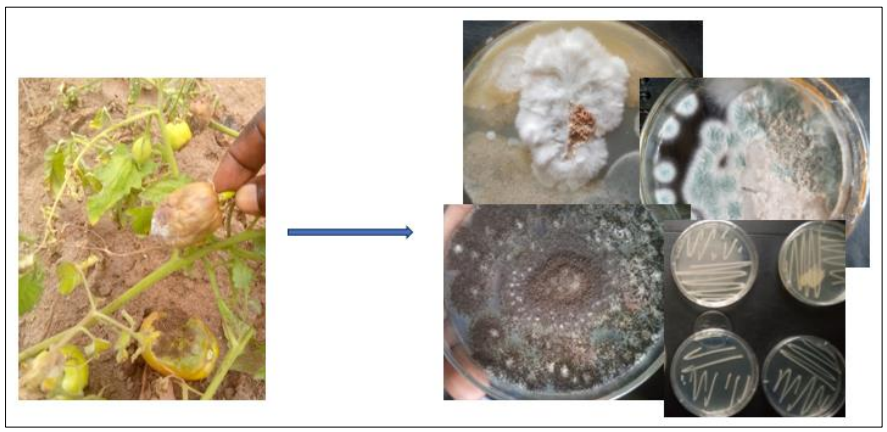

Figure 2 Isolation and Maintenance of Microbial Test

3.3. Cultures (Bacteria and Fungi)

The respective biochemical results of the colored bacteria isolate viz; indole production, Voges-Proskauert test, Nitrate reduction, catalase, urease, glucose fermentation, methyl red, oxidase, and acid production tests were used to identify the bacteria cultures while the mycological atlas was used to confirm the fungi after microscopic examinations according to standard methods.

3.4. Antimicrobial Assay

he pigmented extracts from the bacteria were evaluated using the Kirby-Bauer agar well dilution method at 100mg/ml concentration. The various zone of inhibition value of the extracts is presented in Table 1.

Table 3 The Zones of Inhibition by the Pigmented Extracts

Bacteria Extracts	**Zone of Inhibition (mm)**			
	Xantho. sp.	*Clavibacter sp*	*Alternaria sp.*	*Phytophthora sp.*
Serratia sp.	34.5	32.1	27.8	29.7
Exiguobacterium sp.	20.3	18.9	22.3	-
Salinococcus sp.	30.3	34.2	27.9	29.3
Xanthomonas sp.	-	-	12.5	-
Anti-bacteria Agent	38.3	39.5	-	-
Anti-fungi	-	-	29.4	32.3

According to the results in this study, pigment extract of *Serratia sp* had inhibition zones of 34.5mm and 32.1mm against *Xanthomonas sp*. and *Clavibacter sp*. bacteria and 27.8mm and 29.7mm against *Alternaria alternata and Phytophthora infectans* respectively. While *Salinococcus sp*. had 30.5mm and 34.2mm against the bacteria species and 27.9mm and 29.3mm against the fungi respectively. These values were higher than several researches reported earlier. This difference might be a result of the extraction solvent used. According to findings such as Clarice *et al*., 2017, methanolic extracts produced better antimicrobial results than aqueous extracts. Other factors such as the concentration of extract used and the length of infusion might have accounted for the disparity. However, the trend of the values agrees with the findings of Muhammad *et al.,* 2018.

3.5. The Minimum Inhibitory Concentrations (MIC)

The results of the minimum inhibitory concentrations are shown in Table 4.

Table 4 Minimum Inhibitory Concentrations (MIC)

Bacteria Extracts	**Zone of Inhibition (mm)**			
	Xantho. sp.	*Clavibacter sp*	*Alternaria sp.*	*Phytophthora sp.*
Serratia sp.	10	20	20	20
Exiguobacterium sp.	20	20	20	
Salinococcus sp.	20	20	20	20
Xanthomonas sp.			20	
Anti-bacteria Agent	10	10		
Anti-fungi			5	5

The result shows the minimum inhibitions of the respective isolate by the extracts. The inhibitory concentration was in the range of 10 and 20mg/ml. There was no effect against Xanthomonas sp., *Clavibacter sp*., and *Phytophtora sp*. by the extract prepared from *Xanthomonas sp.* Similarly, the extract of *Exiguobacterium sp*. had no effect on *Phytophtora sp.* at the concentration used. Previous studies have indicated that bacteria secondary metabolites and pigments in particular have immense importance in the treatment of various diseases which was attributed to having properties such as anticancer, antibiotic, and immunosuppressive compounds. The antimicrobial activity was demonstrated in this work with outstanding results. Mohana *et al*., 2013 studied the antibacterial activity of various bacterial pigments against two human pathogenic bacterial strains, *S. faecalis* and *Staphylococcus aureus*.

4. Conclusion

From the history of the existence of humans, plants, and microbes have been known to serve as the primary sources of natural pigments. It is also been noted that microbial-derived pigments offer several advantages over plant-derived

pigments, including enhanced stability, cheap cost, excellent yields, and the convenience of downstream processing. As a result of these attributes, microbial pigments have been a favorable alternative and choice for synthetic drugs.

The pigment extracted from the pigmented microbes including *S. Marcescens* was a success given the effects they produced against the test organisms, which thus agrees with previous work and reports that the extracts contain antimicrobial substances of pharmaceutical relevance. Several pigmented microbial extracts could be produced from native bacteria according to the findings in this work and was demonstrated that the pigment-producing bacteria could be a source of natural ingredients and that these bacteria are present in our native environments including the soil.

Recommendations

Since the antibacterial activities of bacteria pigments like prodigiosin are well-established, it is now the right time for the small-scale industry to start trial production and upscaling for larger applications.

As a hydrophobic molecule, the mechanisms used to introduce most of the extracts into susceptible microbes have to be modified to suit the particular target.

There is a need for further research to determine the spectra range of each extract in order to make them more specific in activity and efficiency. Where the activity and constituents are known, their efficacies could be improved by formulating them with other extracts of known compositions or with known antimicrobial agents.

There is the need to also carry out docking analysis in order to further explore the wider mode of action of the compound using a system like *biopredicta module of the V life MDS 4.3*.

Modern drug discovery promising alternatives to conventional ones. It is obvious that microbial natural products such as those obtained from their pigments could play a significant role in overcoming the present drug resistance threat. in the health care system. Therefore, all efforts should be geared towards upscaling the production of drug constituents from these important microbial products on a large-scale bases through advanced fermentations. Since the producer microbial component can be engineered and the metabolic pathways tailored to optimize production, more contribution supplies can be made to the drug production chain in Nigeria and beyond.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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