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Production, Optimization and Pigment Production Potential of *Serratia marcescens* (GBB151) Isolated from the Industrial Effluent

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Abstract: This research was carried out to isolate pigment producing bacterium from industrial effluent. It also optimized and characterized the pigment produced by the bacterium. A total of twenty pigments producing bacteria were isolated based on different colours exhibited by the colonies on Nutrient agar and one was selected for further study based on its appreciable pigment production. The optimal conditions for pigment production were determined by varying the pH, temperature, nitrogen sources and carbon sources. Characterizations of the pigment were carried out using thin layer chromatography (TLC), FT-IR spectroscopy and Gas Chromatography-Mass Spectrometry (GCMS). The potential isolate was identified using 16s rRNA sequencing and confirmed as *Serratia marcescens.* The optimum pigment production was obtained at 30th hour, temperature of 30°C and optimum pH of 7.0. The best nitrogen and carbon source for production were found to be peptone and maltose with pigment production of 0.668 mg/l and 0.764 mg/l respectively. TLC analyses of the extracted pigment revealed the presence of a reddish single spot. Also, FTIR analysis indicated the presence of O-H and alkene functional group in the pigment. (GC-MS) analysis identified the active components in the pigment as n – haxadenoic acid (20.90%), phenol 2, 4-bis (1, 1 – dimethyl) (17.83%), octadecenoic acid (9.81%) and cis – vaccenic acid (8.53%). The colour of the pigments was stable over a wide range of pH and the pigment also performed excellently as a dye. The obtained results showed that *Serratia marcescens* pigment could find application in industrial processes.

Keywords: Industrial Effluent, *Serratia marcescens*, Isolate, Pigment

1. Introduction

The interest in pigments production from natural sources due to a serious safety problem with many artificial synthetic colourants is becoming popular. Colours and flavour are the immediate signals perceived by the optical and chemical senses of humans and these determine to a large extent whether a certain food is appealing. Colour plays an important role in enhancing the aesthetic appeal of a number of foods [1] and other materials of commercial interest. The use of food colourants as an additive in the food industry is a significant factor for both food manufacturers and consumers in determining the acceptability of processed food products [2]. Colours occur naturally in nature and it has long been known that microorganisms of the genus monascus produce red pigments, which can be used for colouring foods [3]. Food grade pigments like carotenoids, melanin, flavins, quinines and specifically monascins, violacein and indigo are produced by microorganisms [4]. Some bacteria produce pigments as part of their normal metabolism. The specific colour of the pigment is characteristic for each bacterium. Several organisms can produce pigments (often termed bio-pigments) which are important classes of their secondary metabolites. Microbial pigments are of industrial interest because they are

often more stable and soluble than those from plant or animal sources [5]. The rapid growth of microorganisms can enhance high productivity, and can produce a product continually. Most bacteria and fungi are widely studied for their potential as a source of food colorants. Recent increasing concern on the use of edible coloring agents has banned various synthetic coloring agents, which have a potential of carcinogenicity or teratogenicity [6]. Natural pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH [7]. Although there are a number of natural pigments, only a few are available in sufficient quantities to be useful for industry because they are usually extracted from plants [8]. Due to high cost of currently used technology of pigment production on an industrial-scale, there is a need for developing low cost process for the production of pigments, which could replace synthetic pigments. Thus, the main objective of this study was to isolate some bacterial species from industrial effluent, screen them for pigment-production, characterize and investigate optimal conditions of growth and pigment production.

2. Materials and Methods

2.1. Collection of Samples

Effluent were collected from the oxidation ponds of International Breweries Plc (IBPlc), Ilesa, Osun State (07.624855′), 004.786286′) using a 100 ml sterile bottle by submerging the bottle to a depth of about 20 cm, with the mouth facing opposite the water currents. The samples were labelled, put in ice flakes and transported immediately to the laboratory for analysis.

2.2. Isolation of Bacteria

Pour plate technique was adopted for the isolation of pigment - producing bacteria. Water samples were mixed thoroughly, and then ten - fold dilutions were prepared with sterile distilled water ranging from 10^{-1} to 10^{-6}. Exactly 1 ml of the diluted samples from 10^{-3} to 10^{-6} were inoculated according to the pour plate method in three parallel replicates on Nutrient agar and glycerol - supplemented composed agar. The cultured plates were incubated aerobically at 30 and 37°C for 24 – 48 h. After incubation, plates were examined and representative colonies were isolated from each plate for further identification. The twenty representative colonies were maintained on Nutrient Agar (NA) slants at 4°C.

2.3. Screening for Pigment Production

0.5 MacFarland standards of 0.10 optical density (10^8 CFU/ml) of the isolates were prepared and used for inoculation of the pigment production media. The media contained a sterile nutrient broth (100 ml) in 250 ml conical flask inoculated with 1.0 ml of the bacterial cells suspension and incubated in a rotary shaker with the speed of 120 rpm for 24 -48 h for pigment production [9].

2.4. Characterization and Identification of Isolates

Twenty bacterial isolates were screened for their ability to produce pigment. The best strain was selected for further study and characterized. The best selected pigement-producing strains were identified based on their cell morphology, cultural and biochemical characteristics and identified using Bergey's Manual of Determinative Bacteriology [10] and the molecular characterization by 16S rRNA gene sequencing was conducted.

2.5. Optimization of Pigment Production

The optimal conditions for the pigment production were determined by subjecting pigment production to various physiological conditions such as incubation time, carbon sources, nitrogen sources, temperature and hydrogen ion concentration (pH).

2.5.1. Effect of Period of Incubation Time on Pigment Production

To study the effect of incubation period on pigment production, the flasks were placed on a rotary shaker at 120 rpm and 28 ± 2°C for incubation. Two flasks were taken out for analysis at each time interval of 0, 6, 12, 18, 24, 30, 36, 42 and 48 h. The pH, optical density (at visible/ UV light range), total viable bacteria count in CFU/ml and yield (mg) of pigment produced per ml of the broth culture were determined at various time interval.

2.5.2. Effect of Carbon and Nitrogen Sources on Pigment Production

The effect of different sugar substrates for pigment production by the bacterial isolates was determined by cultured the isolates in the different sugars such as maltose, glucose, lactose, starch and sucrose (1%) in minimal broth. Also, different nitrogen sources (casein, peptone, ammonium nitrate, ammonium chloride and ammonium sulphate in composed minimal broth) were investigated for their effect on pigment production. The nitrogen sources were added at 1% concentration [11].

2.5.3. Effect of Temperature and pH on Pigment Production

Flasks containing 100 ml of growth medium for pigment production were inoculated. The flasks were incubated at temperature ranging from 25-45°C at an interval of 5°C for each temperature and for the effect of pH, the basal medium was prepared as describe above by varying pH values ranging from 5-10 at 0.5 interval which was used to prepare culture medium. The effect of pH production was studied in 250 ml conical flasks containing 100 ml of the growth medium for pigment production. The sterile medium was adjusted to different pH and inoculated with standard bacteria suspension. [11]

2.6. Pigment Extraction

Following incubation, the liquid cultured medium was

centrifuged at 7500 rpm for 20 min. supernatants were discarded and the bacterial cell pellets containing the pigments were extracted with 90% (v/v) methanol until the pellets become colourless [9].

2.7. Pigment Estimation

Pigment estimation was done as described by [12]. The methanolic extracts obtained were concentrated using rotary evaporator at 50°C with chiller temperature set at below 10°C. The concentrated pigment was then transferred to a pre-weighed glass Petri-dish prior to drying for 3 d at 60°C. After drying, the weight of the Petri-dish with the dried pigment was taken as final weight. The pigment yield was then calculated as the difference in the final weight and the initial weight of the empty Petri-dish.

2.8. Characterization of Pigment

2.8.1. Thin Layer Chromatography (TLC) Analysis of the Pigments

The methanol extracts of the bacterial isolates were spotted using capillary tubes on precoated TLC Silica gel plates 60 F_{254} (MERCK, Germany), 20 x 20 cm with 0.2 mm thickness [13]. The plates were allowed to dry and then transferred into a chromatography tank already saturated with chloroform: methanol: acetone (4:2:3 v/v) solvent system.

2.8.2. Fourier Transform Infra-Red (FTIR) Analysis

The FT-IR spectra of sample were recorded in order to characterize the presence of functional groups in isolated bioactive. The samples were analysed at the Centre for Energy Research and Development (CERD), Obafemi Awolowo University, Ile-Ife, Nigeria. The model of the machine used for the FTIR was NICOLET IS5. The analysis conditions used were 16 scans at a resolution of cm^{-1} measured between 400 and 4,000cm^{-1}.

2.8.3. Gas Chromatography - Mass Spectrometry (GCMS) Analysis

The GC-MS analysis of the sample was carried out on Agilent chromatography GC (Model 7890A series) fitted with detector VL-MSD (Model 5975C) and Hewlett Packard 7688B injector series. The GC was fitted with a varian Agilent column (30 × 0.320 mm, film thickness 0.25 µm). The injector GC oven temperature started at 80°C for 2 min then rose at 5°C /mm to 120°C and held for 2 min and finally increased at 10°C /min to 240°C. Exactly 1.0 µl of the sample was automatically injected into the column with the injector temperature at 250°C and inlet pressure of 8.021 Psi. The standard septum purge flow was 3 ml/mm splitless.

2.8.4. Stability of Crude Red Pigment

The pigment produced by the isolate was tested for stability at various pH values ranging from very acidic (1.2) to highly basic (13).

2.8.5. Water and Organic Solvent Solubility Test

Distilled water (1.0 ml) was added into cryovials for different pigmented bacterial isolates. Each bacterial isolate was scrapped lightly and introduced into the water using inoculating loop. The bacterial cells were thoroughly mixed by vortexing using Velp Scientific Vortex Wizard (Neutec Group Inc.) for 10 s. The mixture was spun in the micro centrifuge for 5 min at 7,500 rpm. The organic solvents used for this test were methanol, ethanol, acetone, hexane, ethyl acetate, chloroform and butanol. The organic solvent (1.0 ml) was introduced to the cell pellets in the cryo vial of water-insoluble pigment after discarding the water supernatant. The mixture was vortexed to allow the cells to mix thoroughly with the solvent. Then, the mixture was centrifuged at 7,500 rpm for 5 min.

2.9. Applications of Bio-Pigment (Textile Dyeing)

An area of 2 cm^2 of each fabric (satin, silk, nylon, cotton and polyester) was soaked in 4 ml of the red, yellow and green pigment in different test tubes and the tubes were incubated for 48 h at room temperature. The fabrics were dried and cut into smaller pieces which were then treated with tap water, tap water and detergent, warm water (45°C), warm water and detergent for 1 h in respective test tubes [11]

2.10. Statistical Analysis

All values were expressed as means and standard errors of means (SEM). The students' T-test was used for comparison of the experimental groups. The level of significance was set at $P < 0.05$ using one way analysis of variance (ANOVA).

3. Results

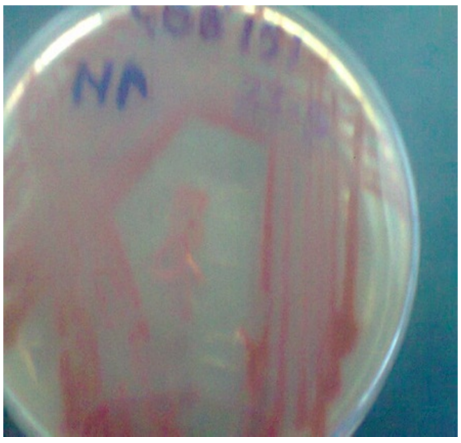

Figure 1. *Petri plate showing growth of S. marcescens (GBB151) on nutrient agar.*

A total of twenty bacterial strains were isolated from the effluents. One strain was selected for further studies based on its appreciable pigment production (figure 1). Cultural,

morphological, biochemical and physiological characteristics of the isolate were examined and it was identified as a strain of *Serratia marcescens* while the molecular characterization by 16S rRNA gene sequencing also confirmed the isolate to be *Serratia marcescens* with maximum of 98% to other *Serratia marcescens* spp on NCBI website (Table 1). The isolate strain was subjected to pigment production studies and it was observed that the pigment production correlates with the rate of bacterium growth. Maximum pigment production by *Serratia marcescens* was obtained at 30th hour of incubation. (Figure 2).

Table 1. *Sequence Identity of GBB151 with Serratia marcescens.*

	Serratia marcescens							
ACCESSION	**LC130340**	**KR262852**	**KR133279**	**KM099142**	**KJ721215**	**KM093865**	**KJ729142**	**KF080166**
% IDENTITY	96%	96%	96%	96%	96%	96%	96%	96%

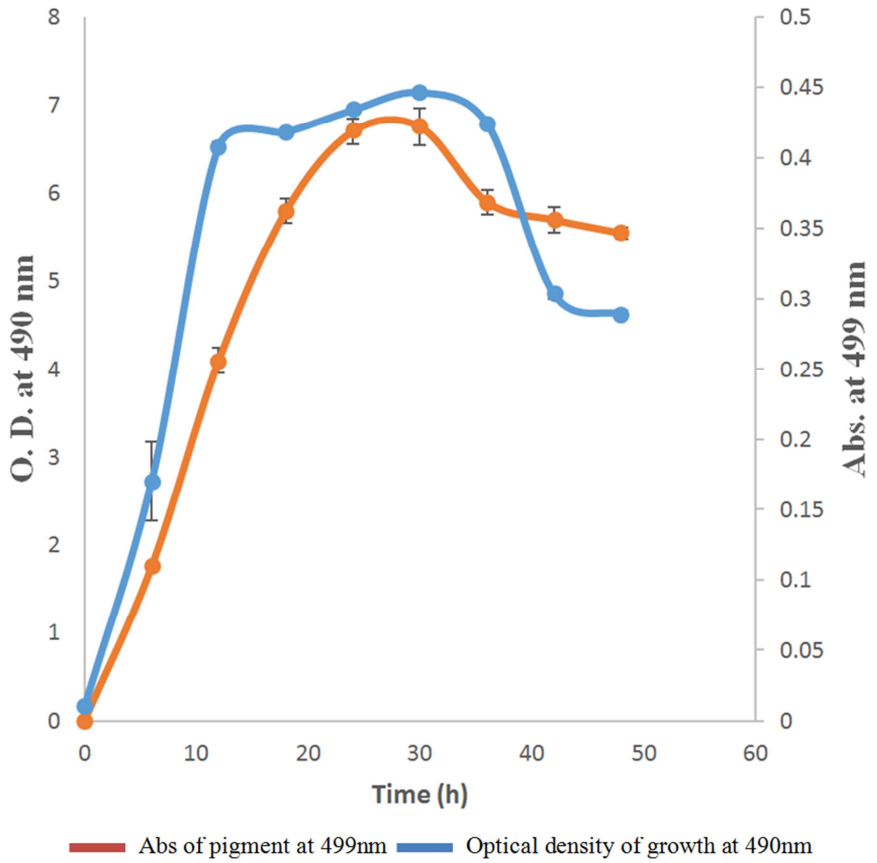

Figure 2. *Time Course of Cellular Growth and Pigment Production of Serratia marcescens (GBB151).*

The present study investigated the best carbon source for pigment production. Maltose was found to be the best carbon source with pigment estimation of 0.764 mg/ml, this was closely followed by starch, then lactose and sucrose with mean absorbance values of pigment of 0.684, 0.662 and 0.647 respectively as shown in Figure 3. Pigment evaluation using different nitrogen sources indicated peptone and casein to be the best nitrogen source for optimum pigment production by *Serratia marcescens* with estimation of 0.668 mg/ml and 0.414 mg/ml respectively while ammonium nitrate gave the least activity. (Figure 4). The optimum pH and temperature for pigment production by *Serratia marcescens* were 7.0 and 30°C with pigment estimation of 0.41 and 0.490 mg/ml respectively as showed in Figure 5 and 6.

Infrared spectroscopy is often used to detect the presence of functional groups in organic compounds. The infrared (IR) spectral analyses of GBB151, show bands at 3.500 cm^{-1} which indicates the presence of O-H functional group, 2991 and 2813 cm^{-1} signify Sp3 C-H of saturated part of the molecules, 1636 cm^{-1} shows the presence of C=C (alkene) while bands at 1034, 1032 and 1014 cm^{-1} account for the presence of C-O functional moiety (figure 7). GC-MS analysis revealed a total of sixty five compounds in the red pigment of GBB151. The chromatogram (Figure 8) shows four prominent peaks identified as n – haxadenoic acid (20.90%), phenol 2, 4-bis (1, 1 – dimethyl) (17.83%), octadecenoic acid (9.81%) and cis – vaccenic acid (8.53%). These compounds constituted about 58.07% of the GBB151 pigment while the minor compounds made up the remaining 41.93% among which were pyrrole -2-carboxyaldehyde and pyrrolo (1,2-a) pyrazine-1,4-dione (precursors of prodigiosin). TLC analyses of the extracted pigment revealed the presence of a reddish single spot (figure 9) and the retardation factor (R_f) was calculated to be 0.87.

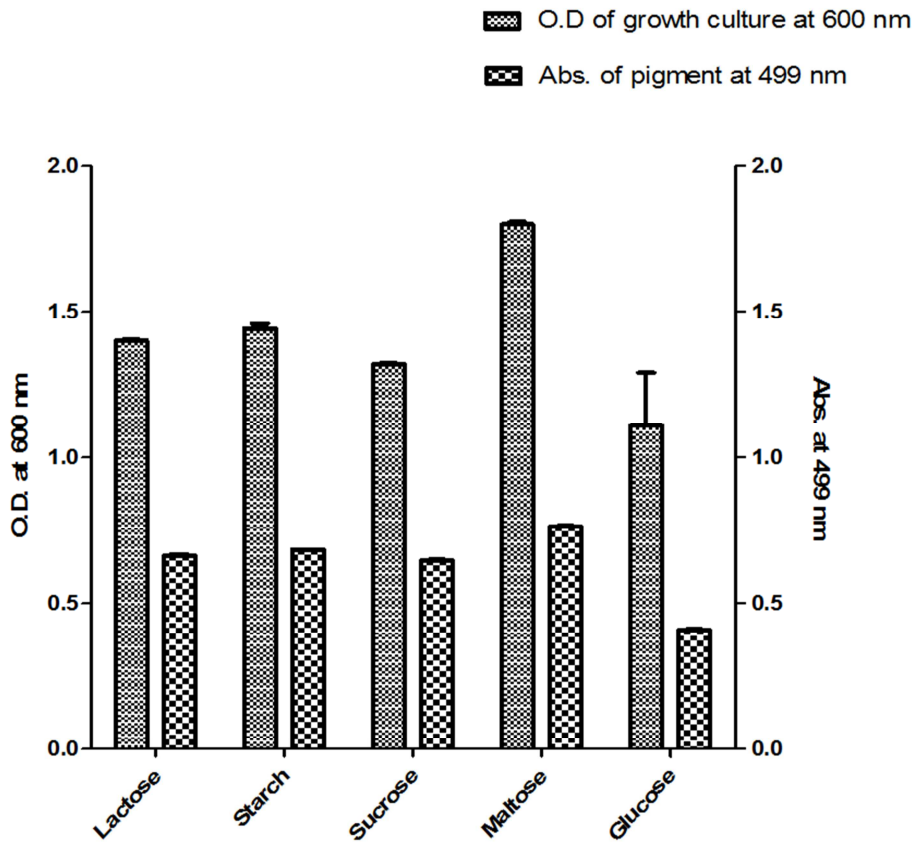

Figure 3. *Effect of Different Carbon Sources on Cellular Growth and Pigment Production of S. marcescens (GBB151).*

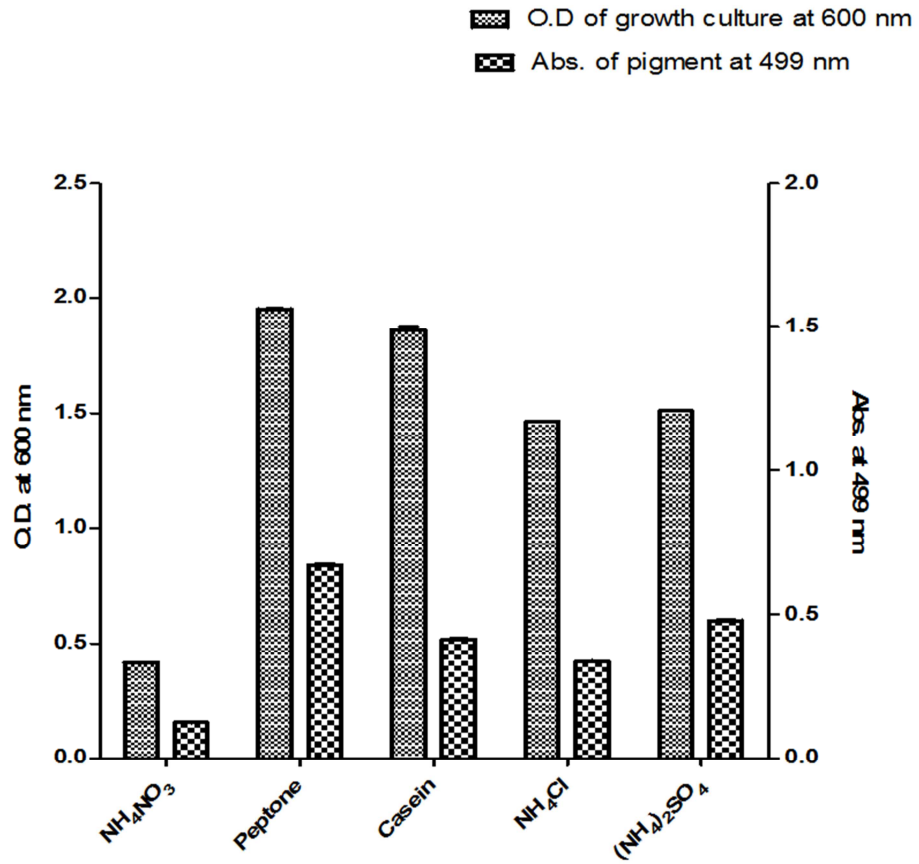

Figure 4. *Effect of Different Nitrogen Sources on Cellular Growth and Pigment Production of S. marcescens (GBB151).*

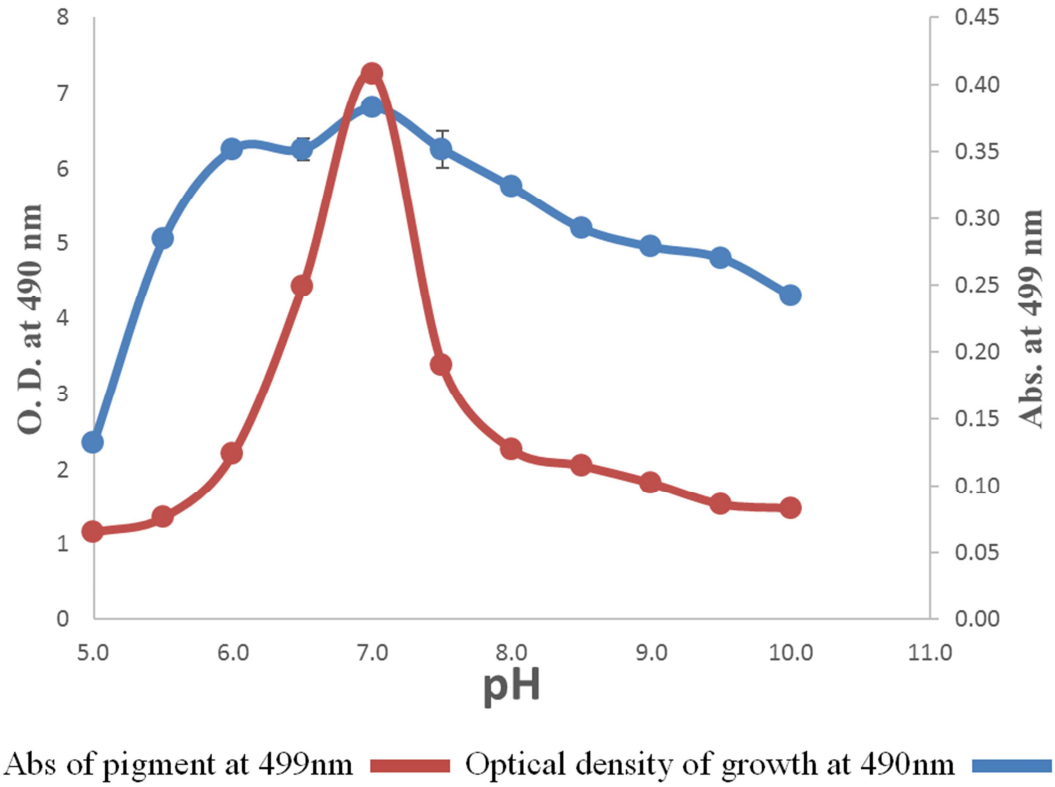

Figure 5. *Effect of pH of Culture Medium on Cellular Growth and Pigment Production of S. marcescens (GBB151).*

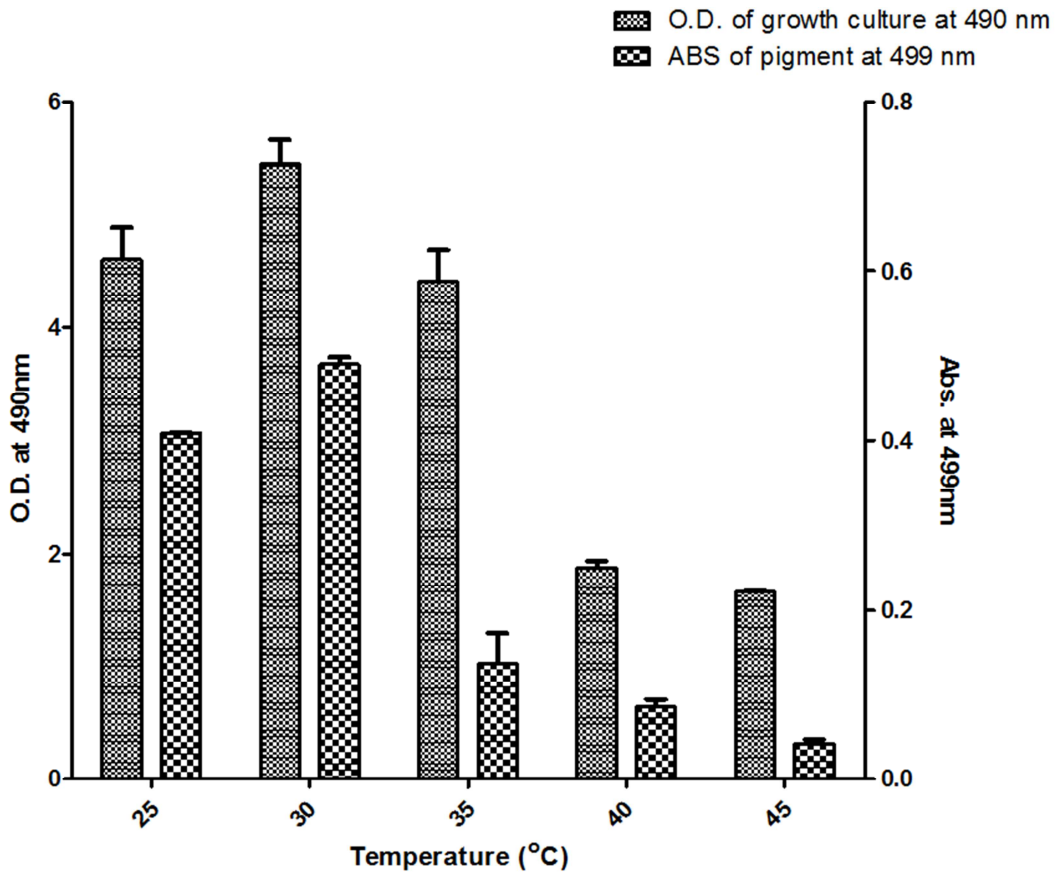

Figure 6. *Effect of Incubation Temperature on Cellular Growth and Pigment Production of S. marcescens (GBB151).*

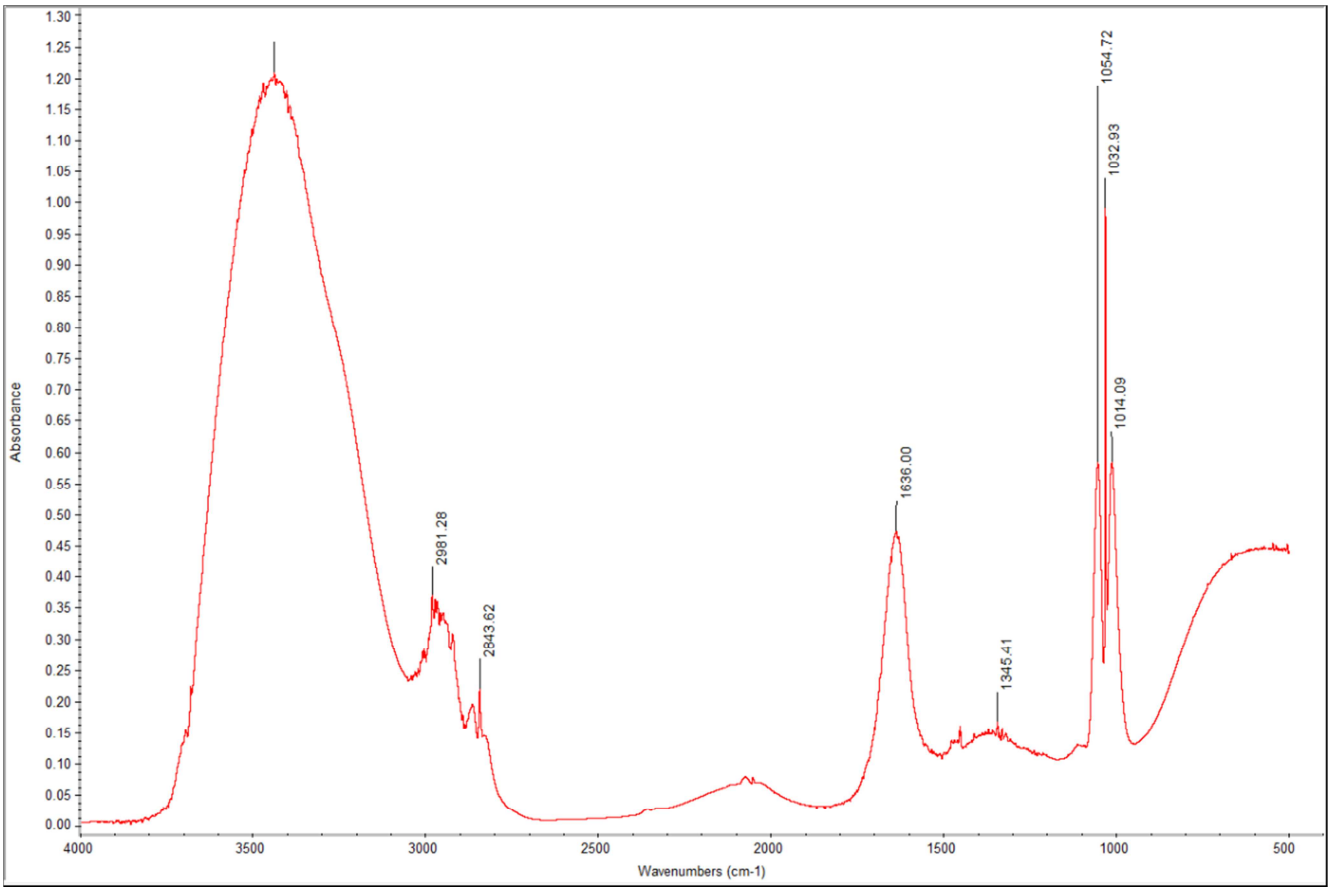

Figure 7. *FTIR Spectrum of Red Pigment Produced by S. marcescens (GBB151).*

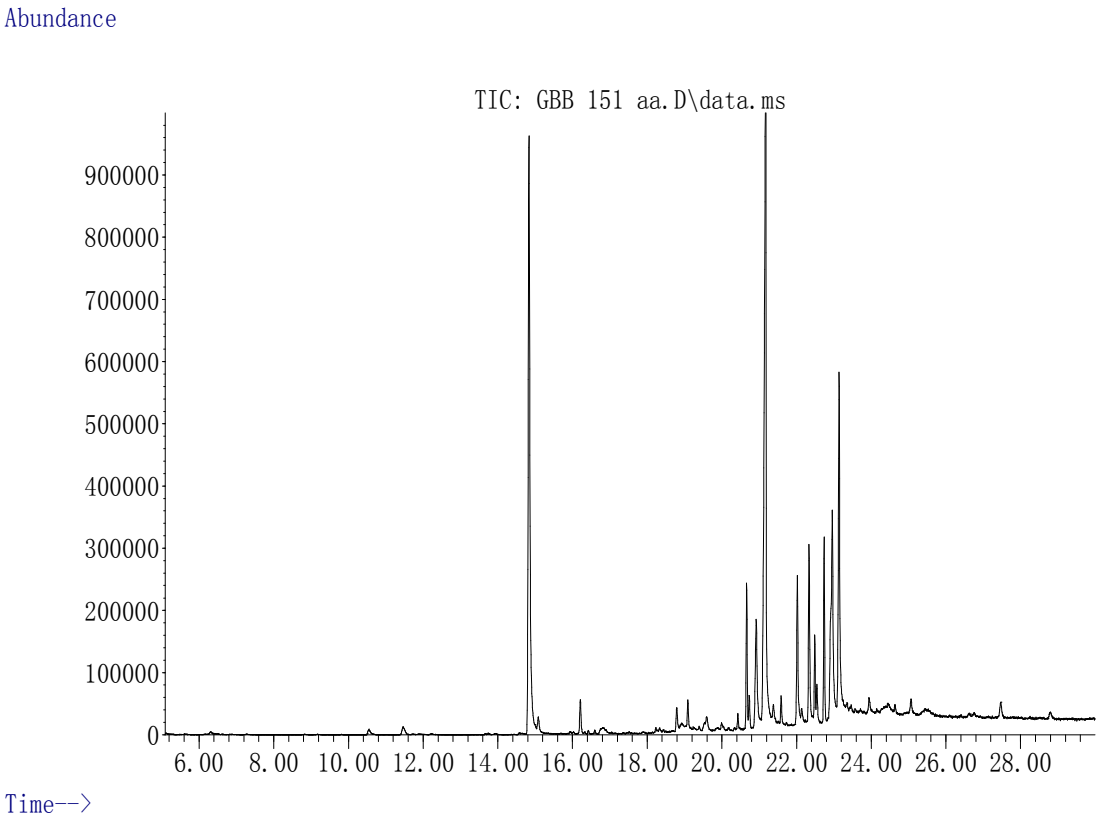

Figure 8. *Chromatogram of Red Pigment by S. marcescens (GBB151).*

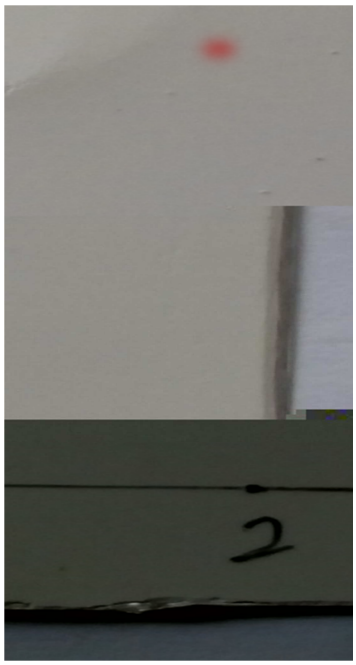

Figure 9. *Thin Layer Chromatography (TLC) Plate of Red Pigment of GBB151.*

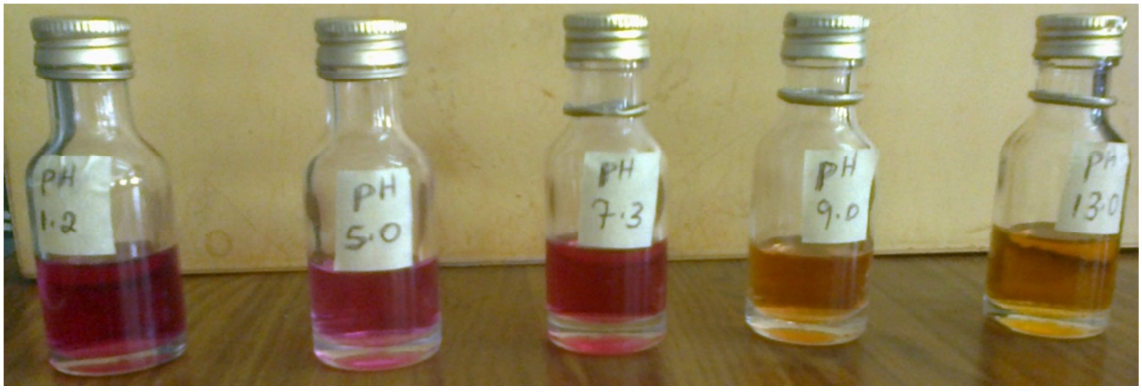

Figure 10. *Variations in Colour of the Red Pigment by GBB151 at Different pH values.*

Figure 11. *The Different Fabrics after Dyeing in Red (Prodigiosin) Pigment produced by Serratia marcescens.*

Stability study showed the pigment to be highly stable over a wide range of pH (3-8) but decrease in colour intensity was noticed at higher alkaline pH values (9-13). The colour variations of prodigiosin at different pH values are shown in figure 10. GBB151 pigments was not soluble in water but highly soluble in methanol, ethanol, and acetone. Also, the effectiveness of the red pigment (prodigiosin) produced by *Serratia marcescens* as a dye for each of the fabrics tested shows that the red pigment performed excellently as a dye on satin, closely followed by cotton and polyester while it moderately coloured nylon and silk fabrics (figure 11).

4. Discussion

In this study, *Serratia marcescens* with the ability to produced pigment was cultured from the effluent of International Breweries Plc (IBPlc), Ilesa and was identified. Pigment production ability have been identified in a variety of bacterial species such as *Monascus, Streptomyces* [14]. *Streptomyces fradiae* [15]. Production of pigment by

bacterium is often dependent on growth of the bacterium in the appropriate media composition. The pigment produced in this study is intracellular in nature, the bacteria accumulated some cell components before the release of pigment in the log phase, followed by decline in production during the stationary and death phase respectively. This pattern of growth was observed in *serratia marcescens* as pinkish-red pigment (of absorbance 0.447 measured at a wavelength of 499 nm) at 30 h of incubation with gradual decline. The decline in pigment production may be due to exhaustion of the nutrients or accumulation of other products or metabolites which are both inhibitory to the growth of the bacterium and pigment production. This result corroborates with the findings of [16] on the influence of environmental condition on the production of pigment by *Serratia marcescens*. They observed that the peak of pigment production was achieved at late stage of growth and that there was a progressive decline in the yield of pigment after 30 h of incubation.

The optimization of culture media and environmental conditions is essential for effective production as it tends to reduce cost of production by increasing yield [17]. The optimum nutritional requirement for pigment production was determined to obtain maximum pigment production by *Serratia marcescens.* The present study investigated the best carbon source for pigment production. Maltose was found to be the best carbon source with pigment estimation of 0.767 mg/ml. This result was in agreement with the findings of [16] that polysaccharides or disaccharides were better than glucose (monosaccharide) as carbon sources in production of secondary metabolites by some bacteria. The effect of different nitrogen sources on pigment production by *Serratia marcescens* was evaluated using both organic and inorganic nitrogen sources. Among the five nitrogen sources, peptone was the best substrate for maximum growth and pigment production. This result is in agreement with that of [18] who submitted that *Serratia marcescens* proliferated and produced maximum pigment in presence of peptone than any other nitrogen source. The optimum pH for the pigment production by *Serratia marcescens* was achieved at 7.0 with pigment estimation of 0.438 mg/ml as showed in (Figure 5). At lower pH values of 5.0-6.0, there was little growth with not so much visible pigment production. This result was in agreements with the work of [18] who discovered from their work on ‘assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* that the maximum pigment production was obtained at a pH of 7.0. It was established by [11] that maximum amount of prodigiosin by a new strain designated as *S. marcescens* NY1 was produced at the neutral pH of 7.0 Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this usually varies from one organism to another [19]. The optimum temperature for the production of pigment by *S. marcescens* was observed to at 30°C with 0.490 mg/ml pigment estimation as shown in Figure 6. At temperature of 25°C, there was a substantial growth and pigment production. These results agree with the findings of [11] who worked on production of prodigiosin from *Serratia marcescens*. There was a gradual decrease in growth and pigment production with the increase in temperature from 30°C. Thin layer chromatography (TLC) is often the first step in identifying compounds present in such samples [20]. In the present study, there was presence of brilliant red and light blue spots on the TLC plate with R_f values of 0.87 for *S. marcescens* pigment. The R_f value of GBB151 was the same as that of [21] who investigated the optimization of prodigiosin production by *Serratia marcescens* and discovered the R_f value of prodigiosin to be 0.87. The infrared spectra of the red pigment (GBB151) showed that it has several degree of similarity to the spectra of prodigiosin [22]. The similar peaks obtained for the red pigment were 3500, 2981.28, 2843.62, 1636.0, 1345.41, 1054.72, 1032.9372 and 1014.09 cm^{-1}. From these peaks, it was suggested that the main functional groups for the red pigment of isolate GBB151 (*Serratia marcescens*) were pyrrole, methylene, alkane, alkene and alcohol (hydroxyl). A total of 65 components were detected in the GBB 151 pigment using GC-MS analytical methods. The GBB 151 sample contained a complex mixture consisting mainly of oxygenated mono- and sesquiterpene hydrocarbons. The major constituents detected in GBB 151 were n-Hexadecanoic acid (21.90%), Phenol, 2, 4-bis (1, 1-dimethylethyl) (17.83%), Octadecenoic acid (9.81%), Cis-vaccenic acid (8.53%), n-Nonadecanol-1 (4.04%), Cis-9-Hexadecenoic acid (3.59%). Stability study showed the pigment to be highly stable over a wide range of pH (3-8) but decrease in colour intensity was noticed at higher alkaline pH values (9-13). The colour variations of prodigiosin at different pH values are shown in figure 10. GBB151 pigments was not soluble in water but highly soluble in methanol, ethanol, and acetone.

The dyeing of satin, cotton, silk, polyester and nylon fabrics with the red pigment (prodigiosin) as natural textile dye indicated that the colour strength and dye uptake were high with satisfactory fastness properties of the dyed fabrics. This is in agreement with the findings of [23], in their work on ‘use of a natural dye from *Serratia marcescens* subspecies *marcescens* in dyeing of textile fabrics. The highest dye uptake was found in satin, followed by cotton and polyester and then silk and nylon. The differences in the dye uptake by these fabrics can be attributed to the chemical compositions of the various fabrics and the reaction of the pigment to these chemicals. Similar observations of the red pigment (prodigiosin) exhibiting pink colour on dyed fabrics was reported by [18], in their work on ‘assessment of process parameters influencing the enhanced production of prodigiosin from *Serratia marcescens* and evaluation of its antimicrobial, antioxidant and dyeing potentials. In conclusion, it has been established in this study that pigment-producing bacteria were isolated from the environment and modified into new strains that were able to produce higher yields of the pigments. The extracted pigments were also characterized as prodigiosin from *Serratia marcescens*, carotenoid from *Micrococcus luteus* and phenazine from *Pseudomonas aeruginosa.* The various pigments produced could find application in food,

pharmaceutical, and textile industries to replace the synthetic dyes that are not safe for the environment and human consumption.

5. Conclusion

The most significant outcome of this study were high yield and stability of red pigment produced by *S. marcescens* (GBB151) isolated from industrial effluent. Colour stability of the pigment over a wide range of pH opens wide scope of the use of this pigment in food applications. Also, the pigment was tested for textile dyeing applications and showed prominent results for the retention of dye. Therefore, the pigment produced by *S. marcescens* could find application in food, pharmaceutical, and textile industries to replace the synthetic dyes that are not safe for the environment and human consumption. However, further studies are needed to increase pigment production potential through genetic engineering.

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