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# Isolation, Characterization and Optimization of Pigment-producing Bacteria from Different Sources in Ogwa, Esan West Local Government Area, Edo State, Nigeria

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**ABSTRACT:** *Staphylococcus* species have capability to produce pigments such as carotenoids, melanin and staphloxanthin which can be used in the applications of industries such as food, pharmaceuticals and cosmetics. Hence, the objective of this paper was to isolate, optimize, characterize and identify of pigment-producing bacterial from soil, water, food around Ogwa, Esan West Local Government Area of Edo State, Nigeria using appropriate standard techniques. The microbial load of the bacterial isolates ranges from $2.20x10^4$ cfu/ml to $7.0x10^4$ cfu/ml. All the isolates produced a yellow-colored pigment. During optimization studies, maximum pigment production was observed at pH range of 5 to 7, the temperature of 30, 37 and 42 ℃, inoculum size up to 1% with NaCl concentration of 0.5%, OD at 540 nm respectively. It was observed that peptone worked best for enhancement of pigment production. This findings showed that the bacterial isolates were able to produce pigments under the influence of different nutrition and environmental variability implementing that the bacteria are potential sources for the synthesis of pigments in industries. Hence, the objective of this paper is to isolation, optimization, characterization and identification of pigment-producing bacterial from soil, water, food around Ogwa, Esan West Local Government Area of Edo State, Nigeria

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*Staphylococcus* species are round shaped (cocci), gram-positive bacteria they are usually identified in various environments which include soil, water, food and skin of animals and humans (Kim *et al*., 2017). Pigments are natural substances that gives color to living things such as plants, animals, fungi, bacteria, algae and yeast (Yadav *et al*., 2021). Large amount of *Staphylococcus* species has capability to produce pigments such as carotenoids, melanin and staphloxanthin which can be used in the applications

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of industries such as food, pharmaceuticals and cosmetics (Nurjahan *et al*., 2020). The bacteria that have the ability to produce colours are numerous and are found everywhere. *Staphylococcus species* was identified by the scientists for it golden-yellowish colour when cultured (Bose *et al*., 2012). There are many *Staphylococcus* species strains pigments that are attached to the membrane, which can help the cell to resist certain substances that can harm it. These pigments can also act as antioxidants, and help to change how fluid the membrane is. Characteristics that can affect how well the body's natural immune system can fight off infections (Bose *et al*., 2012). These pigments act as a protective agent for oxidative damage. Scientists has been studying the optimization of growth conditions for pigment-producing *Staphylococcus* species by finding the best conditions for their growth, they can increase the number of pigments they produce. These bacteria could be used as a natural source of color. Temperature plays a major role in the multiplication of *Staphylococcus* species that can produce colours. Various species differ in temperature optima for maximum pigment production. Research shows that *Staphylococcus aureus* produces the largest quantity of yellow pigment at 30ºC (Kim *et al*., 2017). pH parameter also affects pigment production which range varies in different *Staphylococcus* species. For example, *Staphylococcus saprophyticus* produced the largest quantity of pigment at a neutral pH of 7.0 (Kim *et al*., 2017). The availability and composition of nutrients significantly influence pigment production in *Staphylococcus* species, concentration of carbon, types and nitrogen sources along with the presence of specific minerals can impact the production of pigment (Lee *et al*., 2017).

The presence of oxygen, exposure to light and agitation can all impact the production of pigments by *Staphylococcus* species (Saravanan *et al*., 2020). Oxygen availability affects the growth and metabolism of *Staphylococcus* species, which can influence pigment production. *Staphylococcus saprophyticus* for example produces more pigment when oxygen and light is available (Kim *et al*., 2018). Additionally, agitation can influence the availability of oxygen and nutrients, which can ultimately affect pigment production (Kumar *et al*., 2020). Natural pigments have various uses in different sectors such as food, cosmetics and pharmaceuticals. However, the amount and quality of these pigments depends on many factors such as the kind, amount of carbon, nitrogen sources, pH, temperature, salinity and aeration. Therefore, it is necessary to find the best growth conditions for these bacteria to increase the production and quality of the pigments. The finding of the best growth conditions can also help to lower the cost and environmental impact of the pigment production process. By doing so, this study will contribute to our understanding of microbial pigmentation, paving the way for potential biotechnological applications and advancements in various industries

## MATERIALS AND METHODS

*Sample Collection:* Different samples from environmental sources (soil, water, food) were collected in sterile containers and transported to the laboratory for microbiological evaluation. The research work was conducted in Microbiology Laboratory, Glorious Vision University, Ogwa, Esan West local government area of Edo State, Nigeria. It is about at latitude 6˚30 20. 16" North, and longitude 6˚12 30. 24" East.

*Isolation:* For serial dilution, a milliliter was measured and immediately poured into tubes holding sterile distilled water. To guarantee that the colonies were evenly distributed across the agar surface, fifteen milliliters of the prepared molten agar were aseptically put onto the plates after it had cooled. The agar was then gently spun in both clockwise and counterclockwise directions. After that, the agar was left to solidify. MacConkey agar plates and nutrients were incubated for 24 hours at 37°C in an inverted orientation (Olutiola *et al*., 1991; Okanlawon *et al*., 2024).

*Identification and Characterization:* Bacterial isolates were identified based on their colony appearance, cellular structure, and biochemical properties. The identification of the bacterial isolates was carried out following the guidelines in Bergey's Manual of Determinative Bacteriology (Okanlawon *et al*., 2023).

*Enrichment of pigment-producing Staphylococcus aureus:* The isolates were cultured in hundred millitres sterile nutrient broth, then two percent glycerol was added to the Mannitol salt agar to enhance pigmentation while isolates were added into another broth without glycerol to observe the effect of glycerol in pigmentation process. After this process, the inoculated broth was kept on a shaker for incubation at 100rpm for three days and was later checked for pigmentation (Shahmoradi and Nasir, 2016).

*Extraction of pigment:* The golden-yellow colony underwent a 3-day incubation period on a rotary shaker before being centrifuged for 15 minutes at 5,000 rpm. After discarding the supernatant, the cells were centrifuged once more for ten minutes at 2,000 rpm after being washed with sterile distilled water.

Next, the pellet was combined with four milliliters of ethyl acetate and carefully mixed. For fifteen minutes, the suspension was incubated in a water bath at 60°C to extract all visible pigments. After centrifuging this combination a second time for 15 minutes at 2,000 rpm, the extracts were examined by using a spectrophotometer to scan absorbance between 200 and 800 nm in wavelength range (Clauditz *et al*., 2006).

*Optimization of growth conditions*

*Temperature:* A loop full of *Staphylococcus aureus* was inoculated into nutrient agar plates. The inoculated plates were divided into 3 different experimental groups, each representing a different temperature condition. The first plate was incubated at the optimal growth temperature for *Staphylococcus aureus*, which is typically at 37 ℃. The second plate was incubated at a lower temperature at 30 ℃, to determine the effect of suboptimal temperature on growth and pigment production. And the third plate was incubated at a higher temperature at 42 ℃, this was to check the action of elevated temperature on growth and pigmentation. All plates were incubated for a specific period of 24 hours, to allow sufficient time for pigmentation. Pigmentation, growth characteristics including colony size and shape were monitored and record at regular intervals during incubation period. After the incubation period, the plates were observed for the presence of pigmentation, including the intensity and color of the pigment produced (Sethuraman and Muthaiyan, 2019).

*pH optimization:* A nutrient agar growth medium was prepared and poured into 3 different petri dishes, then the *Staphylococcus aureus* strains was inoculated into the different petri dishes, and the first plate optimal pH typically for *Staphylococcus aureus* growth was adjusted to 7.0 using a pH meter or pH indicator strips. The second plate was adjusted to a lower pH at 5.0, to determine the action of acidic conditions on growth and pigment production. While the third plate was adjusted to a more higher pH level than the usual standard to around 9.0, to assess the impact of alkaline conditions on pigmentation process. All three plates were incubated for at 24 hours at 37 ℃. After incubation period, the colours extracted of *Staphylococcus aureus* under various pH values was compared (Clauditz *et al*., 2006).

*0.5% NaCl optimization:* In a 100 ml flask with 0.5% NaCl, an equivalent volume of the bacterial isolate was added, and it was then inoculated with 50 ml of sterile nutritional broth to find the ideal NaCl content for the chosen colored colony. It was then incubated for three days at 37 °C on a rotatory shaker. In order to test for maximum pigment synthesis, the O.D. was obtained at 540 nm (Clauditz *et al*., 2006).

*Peptone optimization:* The chosen coloured organism was given a comparable quantity of the isolate of bacteria and inoculated with 0.5 to 1% peptone in 50 ml of sterile nutrient broth in a 100 ml flask in order to find the ideal peptone concentration. It was then incubated for three days at 37 °C on a rotatory shaker. In order to test for maximum pigment synthesis, the O.D. was obtained at 540 nm (Clauditz *et al*., 2006).

## RESULTS AND DISCUSSION

*Staphylococcus species* are capable of producing pigments that are attached to the membrane, which can help the cell to resist certain substances that can harm it. These pigments can also act as antioxidants, and membrane fluidity. The total of five (5) isolates were collected from various environmental areas such as Soil and water. The bacteria species isolated were then screen for the production of the yellow colour. This findings correlate with report that observed similar pigmentation in bacteria from the samples and they can be important sources for the production of colour in the large potential for food supplements and as antioxidants (Aishwarya and Binita, 2018). For further identification and characterization, the isolates were subjected to biochemical and morphological identification. Some were creamy, circular, and flat and pigment producing developed on the Mannitol Salt Agar media. Similar to the results from Mukherjee *et al*. (2012) who reported that the texture and characterization of the soil isolates where round, creamy, entire and flat. The microbial load of bacterial isolates from environmental sources is shown on table 1. A total of five (5) isolates were obtained and cultured on Mannitol Salt Agar plates. The microbial load from these isolates ranges from $2.20x10^4$ cfu/ml to $7.0x10^4$ cfu/ml.

**Table 1:** Microbial load of bacterial isolates

| Isolate code | Microbial Load (cfu/ml) |
|---|---|
| **S 1** | $2.20x10^4$ |
| **S 2** | $5.9x10^4$ |
| **S 3** | $6.5x10^3$ |
| **S4** | $7.0x10^4$ |
| **S5** | $5.3x10^4$ |

*Legend: S1- S5 = Sample 1 to 5*

The biochemical characterization of the bacterial isolates from environmental sources is presented in table 2. All the organisms were gram-positive, catalase yield positive while oxidase negative, methyl red all positive, voges proskauer positive, indole all negative, coagulase all positive, nitrate reduction all positive, sodium chloride all positive, from starch hydrolysis to gelatin hydrolysis to casein hydrolysis all yielded a

negative response while citrate utilization, glucose, fructose, maltose and mannitol sugar test all yielded positive, Xylose test was all negative, lactose and sucrose were all positive. The outcomes of the tests of the yellow coloured bacterial isolates were tested positive for gram stain reaction, positive for catalase, showed negative for oxidase, fructose, maltose, mannitol, lactose, sucrose showed positive but xylose negative. Several studies have documented that most of the pigment producing bacterial selected include *Staphylococcus aureus* which are Gram positive with cocci shape (Mukherjee *et al*., 2012).

**Table 2:** Biochemical characteristics of bacterial isolates

| Grams reaction | Cellular | Catalase | Oxidase | Methyl Red | VP | Indole | Nitrate Red | Citrate | Coagulases | Starch | Gelatin | Glucose | Fructose | Maltose | Sucrose | Lactose | Probable identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | C | + | - | + | + | - | + | + | + | + | + | + | + | + | + | + | *Staphylococcus aureus* |

The pigment produced by the bacterial isolates is presented in table 3. A total of five (5) samples where isolated and they all yielded a pigment color of "Golden Yellow". Several nutritional materials have a direct action on the organisms that can produce. Ramendra *et al*. (2016) documented the ability of carbon and nitrogen sources which can upgrade the effective synthesis of pigment at various conditions.

**Table 3:** Pigment produced by the bacterial isolates

| Isolate code | Pigment |
|---|---|
| **S1- S5** | Golden Yellow |

*Legend: S1- S5 = Sample 1 to 5*

The (3) different pH levels (5, 7 and 9) were used to observe which was more optimal for the growth of the pigmentation and they ranged from 0.112 OD at 540nm to 1.777 OD at 540nm with a bar chart representation of the result in figure 1.

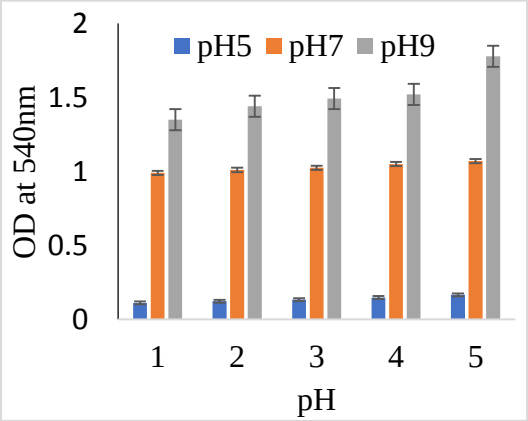


**Fig. 1:** Bar chart representation of the pH optimization of the pigment produced by bacterial isolates

The measure of acidity and alkalinity can have effect on the development of pigmentation by the bacterial species. The results obtained showed the ability of the organism to survive in an array of pH values (5.0 – 9.0) and optical density at 540 nm ranging from 0.112 - 1.777. The slightest of change in pH can solely affect the concentration of the colour which might makes it entirely different from others. The findings of Hizbullahi *et al*. (2018) showed that *Staphylococcus* species can produce yellow colouration from different samples even at higher pH of 7.0. The Temperatures at (30, 37 and 42) degree Celsius were used for incubation to determine the optimal growth for the pigmentation of the bacterial isolates and they ranged from 0.988 OD at 540nm to 3.560 OD at 540nm with a bar chart representation of the result presented in figure 2.

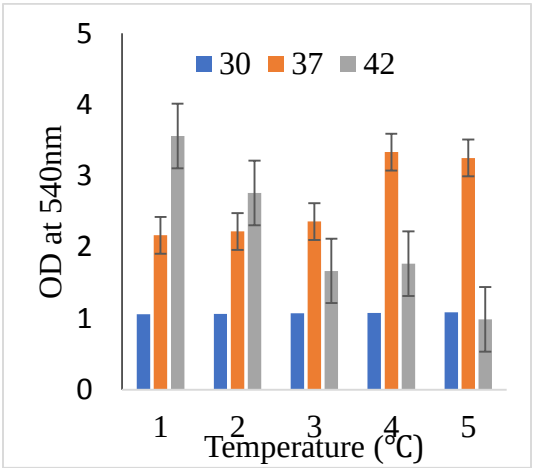


**Fig. 2:** Bar chart representation of the temperature optimization of the pigment produced by bacterial isolates

The optimal temperature for the synthesis of pigment in these bacterial isolates is very essential for characterization. The pigment was produced between 30, 37 and 42 ℃ with OD value of 0.988 - 3.560 at 540 n in this study. The results tallies with results from (Usman *et al.,* 2018), that showed that colours produced were obtained at a wavelength within 200-800 nm. The temperature increment from 30 to 42 ℃ favored the production of yellow pigments. Similarly, Kumar *et al*. (2015) showed that the pigment produced by bacterial species were obtained at 30 to 37 ℃ and they concluded that this range is the best for optimal

pigmentation yield. The production and growth of pigments started to reject moderately as temperature rose far above the optimal values required. In Goswami *et al*. (2010) study showed that 45 ℃ as the highest temperature necessary for pigmentation in bacteria species. It is discovered that temperature is the most effective condition that determine pigmentation in microbes (Kumar *et al*., 2015).The addition 0.5% of NaCl was placed on five (5) different bacterial isolates to enhance the pigmentation and ranged from 0.86 OD at 540nm to 1.045 OD at 540nm with a graphical representation of the result presented in figure 3. In this study, the pigments on the bacterial isolates were produced at a salt concentration of 0.5% NaCl (OD at 540 nm ranging from 0.86 - 1.045 respectively). This findings correlated with the research conducted by Aishwary and Binita (2018), they observed that the production of pigmentation on the bacterial isolates was enhanced at 0.5% NaCl concentration. In several reports on improving the factors necessary for pigmentation by bacterial strains from water bodies which indicates the highest yield at salt concentration of about 30% NaCl (Khanafari *et al*., 2010), showing that bacteria differ in their capacity to synthesize colours at various salt concentrations.

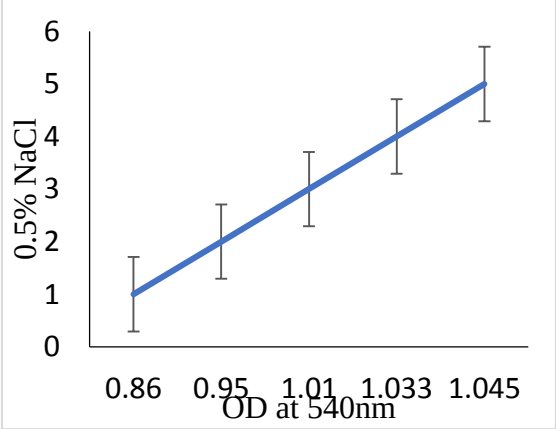


**Fig. 3:** Graphical representation of the 0.5% NaCl optimization of the pigment produced by bacterial isolates

The addition of peptone was placed on five (5) different bacterial isolates to enhance the pigmentation and ranged from 0.645 OD at 540nm to 1.566 OD at 540nm with a graphical representation of the result presented in figure 4. The chosen nitrogen source for this study was peptone, which did put a maximum effect on the pigmentation processes. However, the optical density value was obtained at wavelength 540nm for peptone to enhance the bacterial isolate pigmentation ranged from 0.645 to 1.566. This study correlates with study conducted by Ramendra *et al*. (2016) that observed peptone as a nitrogen source which show maximum effects on the pigmentation by the bacterial isolates.

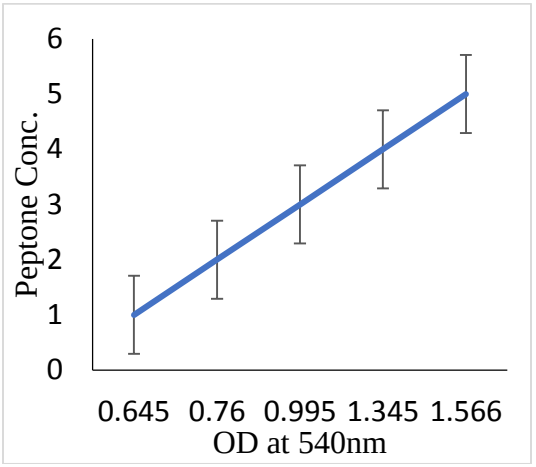


**Fig 4:** Graphical representation of the peptone optimization of the pigment produced by bacterial isolates

*Conclusion:* The pigments producing bacteria were successfully isolated, this study deals with optimization of pigment extracted from bacteria species from environmental sources such as soil and water. However, the potential of the isolates was explored, revealing its ability to be implemented in the medical and industrial fields due to its antimicrobial properties.

*Declaration of Conflict of Interest:* The authors declare no conflict of interest

*Data Availability Statement:* Data are available upon request from the corresponding author

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