Original Research Article

STUDIES ON BIOCOLORANTS PRODUCTION BY PIGMENT-PRODUCING BACTERIA ISOLATED FROM SOIL

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ABSTRACT

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The use of synthetic organic colors has been acknowledged for many years as the most reliable and economical method of restoring some of the food's original shade to the processed products. However, from the health safety point of view, they are not accepted by consumers because they produce skin allergies, less stable and also produce highly toxic wastes that pose a threat to the environment. In this study, bacteria from different soil were screened on nutrient agar for pigments production. Three (3) isolates that showed purple, orange and blue-green pigment were selected for pigment productions. These isolates were purified and identified molecularly as *Chromobacterium violaceum, Pseudomonas aeruginosa* and *Salinococcus roseus*. The phylogenetic analyses of bacterial isolates were carried out using Molecular Evolutionary Genetics Analysis (MEGA 6 software). Ethanol, methanol and chloroform were used for pigments extraction and extracted pigments were characterized using Ultraviolet-Visible spectroscopy, Fourier Transformed Infrared (FTIR) spectroscopy and thin-layer chromatography (TLC).The stability of the pigments was also determined toward pH and temperature. The effects of growth medium, pH, temperature, incubation time, shaking and static conditions on pigments production was also determined. It was observed that *Chromobacterium violaceum* produced highest purple pigment in nutrient broth at pH 8 for 96 hours of incubation at 35^{o}C under shaking condition. The *Pseudomonas aeruginosa* produced green pigment in nutrient broth at pH 7, 72 hours of incubation at 37^{o}C under shaking condition. The *Salinococcus roseus* produced highest orange pigment on nutrient broth at pH 7, after 96 hours of incubation at 40^{o}C under shaking condition. The sequence analysis of 16SrDNA showed maximum identity of 100% to *Salinococcus roseus* and *Pseudomonas aeruginosa.* The characteristics of the pigments corresponded to that violacein, pyocyanin and zeaxanthin based on their FTIR, UV-visible spectroscopy and TLC results. It was found that all the pigments showed good stability at the temperatures of 200 ^{o}C and fairly stable at lower pH (2). It therefore concluded that the soil could be the source for isolating pigment-producing bacteria that would offer various industrial applications such textile industries.

Keywords: Pigments; FTIR, Bacteria; *Pseudomonas aeruginosa.;* Pyocyanin; UV-Visible spectroscopy

INTRODUCTION

Pigments are compounds with uniqueness of importance to many industries. In the food industry, they are used as additives, antioxidants, color intensifiers, etc. Pigments come in a wide selection of colors, some of which are water-soluble. The terms 'pigment and color' are generally applied for the food coloring matters, sometimes indistinctly [1]. The color determines the acceptance of a product and has paramount influence on human life. Many synthetic colorants used in

UNDER PEER REVIEW

foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing pose various hazardous effects such as allergies, tumor, cancer and severe damages to the vital organs [2]. Moreover, the effluent of synthetic dyes poses serious threat to the environmental 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 [3].

The recent awareness in human safety and environmental conservation has made fresh enthusiasm for natural sources of colors. Natural colorants or dyes derived from flora and fauna are believed to be safe because of non-toxic, non-carcinogenic and biodegradable nature [4]. Traditional sources of colorants include natural products such as flavonoids and anthraquinones produced by plants and animals. For example, carminic acid, a deep red anthraquinone, produced by scale insects, is now used as a pigment in paints, crimson ink, cosmetics and food colors [5].

As the present trend throughout the world is shifting towards the use of eco-friendly and biodegradable commodities, the demand for natural colorants is increasing day by day. Natural pigments are sourced from ores, insects, plants and microbes. Among the microbes, bacteria have immense potential to produce diverse bioproducts like pigments. The production and application of bacterial pigments as natural colorants have been investigated by various researchers [6][7]. Bio-pigments produced from microorganisms are preferred over those from plants because of their stability [8] and availability for cultivation throughout the year [9]. Bacterial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications [10]. Most of the bacterial pigment production is still at the research and development stage. Hence, work on the bacterial pigments should be intensified especially in finding cheap and suitable growth medium, which can reduce the cost and increase its applicability for industrial production [7].

Pigments of various colors are synthesized to protect the cells of microorganisms from injurious effect of light rays of visible and near ultraviolet range [11]. These pigments are synthesized by various types of microorganisms as secondary metabolites and not often found in all types of organisms [12]. An important group of organic constituents of bacterial protoplasm is that of pigments. Some of these, like prodigiosin, pyocyanin, violacein, phenazine, pulcherrimin, iodinin, indigoidine and melanin, are metabolic by-products formed under special circumstances

UNDER PEER REVIEW

[13]. This study was aims to isolate and identify pigment producing bacteria from soil and determine various growth and cultural conditions for highest pigment production and also to determine the stability of the pigments produced.

MATERIALS AND METHODS

Study Area

This study was carried out in Sokoto Metropolis, Sokoto State which is located in the Northwestern part of Nigeria. Sokoto metropolis comprises of Sokoto North, Sokoto South, Part of Wamakko, Dange–Shuni and Kware Local Government Areas. The metropolis is the seat of the Government of Sokoto State and the political capital of the State. The State lies within latitude of 12^{o}N and 13.58^{o}N and longitude 4.8 and 60-54^{o}E bounded in the North and West by the Niger Republic, the South by Kebbi State and East by Zamfara State. It covers a land area of 26, 648.48KM [14].

Collection of Soil Samples

Different types of soil samples were collected from different sites within Sokoto metropolis. Different soil conditions such as organic waste soil, river site soil, garden soil, road side soil, mechanic workshop soil, dustbin soil and farm soil were taken into consideration for the site selection and sample collection. Fifty gram (50g) of soil samples were collected in the morning around 7:30 am, by excavating the surface at a depth of 1.5 cm and transferred into sterile container and labeled accordingly. The containers were placed on ice in a cooler and transported to Microbiology laboratory, Sokoto State University [15].

Isolation of Pigment-Producing Bacteria

Isolation of pigment-producing bacteria was done following the method [16]. The soil samples collected were serially diluted and plated on nutrient agar and incubated at 35°C for 48 hours. Following incubation, only pigmented colonies were selected and propagated on the same medium and pure cultures obtained were used for further studies. Individual colonies of bacteria which varied in shape and pigments were stored on the nutrient agar slant at 4°C and sub-cultured every 2 weeks

UNDER PEER REVIEW

Morphological and Biochemical Identification of Pigment-Producing Bacteria

Gram staining reaction and microscopic studies were performed for the isolates after 48 hours incubation at 37^{o}C. The biochemical tests performed were Simmon's Citrate test, Indole test, Methyl Red (MR), Voges Proskauer (VP), Oxidase and Catalase tests, Coagulase test, Urease test and TSI for Identification accordingly [17].

Molecular Characterization of Selected Isolates using 16SrDNA Sequence Analysis

Amplification of 16S rDNA

Polymerase Chain Reaction (PCR) was carried out in 200 µL reaction containing template DNA, forward primers (5'-AGAGTTTGATCMTGGCTCAG-3'), Deoxyribonucleotide triphosphate (dNTPs) and Taq polymerase. The reaction was cycled 35 times as 94^{o}C for 30 seconds, 58^{o}C for 30 seconds, 72^{o}C for 1 min 30 seconds followed by final extension at 72^{o}C for 10 minutes. The PCR products were analyzed on 1% agarose gel in 1× TBE or Tris/Borate/EDTA buffer, run at 100V for 45 hours. Gel was stained with Ethidium bromide and photographed [18].

Sequencing and Phylogenetic Analysis

Sequencing was done as per manufacturer instructions. The sequence was aligned with corresponding sequences of 16SrDNA from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast [19]. Multiple alignments were generated by the CLUSTAL W program and phylogenetic tree was constructed by neighbor-joining algorithm using MEGA 6 Software [18].

Optimization Studies

The optimization studies were carried out in accordance to method used [20]. 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 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 °C and 45 ^{o}C) and effect of shaking/static conditions was determined on pigment-producing bacteria for highest pigment production.

Production and Extraction of Pigment

The isolates were grown in Elemlayer flask containing 250 ml nutrient broth at 37 ^{o}C for 72 hours. The observation of orange pigmentation in a broth indicated pigment production. The extraction of orange and purple pigments was done by centrifuging the culture broth at 4,000

UNDER PEER REVIEW

rpm for 15 minutes, the supernatants was discarded. The orange pigment cells were washed using deionized water and further extracted by addition of 50 ml of ethanol to purple pigment and 50 ml of ethanol to orange pigment. The extracted pigments were then subjected to 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 [21].

Fourier Transform Infrared (FTIR) Spectroscopy

The concentrated pigments were 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 [7].

RESULTS AND DISCUSSION

The results presented in Table 1 showed list of pigment-producing bacteria isolated from different soils. Bacteria with different pigmentation such as blue green pigmentation, orange pigmentation, yellow pigmentation and purple pigmentation were observed.

Table 1: Pigment-Producing Bacteria Isolated From Different Types of Soil Samples

S/n	Bacterial ID	Sample Type	Pigment Produced
1	SP1	Organic waste soil	Blue green
2	SP2	River site soil	Yellow
3	CH1	Garden soil	Purple
4	SP4	Road site soil	Yellow
5	SP7	Abattoir soil	Orange
6	SP10	Mechanic workshop soil	Yellow
7	SP13	Sewage soil	Yellow green

UNDER PEER REVIEW

8	SP14	Garden soil	Blue green
9	SP9	Farm soil	Orange
10	CH10	Dustbin soil	Yellow

Table 2 presents the results of morphological and biochemical characteristics of pigment-producing bacteria isolated from soil. It was observed that all the isolates were Gram-negative rods, catalase positive, citrate positive and motility positive. The CH1 Isolate produced purple pigment, SP1 produced blue green pigment and SP7 produced orange pigment. The isolates were confirmed based on Bergey's Manual of Determinative Bacteriology. The occurrence of *Chromobacterium violaceum* in garden soil indicated that the organisms belong to Rhizobiaceae family which is found in soil. The inhabitation of *Pseudomonas aeruginosa* in organic waste soil indicated that the bacterium is capable of utilizing various organic substances, also the bacterium have the ability to secrete various substances for solubilizing organic compound. The primary reason for pigment production by the isolates might be attributed to photosynthetic process, UV protection and defense mechanisms [15]. A similar result was observed by Mukherjee *et al*. [22] isolated *Pseudomonas aeruginosa* from soil and the bacterium showed green pigmentation. Rokade and Archana [15] isolated violet pigment producing bacteria from garden soil. The bacteria were found to be *Chromobacterium violaceum* and the bacterium showed purple pigmentation on nutrient agar after 24 hour incubation at 30 ^{o}C.

Table 2: Morphological and Biochemical Characteristics of the Isolates

S/n	Biochemical and Morphological Characterization	Bacterial Isolates		
		CH1	SP1	SP7
1.	Gram's reaction	Negative	Negative	Negative
2.	Shape	Rod	Rod	Rod
3.	Pigment	Purple	Blue green	Orange red
4.	Motility	Motile	Motile	Motile
5.	Catalase	+	+	+
6.	Coagulase	-	-	-
7.	Methyl red test	-	-	-
8.	Voges proskauer test	-	-	-
9.	Indole test	-	-	-
10.	Citrate test	+	+	+
11.	Urease test	-	-	-
12.	H_2S	-	-	-
13.	Gas production	+	+	+
14.	Glucose	+	-	+

UNDER PEER REVIEW

15.	Fructose	+	-	-
16.	Lactose	-	-	+

Key: - = Negative, + = Positive

Figure 1 presents the result of gel electrophoresis showing short fragment of PCR products from pigment-producing bacteria. Line 1 shows ladder 100bp, while the remaining line indicates PCR products.

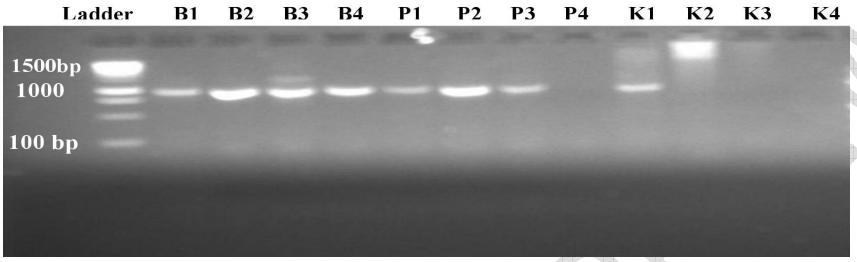

Figure 1: Gel Electrophoresis of PCR Products

Key: B1, P1, K1 = DNA Extraction (by boiling, Phenol-chloroform and DNA kit for isolate CH1), **B2, P2, K2** = DNA Extraction (by boiling, Phenol-chloroform and DNA kit for isolate SP1) and **B3, P3, K3** = DNA Extraction (by boiling, Phenol-chloroform and DNA kit for isolate SP7)

GAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAAGGGA
GCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACG
TCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT
CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC
ACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT
ACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG
AACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGT
GGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGA
CATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCAC
CTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCA
TGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGG
AGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGC
TAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGAC
TGGGGTGAAGTCGTAACAG

Figure 2: DNA Sequences of *Pseudomonas aeruginosa* Isolated from Garden Soil

UNDER PEER REVIEW

GGGATGCGAGTGCTATACATGCAGTCGAACGCGCGGATCAGGAGCTTGCTCCTGTGACGCGAGTGGCGGA
CGGGTGAGTAACACGTAGGCAACCTGCCCATCAGACTGGGATAACCACGGGAAACCGTGGCTAATACCGG
ATAATCCTTTTCCACACAGGTGGGAAAGTTGAAAGGCGGTCTTTTGGCTGTCACTGATGGATGGGCCTGC
GGCGCATTAGCTGGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTCAGGG
AAGAACGCCGACGGGAGTAACTGCCCGTCGGGTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCG
GTTCGTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGCGGACTTGAGT
GCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCG
AAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGGTAAGGGGGTTTCCGCCCCTTTAGTGCTGCAGCT
AACGCATTAAGCACTCCGCCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGAGCATGTGGTTTAATTTCGAAGCAACGCGAAGACCTTACCAATCTTGACATCCTCTGA
CCACCCTGGAGACAGGTTTCCCTTCGGGGCAGAGTGACAGGTGGTGCATGGTGTCGTCAGCTCGTGTCGT
GAGATGTTTGGTTAGTTCCGCACGAGCGCACCCTTATCATAGTGCAGCATCAGTGGCACTCTATGGACAC
TGCGTGACATCGGAGAAGGTGGGGGATGACGTCAATCATCATGCCGTTTAAGATGGTTAACACGGTCTCA
ATGACGGTACAGCAGCTAAGCGCTAGC

Figure 3: DNA Sequences of *Salinococcus roseus* Isolated from Abattoir Soil

Figure 4 presents the phylogenetic tree by neighbor joining of *Pseudomonas* species isolated from garden soil. The sequence of the isolate showed 100% identity to the 16SrDNA gene sequence of *Pseudomonas aeruginosa* (ATCC 10145) when the sequence was blasted against NCBI database.

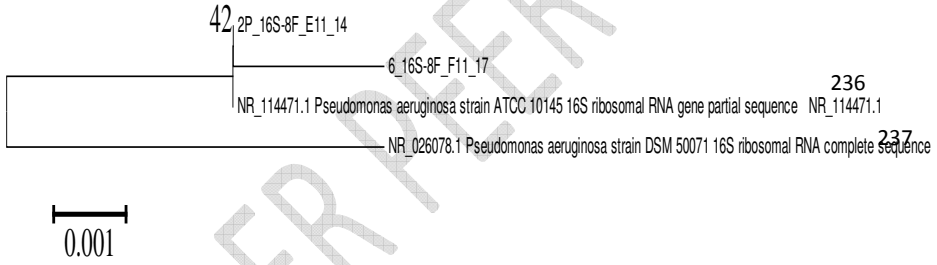

Figure 4: Phylogenetic Tree by Neighbor Joining of *Pseudomonas* specie Isolated from Garden Soil

UNDER PEER REVIEW

Figure 5 presents the phylogenetic tree by neighbor joining of *Salinococcus* species isolated from abattoir soil. 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.

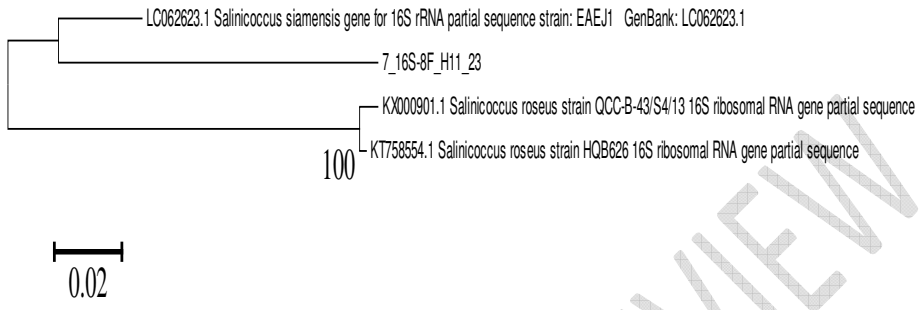

Figure 5: Phylogenetic Tree by Neighbor Joining of *Salinococcus* specie Isolated from Abattoir Soil

The results of effect on growth medium on pigments production is presented in Figure 6 and revealed that nutrient broth favored highest pigmentation on all the isolates than in lactose and Mueller Hinton broth. This might be due to availability of some amino acids required for biosynthesis of the pigment which is present in nutrient broth but absent in lactose both and Mueller Hinton broth. The nutrient broth is a commercially media containing digest of a particular plant or animal protein, which made it available to organisms, as peptides and amino acid to help satisfy the requirements for nitrogen, sulfur, carbon and energy [23]. Similar results reported by Bhat and Marar [24], who observed that the growth and pigment production were higher when the *Salinicoccus roseus* (MKJ 997975) was grown in nutrient broth than in lactose broth medium. Cortes-Osorio *et al.* [25] reported that the *Chromobacterium violaceum* showed highest violocein production on nutrient broth medium. And Laqaa [26] observed pigment production *Pseudomonas aeruginosa* was highest in nutrient broth.

UNDER PEER REVIEW

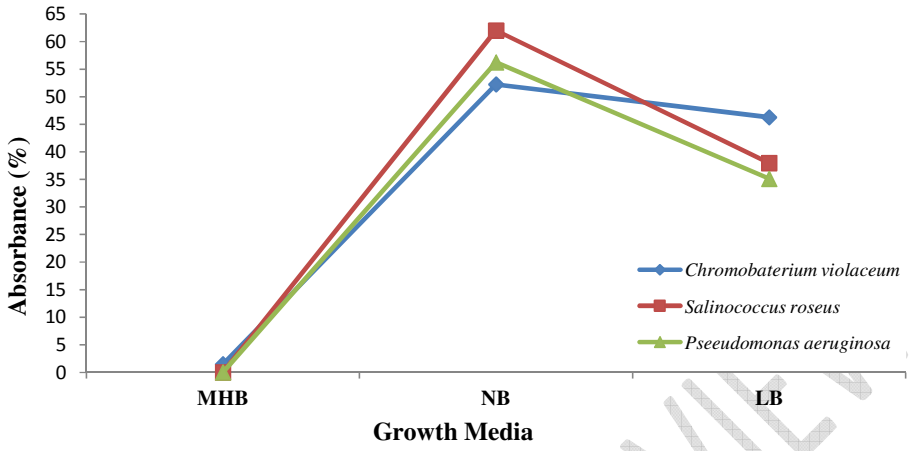

Figure 6: Effect of Different Growth Media on Pigment Production by Pigment-Producing Bacteria Isolated From Soil

The biosynthesis of a pigment is significantly affected by the incubation temperature [27]. The results of effect of incubation temperature on pigment production showed that highest pigmentation was observed at 35^{o}C by *Chromobacterium violaceum*, *Pseudomonas aeruginosa* produced highest green pigment at temperature of 37^{o}C and *Salinococcus roseus* produced highest pigment at 40^{o}C (Figure 7). The variation of pigment production at different temperature by the pigment-producing bacteria might be attributed to enzymes activities during growth and pigment production, as highest activities of enzymes occur at optimum temperature. This implies that *Chromobacterium violaceum*, *Pseudomonas aeruginosa* and *Salinococcus roseus* are mesophilic bacteria requiring optimum temperature ranges between 25 – 45^{o}C. The results from this findings is similar with the finding of Chandran *et al.* [28] who reported that *Pseudomonas aeruginosa* produced highest pigmentation at temperature of 37^{o}C. Cortes-Osorio *et al*. [25] also reported that the maximum production of violacein by *Chromobacterium violaceum* was observed at temperature between 30^{o}C – 35^{o}C.

UNDER PEER REVIEW

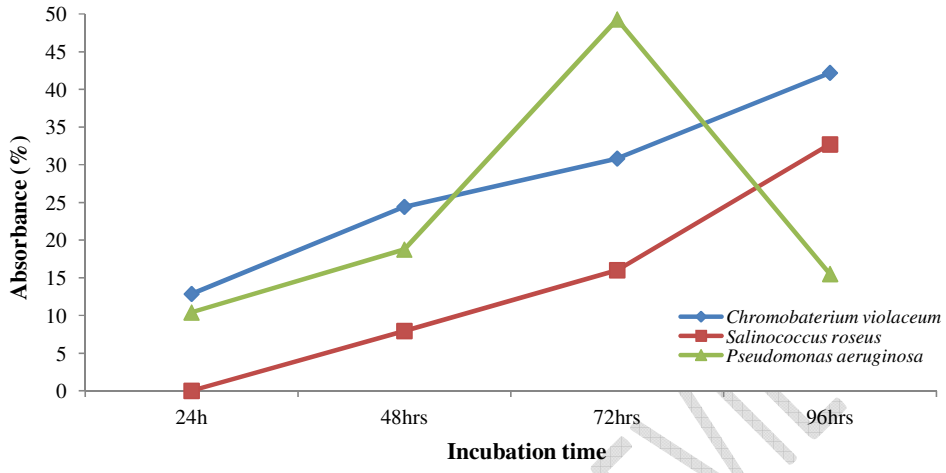

Figure 7: Effect of Incubation Time on Pigment Production by Pigment-Producing Bacteria Isolated From Soil

Figure 8 presents the results of the effect of pH on pigments production by *Salinococcus roseus, Pseudomonas aeruginosa* and *Chromobacterium violaceum*. It was observed that the rate of pigmentation was higher around neutrality. At acidic or alkaline pH, the rate of pigmentation was very slow. The *Salinococcus roseus* and *Pseudomonas aeruginosa* showed highest pigmentation at pH 7 while *Chromobacterium violaceum* showed highest pigmentation at pH 8. The low production of pigments by the isolates 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 [29]. Similar work reported by Chandran *et al.* [28] who observed that *Pseudomonas aeruginosa* produced highest pigmentation at pH 7. Cortes-Osorio *et al.* [25] reported that the highest production of violocein by *Chromobacterium violaceum* occurred at pH 7 and pH 8, which corresponded with results obtained in this study. Also Bhat and Marar [24] reported that *Salinococcus roseus* showed highest pigmentation at pH 8.

UNDER PEER REVIEW

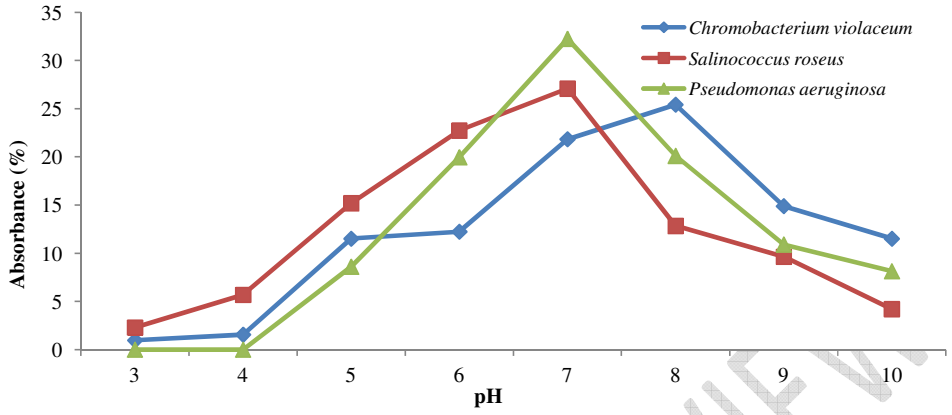

Figure 8: Effect of pH on Pigment Production by Pigment-Producing Bacteria Isolated From Soil

The results of effect of incubation time on pigment production revealed that *Chromobacterium violaceum* and *Salinococcus roseus* showed highest peaked after 96 hours of incubation, while *Pseudomonas aeruginosa* showed highest green pigmentation after 72 hours of incubation (Figure 9). The variation of pigments production by the *Chromobacterium violaceum, Pseudomonas aeruginosa* and *Salinococcus roseus* on incubation time might be attributed to nature of growth of organisms, as some bacteria have shorter generation time than others. The increasing pigment production by *Chromobacterium violaceum* and *Salinococcus roseus* up till 96 hours might be an indication that the organisms did not reached the peak of its growth. Pigment and other secondary metabolites produced by microorganisms have been shown at stationary phase [30]. It might also indicate that at this time there is maximum stress in the growth medium which stimulates highest pigment production. This stress could be as a result of nutrient depletion and accumulation of waste products. The results indicated that 72 hours has the peak period for pyocyanin production by *Pseudomonas aeruginosa* and at 96 hours there was decline of pyocyanin production. This implies that as the numbers of days increased, the number of bacteria also increased which would increase the growth and pigments production. This is in line with findings of Cortes-Osorio *et al.* [25] who reported that highest violacein production by *Chromobacterium violaceum* occurred after 96 hours of incubation. Chandran *et al.* [28] reported that *Pseudomonas aeruginosa* produced highest pigment at optimum temperature of 37^{o}C at 72

UNDER PEER REVIEW

hours. Bhat and Marar [24] reported that the growth and pigment production by the *Salinicoccus* sp. MKJ 997975 was higher in nutrient broth after 6 days incubation.

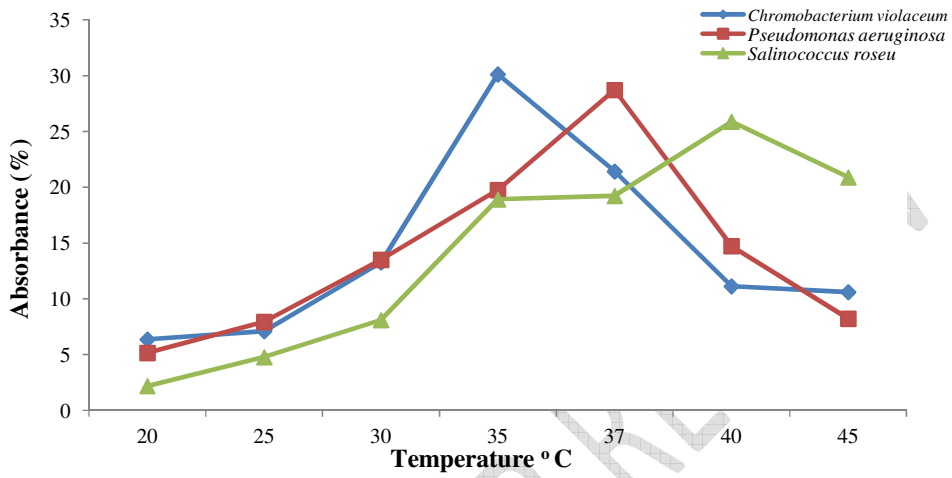

Figure 9: Effect of Incubation Temperature on Pigment Production by Pigment-Producing Bacteria Isolated From Soil

Figure 10 show the effect of static and shaking condition on pigment production. It was observed that the pigmentation on all the isolates was higher under shaking condition while under static condition, the isolates showed lowest pigmentation.

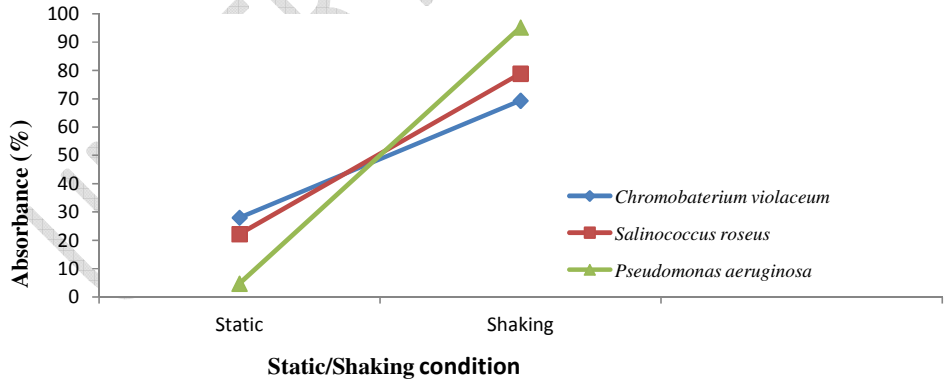

Figure 10: Effect of Static/Shaking Condition on Pigment Production by Pigment-Producing Bacteria Isolated from Soil

Table 3 presents the results of Thin Layer Chromotography (TLC). The thin-layer chromatographic results showed that the pigments exhibited Rf values characteristics to pyocyanin, violacein and zeaxanthin. The Rf value of purple pigment produced by *Chromobacterium violaceum* was noted as 0.44, which corresponded to that of violacein. The Rf value of green pigment produced by *Pseudomonas aeruginosa* exhibited two spots showing Rf value of 0.73 which was similar to pyocyanin and 0.52, which was closed to rhamnolipid and that of orange pigment produced by *Salinococcus roseus* showed single spot with Rf value of 0.82, which corresponded to zeaxanthin. The Rf values of the pigments indicated that the solvent used (n-hexane, methanol and chloroform) in the ratio of 8:2:2 was an ideal solvents for separation and movement of those compounds on silica gel. Popy *et al.* [31] extracted, purified and characterized the green pigment produced by *Pseudomonas aeruginosa* and reported the Rf values of the green pigment range between 0.70 – 0.81 and identified as pyocyanin. Abdul-Hussein and Atia [32] reported that the green pigment produced by *Pseudomonas aeruginosa* was identified as pyocyanin with Rf value of 0.81. Ahmad *et al.* [7] extracted purple pigment produced by *Chromobaterium violaceum* using solvents extraction. The pigment was characterized using TLC and identified as violacein with Rf value of 0.43.

Table 3: Identification of Pigments by Thin Layer Chromotography

Bacterial Isolates	Color of pigments	Spots	Rf value	Rf value as per literature	Compound
Chromobacterium violacein	Purple	1	0.44	0.43	Violacein
Pseudomonas aeruginosa	Green	1	0.73	0.70 – 0.81	Pyocyanin
Salinococcus roseus	Orange	1	0.82	0.82	Zeaxanthin

The results presented in Table 4 show the effect of pH on the stability of pigments. It was observed that the purple pigment produced by *Chromobacterium violacum* turned to dark blue at pH 2, which gradually turned to colorless after 24 hours while at higher pH 13, it changed to green and became colorless after 24 hours. The green pigment produced by *Speudomonas aeruginosa* turned to dark red at pH 2 while at pH 13 turned to light green. The orange pigment produced by *Salinococcus roseus* turned to yellow at pH 2 and remained orange color at alkaline pH 13. The pigments violacein (purple), pyocyanin (green) and zeaxanthin (orange) showed

UNDER PEER REVIEW

good stability toward temperature when exposed to 160 ^{o}C and 200 ^{o}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 pigments violacein, pyocyanin and zeaxanthin can offer various industrial applications such as in dying, textile and food industries. Similar finding by Ahmad *et al.* [7] who reported that the pigments produced from bacteria showed good stability toward temperature ranging from 45^{o}C - 120^{o}C when exposed for one (1) hour.

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
Purple pigment	Control	560nm	Purple	Purple
	pH 2	560nm	Dark blue	Colorless
	pH 13	520nm	Green	Colorless
Green pigment	Control	280nm	Green	Green
	pH 2	460nm	Dark red	Dark red
	pH 13	280nm	Light green	Yellow
Orange pigment	Control	440nm	Orange	Orange
	pH 2	400nm	Yellow	Yellow
	pH 13	440nm	Orange	Orange

Table 5 presents the results of the effect of temperature on the stability of the pigments. It was observed that the purple, green and orange pigments were stable at 160^{o}C and 200^{o}C temperature. The instability of the pigments (violacein, pyocyanin and zeaxanthin) 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 [7].

Table 5: Effect of Temperature on Stability of the Pigments

Pigments	Temperature Condition	Maximum wavelength (ʎmax.)	Color Changed
Purple pigment	Control	560nm	Purple
	160^{o}C	560nm	Purple
	200^{o}C	560nm	Purple
Green pigment	Control	280nm	Dark green
	160^{o}C	280nm	Green
	200^{o}C	280nm	Green

UNDER PEER REVIEW

Orange pigment	Control	440nm	Orange
	160°C	440nm	Orange
	200°C	440nm	Orange

The results presented in Figure 11 show UV-visible spectrum of green pigment produced by pigment-producing bacteria. The green pigment produced by *Pseudomonas aeruginosa* showed highest peak of 270 nm which gradually declined toward visible region. The primary reasons for absorption within ultraviolet region might be attributed to present of some functional group CH_3 and C=C. A similar result was observed by Ohfuji *et al.* [33] who found that the UV-visible spectrum of green pigment produced by *Pseudomonas aeruginosa* was 278 nm. The UV-visible spectrum of purple pigment produced by *Chromobacterium violaceum* showed highest peak at 560 nm. The reason for stronger absorption of the purple pigment within visible region might be attributed to electron conjugated effect, that the conjugated system required lower energy for the electron transition from the orbital. The present of conjugated bond resulted in highest absorption appearing at the longer wavelength region [34]. Similar results reported by Ahmad *et al.* [7] who observed that the purple pigment produced by *Chromobacterium violaceum* had highest absorption spectrum of 573 nm. The highest absorption of orange pigment at 450 nm might be attributed to conjugated bonds of the pigment. This indicated that the orange pigment belong to carotenoid family.

UNDER PEER REVIEW

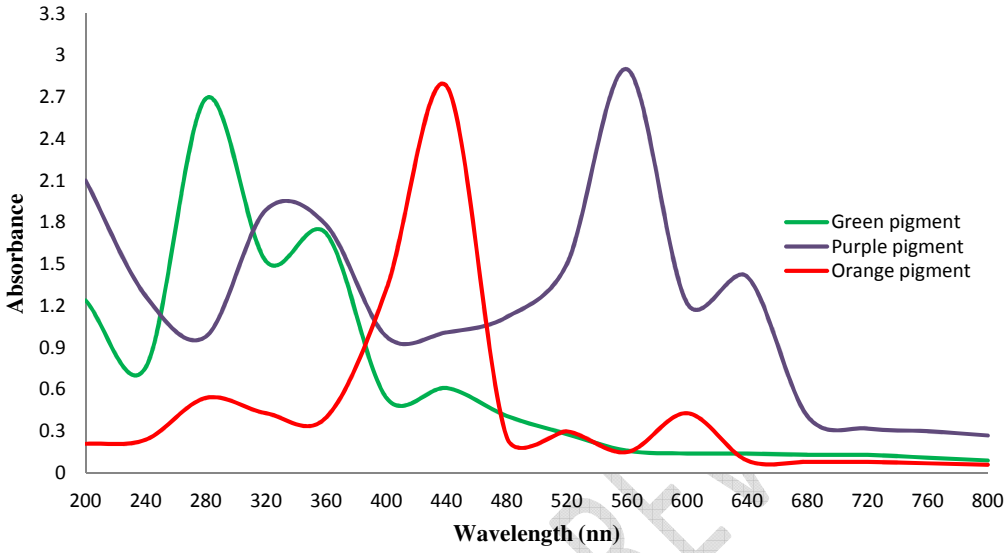

Figure 10: UV-Visible spectrum of Orange, Purple and Green Pigment Produced by Pigment-Producing Bacteria

The results of Fourier Transform Infrared Spectroscopy of purple pigment produced by *Chromobacterium violaceum* revealed the following functional groups and their absorption frequencies: OH (3650cm^{-1}), N-H (3400cm^{-1}), C=O (1620cm^{-1}), C-N (1200cm^{-1}), C-O (940cm^{-1}) and C-H (910cm^{-1}). These functional groups and their absorption frequencies are characteristic of violacein (Figure 11). The FTIR spectrum of green pigment produced by *Pseudomonas aeruginosa* revealed the following functional groups: OH (3620cm^{-1}), CH_3 (2940cm^{-1}), C=C (1650cm^{-1}), C-N (1350cm^{-1}), C-O (1040cm^{-1}) and C-H (980cm^{-1}). These functional groups and their absorption frequencies are characteristic of pyocyanin (Figure 12). The results presented in Figure 16 show FTIR spectrum of orange pigment produced by *Salinococcus roseus*. The results indicated 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 corresponded to that of zeaxanthin (Figure 13).

Economy of Bio-pigment Production

Textile industry will remain the largest consumer of organic pigments and dyes, although there is a growing preference for the bacterial pigments in food industry, pharmaceuticals and cosmetics.

UNDER PEER REVIEW

However, natural pigments may be several times more expensive than synthetic analogs. A unique example is the carotene produced by bacteria which has an approximate cost of US$1000/kg against US$500/kg by synthetic means; although more costly, carotene produced by the bacterial means competes in a market segments [35]

Increasing globalization, restructuring, and internationalization has been a key trend shaping the pigment industry over the past several years. Global demand for organic pigments and dyes is expected to reach almost 10 million tons by 2017 according to Global Industry Analysts. There is an increasing thrust towards the use of natural dyes due to the forbidden use of synthetic compounds (banning of azo dyes in Europe). Market value will benefit from consumer preferences for environmentally friendly products. Development of bacterial strains that can utilize cheap and renewable substrates will make the price of bio-pigments competitive with synthetic pigments. Therefore discovering cheap substrates for pigment production is believed to reduce the production cost. Although the price of bacterial pigment will be relatively higher compared to the synthetic dyes, the production cost can be reduced via the use of agricultural wastes such as pineapple wastes, sugarcane bagasse and molasses as growth medium for cultivation of bacteria, use of locally isolated wild type bacterial strains eliminates the cost for genetic alterations and the use of simple extraction techniques. The bacterial pigments will offer good opportunities due to their enhanced environmental acceptability and superior performance characteristics, classical or conventional grades are expected to continue to dominate the organic market [35].

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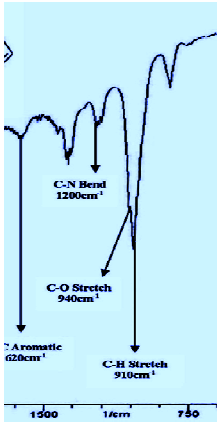

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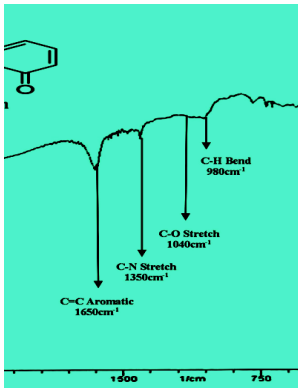
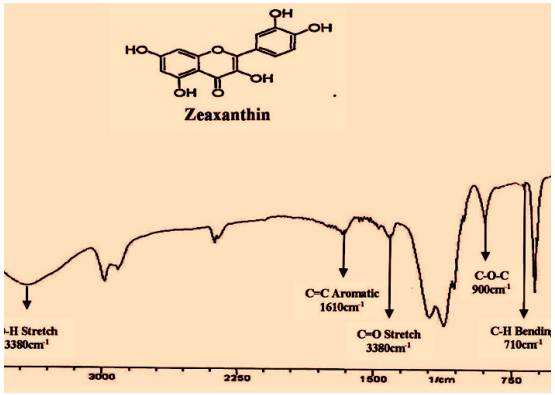


rple Pigment Produced by



FTIR Spectrum of Purple Pigment Produced by *erium violaceum*

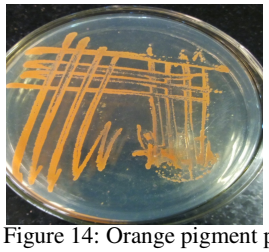


Figure 14: Orange pigment producing bacteria

Figure 15: Extracted orange pigment

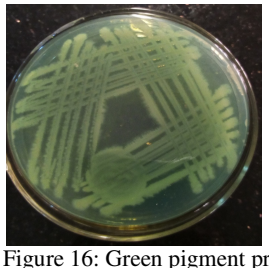


Figure 16: Green pigment producing bacteria

Figure 17: Extracted green pigment

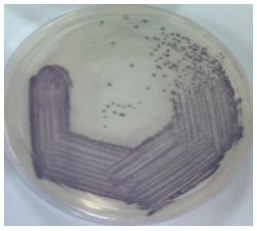


Figure 18: Purple pigment producing bacteria

Figure 19:Extracted purple pigment

Conclusion

The results obtained from this study serve as an important insight for production of bio-color from soil inhabiting bacteria. The bacteria were identified as *Chromobacterium violaceum, Pseudomonas aeruginosa* and *Salinococcus* roseus and were found to produce purple, green and orange pigments. Based on the optimization studies, the parameters such growth medium, pH, temperature, incubation time and shaking/static condition were to have effects on pigment production by pigment-producing bacteria. Based on the Thin Layer Chromotography, UV-Visible spectroscopy and Fourier Transform Infrared (FTIR) Spectroscopy results revealed closed characteristics to that as violacein (purple pigment), pyocyanin (green pigment) and

zeaxanthin (orange pigment). The pigments were found stable when heated for 10 minutes at 200^{o}C. It's therefore recommended that more studies on bio-color productions should be intensify on bacteria and fungi because they are less toxic, non carcinogenic and easily biodegradable than the synthetics counterpart and determining the characteristics and nature of bacterial pigments is critical for industrial applications of the bio-colorants.

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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