Production and characterization of orange pigment produced by Halophilic bacterium *Salinococcus roseus* isolated from Abattoir soil

**Abstract**

There was little information about the nature of orange pigment produced by *Salinococcus roseus*. Therefore this study aimed to optimize and characterize the orange pigment produced by *Salinococcus roseus* isolated from abattoir soil. The soil collected from abattoir was screened for isolation and identification of orange pigment-producing bacteria. The resulting orange pigmented colonies were subjected to biochemical and molecular identification. The phylogenetic analysis of bacterial isolate was carried out using MEGA 6 software. Ethanol was used for pigment extraction and extracted pigment was characterized using UV-Visible spectroscopy and Fourier Transformed Infrared (FTIR) spectroscopy. The stability of the pigment was also determined toward pH and temperature. The sequence analysis of 16SrDNA of the isolate showed maximum identity of 100% to *Salinococcus roseus*. Of various parameters optimized, a temperature of 37°C, pH 7, nutrient broth, 96 hours incubation and under shaking condition of 100rpm/min was found to be optimum for orange pigment production. The UV-Visible spectroscopy at wavelength of 440nm showed characteristic corresponds to zeaxanthin. The FTIR spectroscopy revealed the presence of following functional groups C-O-C (900cm⁻¹), C-H (710cm⁻¹), C=O (1430cm⁻¹), C=C (1610cm⁻¹) and OH (3380cm⁻¹) correspond to zeaxanthin. The pigment was found stable at pH 13 and at temperature of 200oC. This indicated its suitability for various industrial applications such as textile industries.

**Keywords:** Abattoir, sequence, zeaxanthin, FTIR, *Salinococcus roseus*

**Introduction**

The color determines the acceptance of a product and has paramount influence on human life. Many synthetic colors used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing pose various hazardous effects like allergies, tumor, cancer and severe damages to the vital organs. Moreover, the effluent of synthetic dyes poses serious threat to the environment conservation. Consequently, many synthetic colors have been banned due to their toxicological problems. With the increasing awareness about the toxic effects of synthetic colors and consumer safety, there is an increasing interest in the development of colors from natural sources. Pigments are the chemical substances that absorb the light of visible region. They produced color because of the chromophore, a molecule specific structure which captures the sun energy and causes an excitation of electron from external orbital to higher orbital, where the non-absorbed energy is refracted or reflected to be captured by eye. As the present trend throughout the world is shifting towards the use of eco-friendly and biodegradable commodities, the demand for natural colors is increasing day by day. Natural pigments are sourced from ores, insects, plants and microbes. Among microbes, bacteria have immense potential to produced diverse bio-products and one such bio-product is pigments. Biopigments produced from microorganisms are preferred over those from plants because of their stability and availability for cultivation throughout the year. Bacterial pigment production is now one of the emerging field of research to demonstrate its potential for various industrial applications.

**Materials and methods**

**Collection of soil samples**

Twenty (20) different types of soil samples were collected within Sokoto State Abattoir. Ten gram (10g) of soil samples were collected in the morning around 7:00 am, by excavating the surface at a depth of 1.0 cm and transferred into sterile container and labeled accordingly. The containers were placed on ice in a cooler and transport to Microbiology Laboratory, Sokoto State University.

**Isolation of orange pigment-producing bacteria**

The soil samples collected were serially diluted and plated on nutrient agar and incubated at 35°C for 48 hours. Following the incubation only orange colonies were selected and propagated on the same medium to obtained pure cultures used for further studies.

**Morphological and biochemical characterization of the isolates**

Gram staining reaction and microscopic studies were performed for the isolate after 48 hours incubation. The biochemical tests performed were Simmons’s Citrate test, Indole test, Methyl Red (MR), Voges Proskauer (VP), Oxidase and Catalase tests, Coagulase test, Urease test and TSI for Identification according to Bergey’s Manual of Determinative Bacteriology.

**DNA extraction using boiling method**

For DNA extraction, single colonies growing on solid media were removed with a sterile plastic tip and resuspended in 100μl of sterile molecular grade water in a micro centrifuge tube and vortex for 1 minute.
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Optimization studies were carried out in accordance to method used by Batt et al.\textsuperscript{10} An affect of growth media (Nutrient broth, lactose broth and Mueller Hinton broth), Incubation period (24, 48, 72 and 96 hours), effect of pH (3, 4, 5, 6, 7, 8, 9 and 10), effect of temperature (25°C, 30°C, 35°C, 40°C and 45°C) and effect of shaking/static conditions was determined on the bacteria for highest pigment production.

DNA extraction using phenol-chloroform method

For DNA extraction, single colonies growing on solid media were removed with a sterile plastic tip and resuspended in 100μL of sterile molecular grade water in a micro centrifuge tube and vortex for 1 minute. 100μL of chloroform-isomyl alcohol were added to the suspensions and, after briefly vortexing for 30 second, the mixture was centrifuged at 16,000xg for 5 min at 4°C. 10μL of the upper aqueous phase were used as a source of DNA template for the PCR applications. The rest of the mixture was stored at 4°C until use (Silva and Silva, 2005).

Polymerase chain reaction

PCR amplification was carried out on DNA using 16SrDNA. All PCR reaction was performed using Thermal Cycler. Each 50μL reaction mixture contained 25μL Mastermix (50 units/ml Taq polymerase, 400μL moles DNTPs, 3mM MgCl\textsubscript{2}) 19μL of ultrapure PCR water, 2μL of forward primer and 2μL of DNA template. Amplification was carried out under the following PCR conditions: Initial denaturation at 95°C for 5 minutes, 30 circles at 95°C for 1 min, an annealing at 55°C for 1 min, elongation at 72°C for 2 minutes, with final elongation step of 72°C for 5 minutes. Successful amplification was confirmed by ethidium bromide fluorescence in 1% agarose gel.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out on extracted DNA to separate the DNA fragments by their size and to visualize the fragments. This is done by weighing 1.0g of agarose and dissolved in 100ml of 1 X TBE buffer and heated in a microwave for 30sec. swirling halfway through. It was allowed to cool and 4μL of ethidium bromide was added and poured into taped tray. Bubbles were removed with the tip of the comb and the comb was inserted and allowed to set for 20 mins. The tape and comb were then removed and the ladder and samples were loaded after mixing 5μL of each sample with 2.5μL loading dye. It was then run at 100V for 40mins. and viewed under UV light.

Sequencing analysis of 16SrDNA

Samples were sequenced after purification. Sequencing was carried out using the same primers for PCR. Analysis of sequences was carried out using Blast search\textsuperscript{3} by aligning sequences obtained with the closest match found in the GenBank, after which phylogenetic analysis was carried out for the blast results.

Results and discussion

Twenty (20) abattoir soils were screen for isolation of orange pigment bacteria, a total of fifteen bacteria were isolated showing different pigmentation only one bacterium showed orange pigmentation and was used in this study (Table 1). The colonies of the isolate were round, convex, smooth, mucoid and orange. The pigmentation and was used in this study (Table 1). The colonies of pigment bacteria, a total of fifteen bacteria were isolated showing different pigmentation only one bacterium showed orange pigmentation and was used in this study (Table 1). The colonies of the isolate were round, convex, smooth, mucoid and orange.

Production and extraction of pigment

The isolate was grown in Erlenmayer flask containing 250ml nutrient broth at 37°C for 72 hours. The observation of orange pigmentation in a broth indicated pigment production. The extraction of pigment was done by centrifuging the culture broth at 4,000rpm for 15 minutes, the cell pellets were discarded. The orange pigment cells were washed using deionized water and further extracted by addition of 50ml of ethanol. The extracted orange pigment was then subjected further analysis.

Characterization of orange pigment

UV-Visible spectroscopy

The extracted pigments were subjected to UV-visible spectrophotometric analysis. The extracted color was analyzed by scanning in a UV-Visible spectrophotometer for determining the maximum absorbance. The scanning range was selected from 200-800 nm and absorbance at an interval of 40nm was measured.\textsuperscript{11}

Fourier transform infrared (FTIR) spectroscopy

The concentrated orange pigment was subjected to FTIR spectroscopy. This is done by mixing the pigment extract with small amount of KBr. The preparation was then pressed in a sample holder and analyzed by computerized Fourier Transform Infrared Spectroscopy system which generates the transmitting spectra showing the unique chemical bonds and the molecular structure of the sample material.\textsuperscript{7}

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**Table 1** Pigment-producing bacteria isolated from Abattoir soil

| S/n | Bacterial ID | Colony appearance       | Number of occurrence |
|-----|-------------|-------------------------|----------------------|
| 1   | SP1         | Blue green pigmentation | 7                    |
| 2   | SP2         | Yellow Pigmentation     | 4                    |
| 5   | SP7         | Orange pigmentation     | 1                    |
| 7   | SP13        | Yellow green pigmentation | 3                  |

**Table 2** Morphological and biochemical characteristics of the isolates

|   | Catalase | Coagulase | Methyl red test | Voges proskauer test | Indole test | Citrate test | Urease test | H2S | Gas production | Glucose | Fructose | Lactose |
|---|----------|-----------|-----------------|----------------------|-------------|--------------|-------------|-----|----------------|---------|----------|---------|
| 1 | +        |           |                 |                      |             |              |             |     |               |         |          |         |
| 2 |          | -         |                 |                      |             |              |             |     |               |         |          |         |
| 3 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 4 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 5 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 6 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 7 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 8 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 9 |          |           |                 |                      |             |              |             |     |               |         |          |         |
| 10|          |           |                 |                      |             |              |             |     |               |         |          |         |
| 11|          |           |                 |                      |             |              |             |     |               |         |          |         |
| 12|          |           |                 |                      |             |              |             |     |               |         |          |         |

Key: - = Negative + = Positive

The neighbor joining Phylogenetic tree analysis of 16SrRNA sequence of *Salinococcus* sp. with other sequences in the database revealed that, the sequence of the isolate showed 100% identity to the 16SrDNA gene sequence of *Salinococcus roseus* (KX000901.1) when the sequence was blasted against NCBI database (Figure 2)(Figure 3).

**Figure 1** Agarose gel electrophoresis of the PCR amplified 16SrRNA gene of the isolate.

**Table 1** Pigment-producing bacteria isolated from Abattoir soil

**Figure 2** The Phylogenetic analysis by neighbor joining tree of *Salinococcus* specie isolated from Abattoir soil.

**Figure 3** DNA Sequences of *Salinococcus roseus*.

The biosynthesis of a pigment is significantly affected by the incubation temperature. The results of effect of incubation temperature on pigment production (Figure 5) showed that highest pigmentation was observed by *Salinococcus roseus* at 40°C. The variation of pigment production at different temperature by the *Salinococcus roseus* might be attributed to enzymes activities during
growth and pigment production, as highest activities of enzymes occur at optimum temperature. This implies that *Salinococcus roseus* is mesophilic bacteria requiring optimum temperature between 25–45°C.

The rate of pigmentation by the *Salinococcus roseus* was higher around neutrality, at pH 7 the isolate showed highest pigmentation with 30% absorbance which gradually decline toward alkaline pH (Figure 7). The low production of pigments by the isolate between pH 2–8 and pH 8–10 might be attributed to enzymes inhibition for the biosynthesis of the pigment at both acidic and alkaline pH. This implies that the bacterial isolates required neutral pH or somewhere around neutrality for growth and pigment production. The growth and type of pigment production by microorganisms is largely affected by the pH of the medium in which the microorganisms grow, therefore slight changes in pH can also alter the rate of growth of microorganisms and pigment production.16 Similar work reported by Bhat & Marar13 reported that *Salinococcus roseus* showed highest pigmentation at pH 7. The influence of static and shaking condition were determined on pigment production by the *Salinococcus roseus*. It was observed that the pigmentation was favor under shaking condition while at static condition *Salinococcus roseus* showed minimum pigmentation (Figure 8). The studied parameters showed that that *Salinococcus roseus*.

The UV-Visible results of the extracted pigments was generated at a wavelength region between 200-800nm. The orange pigment produced by *Salinococcus roseus* showed highest peak of 450nm which is characteristics of carotenoid pigment (Figure 9). The FTIR analysis of orange pigment produced by *Salinococcus roseus* revealed the following functional groups C-O-C (900cm⁻¹), C-H (710cm⁻¹), C=O (1430cm⁻¹), C-C (1610cm⁻¹) and OH (3380cm⁻¹). These functional groups and their absorption frequencies correspond to that of zeaxanthin (Figure 10).
The orange pigment showed good stability toward temperature when exposed to 160°C and 200°C for ten (10) minutes. The reasons for thermal stability of the pigments might be attributed to present of phenolic conjugated double bond in the pigments structure. The thermal stability of pigments implies that the orange pigment can offer various industrial applications such as in dying, textile and food industries. Similar finding by Ahmad et al., who reported that pigments produced from bacteria showed good stability toward temperature ranging from 45°C-120°C when exposed for one (1) hour (Table 3). The results of pigment stability revealed that the orange pigment changed to different color at pH 2 and 13. The instability of the pigments at pH 2 and 13 is attributed to complete destruction or alteration of pigments structure at acidic and alkaline pH. In alkaline condition, excess OH ions from NaOH deprotonates the phenolic group causing the formation of an anion and destruction in the conjugated structure of the pigment (Table 4).

### Table 4 Effect of pH on the stability of pigments

| Pigment     | pH condition | Maximum wavelength (ʎ max.) | Instant color changed | Color changed after 24 hours |
|-------------|--------------|------------------------------|-----------------------|------------------------------|
| Orange pigment | Control       | 440nm                        | Orange                | Orange                       |
|             | pH 2          | 400nm                        | Yellow                | Yellow                       |
|             | pH 13         | 440nm                        | Orange                | Orange                       |

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