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Studies on Pigments Produced by two Bacteria Species Isolated from a Dump Site in Ile-Ife

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Abstract

Pigment producing potential of two different bacteria species isolated from a dump site in Ile-Ife were investigated. The organisms were isolated and identified by sequencing and amplification of their 16SrRNA gene. The pigment produced was characterized and various conditions of productions were optimized. Based on the 16SrRNA gene sequencing, they were identified as *Micrococcus luteus* (G152) and *Pseudomonas aeruginosa* (G153). The isolated strains produced extracellular yellow and green pigment on nutrient agar medium respectively. The optimum conditions for pigment production for both isolates were observed at 30°C, pH 7 and 24th hr. Maltose and peptone were the best carbon and nitrogen source for the pigment production. Under optimized condition, the amount of yellow pigment produced were 524.0 mg/l, 428.0 mg/l and 416.0 mg/l in the first, second and third generations respectively while the green pigment produced were 561.0 mg/l, 557.0 mg/l and 508 mg/l respectively. The infrared (IR) spectral analyses of both pigments showed bands at 3.500 cm^{-1} which indicates the presence of O-H functional group. GC-MS analysis of the G152 pigment showed four prominent compounds identified as octadecanoic acid (20.40%), n-hexadecanoic acid (20.03%), cis- vaccenic acid and pyrrolo (1,2-a) pyrazine-1,4-dione (14.02%) while G153 showed only three prominent compounds 2-Furancarboxaldehyde (26.41 and 23.59%), octadecanoic acid (11.98%) and 1-Hydroxyphenazine. The high pigment producing potential of the two organisms suggests their usefulness in the industrial processes.

Keywords: Pigment, M. luteus, optimization, P. aeruginosa

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Introduction

Synthetic dyes as well as natural pigments have been used extensively in various fields of everyday life such as textile, paper, cosmetic and pharmaceuticals company[1,2]. Colour additives are essential in food industry due to its important attribute in determining acceptability of processed food products[3]. Various synthetic food colourants have been produced but majority of them are harmful to human lives when consumed at high concentrations[4]. The use of natural additives is becoming popular due to the toxicity of several artificial colourants. The demand for natural colourant has increased owing to the consumer awareness, antioxidative and their anticarcinogenicity activity[1,5]. The range of natural colour-shades is still limited when compared to synthetic dyes. Most of the natural colours used in industry are plant extracts. They have several disadvantages such as instability against heat, light, extreme pH and low water solubility. Microbial pigments are a promising alternative source for natural food grade pigments and have a great potential for food application due to their natural colour and safety to use, medicinal properties, nutrient like vitamins. Also, the production of microbial pigment is independent of season and graphical conditions and the yield can be predicted[6,7]. The use of microbial pigments are likely to not only reduce the cost of production but also leading to a cheaper source of natural food colourants among the consumers. Microbial pigment production is now one of the

emerging fields of research. Fortunately, the microorganisms that can produce different pigments are quite common. *Monascus*, *Streptomyces* and *Serratia* have been shown to produce pigments[8,9] . Pigments like melanin, carotenoids, flavins, quinines and indigo synthesized by microorganisms have found application in medical areas[10,11]. 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 a dump site, screen them for pigment-production, characterize and investigate optimal conditions of growth and pigment production.

Methods

Organisms Source and Isolation

The soil samples used were collected from the Botanical Garden of Obafemi Awolowo University Ile-Ife, adjacent to the Biological Science building within the campus (07.52091', 004.525990'). The samples were screened for as many bacteria as possible that showed evidence of pigment production as could be seen in different colours of their colonies.

Isolation

Ten-fold dilutions were prepared and 1 ml of the diluted samples from 10^{-8} was inoculated on Nutrient and glycerol-supplemented composed agar. The plates were incubated at 30°C for 24 h. The pure pigmented isolates were picked stored in a medium containing 50% glycerol.

Screening and Extraction of Pigment

Macfarland standards of the isolates were prepared and used to inoculate the pigment-producing media. The media consist of (100 ml) sterile nutrient broth in 250 ml conical flask inoculated with 1.0 ml of the bacterial cells suspension. The broth mixture was incubated in a rotary shaker with the speed of 120 rpm for 24 -48 h for pigment production. After spinning liquid culture at 7500 rpm, supernatants were discarded while bacterial cell pellets containing the pigments were extracted with 95% (v/v) methanol. The cells (pellets) were washed and streaked on a nutrient agar plate to observe growth and second generation pigment production[12,13].

Molecular characterization of the Isolate

Deoxyribonucleic acid (DNA) extraction from the bacterial isolates was carried out using a modified method of Trindade *et al*[14]. Absorbance was measured at wavelengths of 260 nm and 280 nm. Absorbance quotient was used to estimate DNA purity. DNA concentrations was determined with a Nanodrop spectrophotometer (Beckman Coulter) and adjusted to 25 ng/µl for PCR amplification

Polymerase Chain Reaction (PCR) Amplifications

PCR amplification was performed in a total volume of 25 µl containing 4 µl of the DNA solution, 0.4 µl of 10mM dNTPs, 2 µl of 25 mM MgCl2, 1 µl of 10 pmol each of primer (Forward 5'- CCAGCAGCCGCGGTAATACG -3' and Reverse 5'-ATCGGCTACCTTGTTACGACTTC -3'), 0.24 µl of Taq polymerase (1 U/µl) (Promega USA) and the 5 µl of 5× PCR buffer. Sterile DNase free water was added to make a volume of 25 µl. The PCR amplicons were visualized using 1.5% agarose gel electrophoresis. DNA sequence analysis was carried out as previously described (Fakorede *et al*.[9]

Establishment of Optimum Growth Conditions

Effect of Incubation Time on Pigment Production

Equal volume (1.0 ml) of each standardized cell suspension was inoculated into 250 ml conical flask containing 100 ml nutrient broth. The flasks were placed on a rotary shaker at 120 rpm and 30°C for incubation. Flasks were taken out for analysis at each time interval of 0, 6, 12, 18, 24, 30, 36, 42 and 48 h. The optical density (at visible/ UV light range) and the yield (mg) of pigment produced per ml of the broth culture were determined.

Effect of Temperature and pH on pigment Production:

Flasks containing 100 ml of growth medium were incubated at temperature ranging from 25-45°C and for the effect of pH, the basal medium for pigment production was prepared by varying pH values ranging from 5-10 at 0.5 interval. In both cases, the medium was inoculated with standardized cell suspensions of 18 – 24 h old culture.

Effect of Different Carbon and Nitrogen Substrates on Pigment Production

The isolates were cultivated in the different sugars such as maltose, glucose, lactose, starch and sucrose (1%) in minimal broth (pH 7.0). 1.0 ml of standardized cell suspensions was added to 100 ml of broth in 250 ml conical flasks. After incubation at 30°C for 24h, the amount of pigment produced in each medium was estimated[15]. The same procedure was used for the effect of different nitrogen sources.

Characterization of Pigment

Fourier Transform Infra-Red (FTIR) Analysis

The FTIR analysis of the pigments were carried out at the Centre for Energy Research and Development (CERD), Obafemi Awolowo University, Ile-Ife, Nigeria. The model of the machine used was NICOLET IS5. The conditions used for analysis was 16 scans at a resolution of cm^{-1} measured between 400 and 4,000cm^{-1}.

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 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. 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.

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).

Results and Discussion

Two out of the over hundred strains isolated were selected for further studies. Molecular characterization by 16S rRNA gene sequencing revealed the isolates to be a strain of *Micrococcus luteus* (G152) and *Pseudomonas aeruginosa* (G153) (Table 1 and 2). The isolated strains were subjected to pigment production studies and it was observed that the pigment production correlates with the bacterium growth. *M. luteus* and *P. aeruginosa* produced yellow and green pigments (Figure 1). Pigment production was picked at 24 h for both isolates (Figure 2 and 3). The production of the yellow pigment decreased considerably as the time of incubation progressed from 24 h to 48 h. There was a direct and steady correlation between cell growth and pigment production which was statically significant ($R= 0.905$; $P < 0.05$) implying that more cell growth favours more pigment production. This result corroborates Linawati *et al*.[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.

				Micrococcus luteus			
ACCESSION	FJ357618	KF054946	KF05488	JX42984	KC583187	HM584259	JN545040
% IDENTY	80%	80%	80%	80%	80%	80%	80%

Table 1: Sequence Identity of Isolate G152 with other Micrococcus luteus

				Pseudomonas aeruginosa.			
ACCESSION	HQ377326	KF835840	EU734822	KJ655550	KJ655548	AB915405	KF826470
% IDENTY	97%	95%	94%	94%	91%	96%	96%

Table 2: Sequence Identity of Isolate GB153 with Pseudomanas aeruginosa

Figure 1: The yellow and green pigment of bacterial Isolate G152 and G153 extracted at different time of incubation.

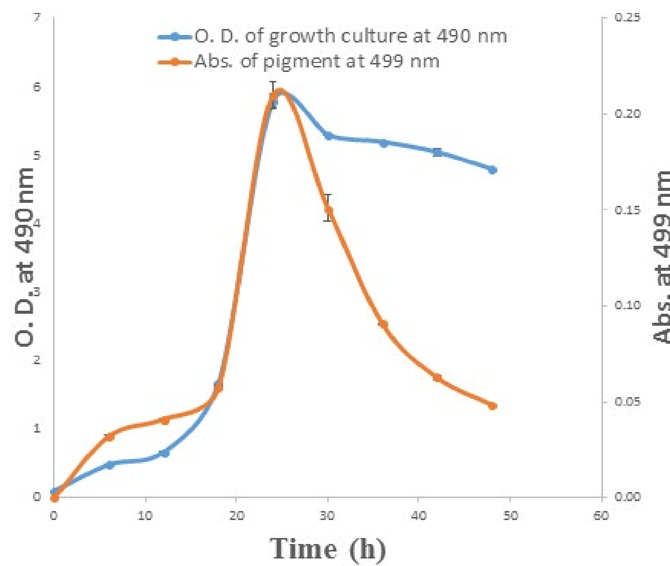

Figure 2: Time Course of Cellular Growth and Pigment Production of Bacterial Isolate G152.

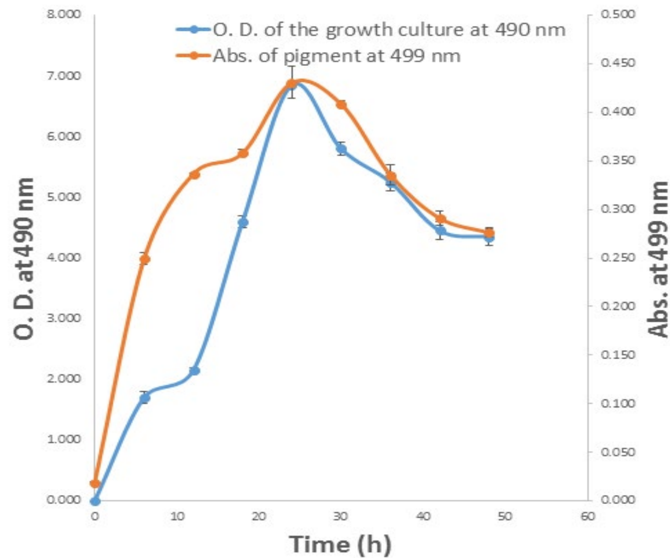

Figure 3: Time Course of Cellular Growth and Pigment Production of Bacterial Isolate G153.

Effect of Incubation Temperature on Cells Growth and Pigment Production.

In both isolates, the incubation temperature had significant effect on the growth and pigment production. For bacterial isolate G152 grew and produced appreciable yellow pigment at 25°C (0.146) and had its peak (0.216) at 30°C (Figure 4). As the temperature increased from 30 to 45°C, there was gradual reduction in cellular growth and sharp decline was observed in the absorbance of pigment production from 0.216 to 0.055. In case of bacteria isolate G153, cellular growth and green pigment production was on the increase from 25 to 35°C. The green pigment produced had mean absorbance values of 0.285, 0.294 and 0.411 (highest) at 25, 30 and 35°C respectively (Figure 5). At higher temperature of incubation of 40 and 45°C, there was reduction in the cellular growth of the isolate and subsequently, there was decline in the production of green pigment with the minimum absorbance of 0.108 at 45°C. Similar trend was reported by Sundaramoorthy *et al.*[15] who worked on production of prodigiosin from *Serratia marcescens*. In their study, they observed a gradual decrease in growth and pigment production with the increase in temperature from 30°C.

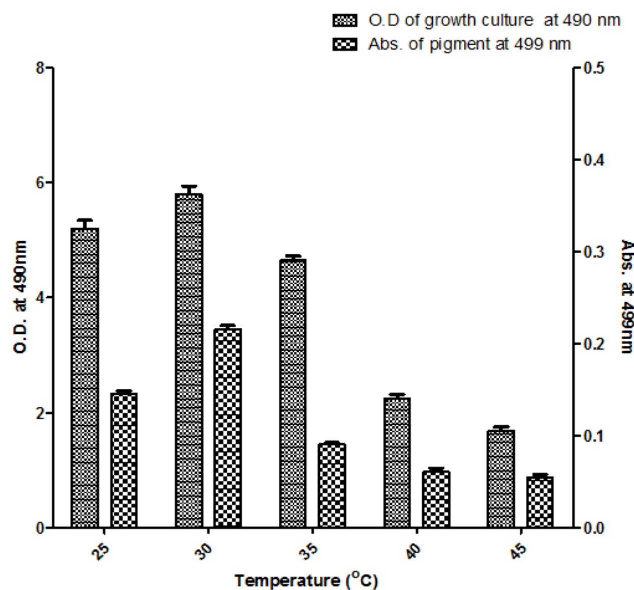

Figure 4: Effect of Incubation Temperature on Cellular Growth and Pigment Production of Bacterial Isolate G152

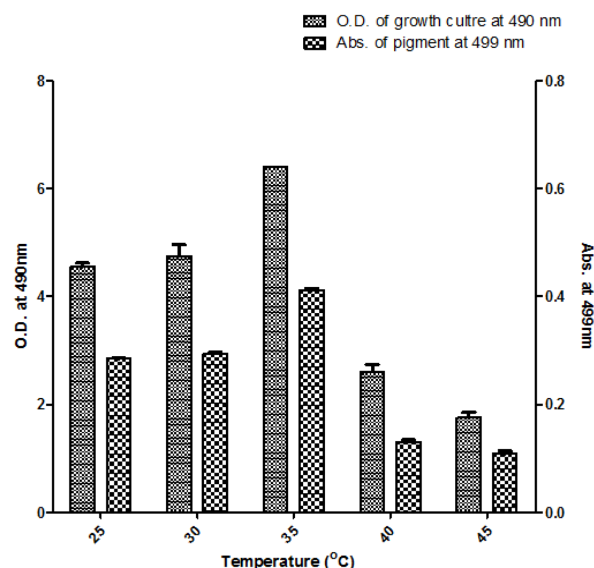

Figure 5: Effect of Incubation Temperature on Cellular Growth and Pigment Production of Bacterial Isolate G153

Effect of Incubation pH on Cells Growth and Pigment Production

The result of the effects of pH on cellular growth and pigment production in the two isolates G152 and G153 revealed that the maximum growth and pigments production were achieved at the neutral pH of 7.0 of the culture media for isolates. (Figure 6 and 7). At acidic pH values, isolate G152 had a low cellular growth with little pigment production (absorbance of 0.002 and 0.016 at 499 nm) but cellular growth increase from pH 6.0-7.0 with yellow pigment production of absorbance of 0.018 to 218. There was gradual decline in absorbance of the pigment produced in the alkaline media from pH 7.0 to 10.0. The decrease in pigment yield might due to the metabolic repression caused by the extreme pH. In case of GBB153, at low pH values of 5.0 and 5.5, cellular growth and pigment were at minimum levels (0.003 and 0.007) which increased drastically at pH 6.0 (0.258) and was maximal at pH 7.0 (0.358). The cellular growth and absorbance of green pigment produced at higher pH values reduced progressively to 0.082 at the pH 10.0. This was in agreements with the work of Gulani *et al*.[17] 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 *Sundaramoothy* et al.[15] that maximum amount of prodigiosin by a new strain designated as *S. marcescens* NY1 was produced at the neutral pH of 7.0.

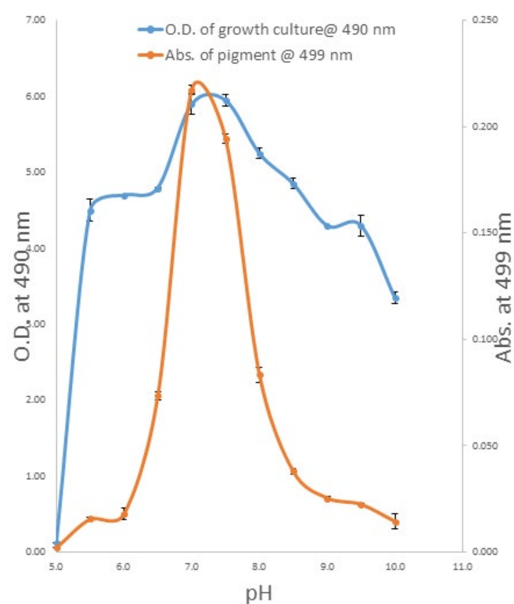

Figure 6: Effect of pH of Culture Medium on Cellular Growth and Pigment Production of Bacterial Isolate G152

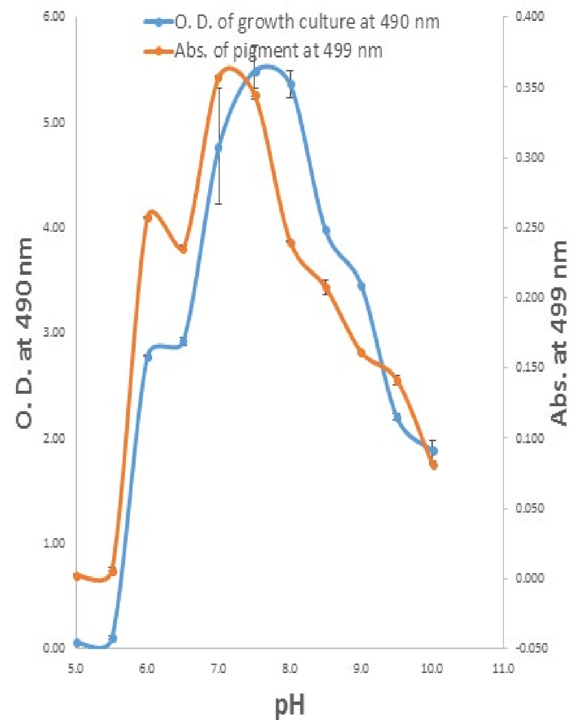

Figure 7: Effect of pH of Culture Medium on Cellular Growth and Pigment Production of Bacterial Isolate G153

Effect of Different Sugar Substrates as Carbon Sources on Cells Growth and Pigment Production.

Bacterial isolate G152 grew and produced yellow pigment maximally in presence of maltose with the mean absorbance of 0.285 (Figure 8). This was followed orderly by sucrose, starch and lactose with mean absorbance values of 0.224, 0.199 and 0.125 respectively. It was found that the isolate had the least cellular growth and pigment in presence of glucose. Also, in isolate G153, maltose was the best carbon source for cellular growth and green pigment production, yielding 0.316 (mean absorbance of pigment at 499 nm). Lactose was the second best with 0.280 while sucrose and starch followed with 0.193 and 0.141 respectively. The isolate grew in glucose but produced pigment poorly with the mean absorbance value of 0.046. (Figure 9). This result corroborate the findings of Linawati *et al*.[6] that polysaccharides or disaccharides were better than glucose (monosaccharide) as carbon sources in production of secondary metabolites by some bacteria.

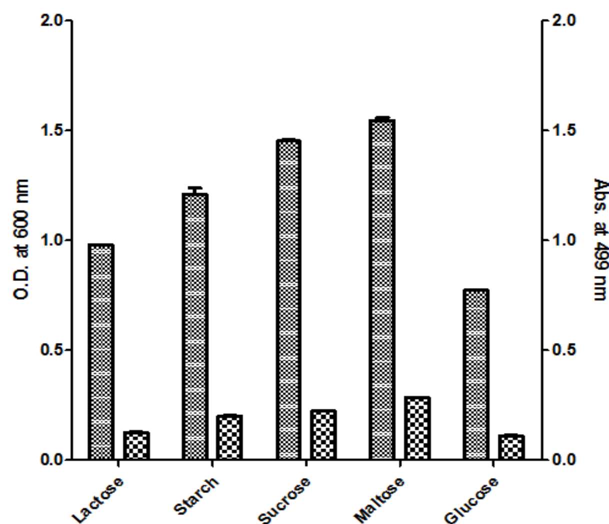

Figure 8: Effect of Different Carbon Sources on Cellular Growth and Pigment Production of Bacterial Isolate G152

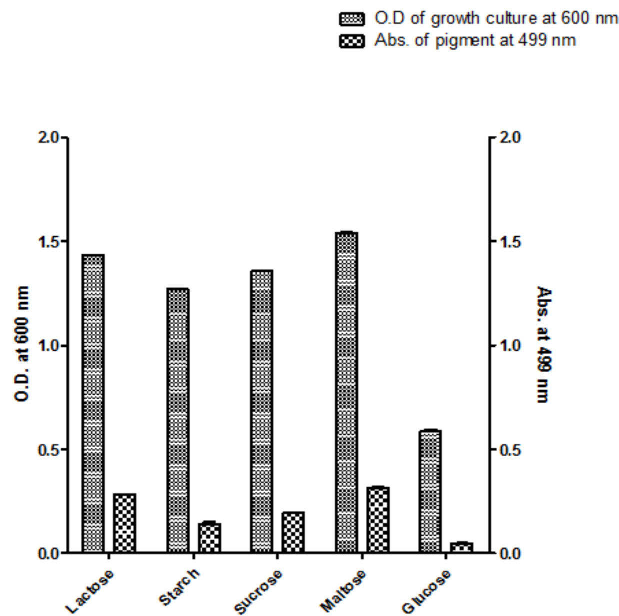

Figure 9: Effect of Different Carbon Sources on Cellular Growth and Pigment Production of Bacterial Isolate G153

Effect of Different Nitrogen Substrates on Cells Growth and Pigment Production

Of the five nitrogen sources, peptone was found to be the best substrate for cellular growth and pigment production in both isolates. In the bacterial isolate G152, peptone gave pigment production with mean absorbance value of 0.293 (figure 10). This was followed by casein (0.124), ammonium sulphate (0.080), ammonium nitrate (0.064) and the least effective nitrogen source was ammonium chloride (0.035). In case of isolate G153, the organism grew and produced green pigment of mean absorbance of 0.228. The isolate did not grow well in the other nitrogen sources and insignificant pigment production was observed with ammonium nitrate having mean absorbance value of 0.063, ammonium chloride (0.030), casein (0.018) and ammonium sulphate was the least with 0.016. (Figure 11). Gulani et al.[17] observed similar pattern. They submitted that *Serratia marcescens* proliferated and produced maximum pigment in presence of peptone than any other nitrogen source.

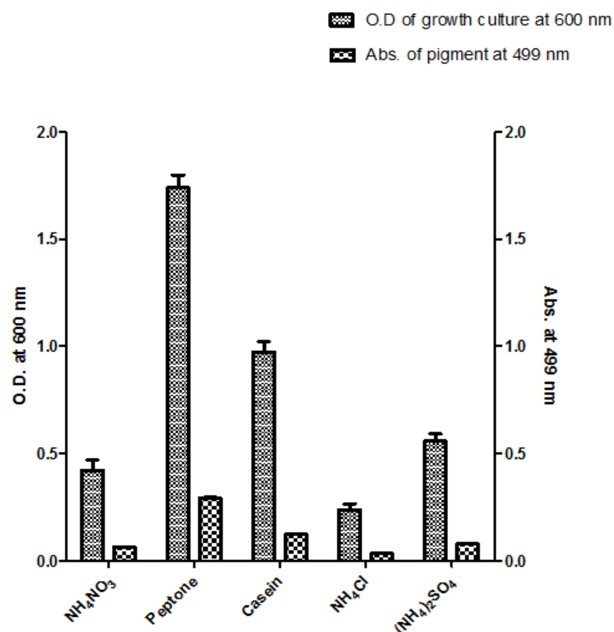

Figure 10: Effect of Different Nitrogen Sources on Cellular Growth and Pigment Production of Bacterial Isolate G152

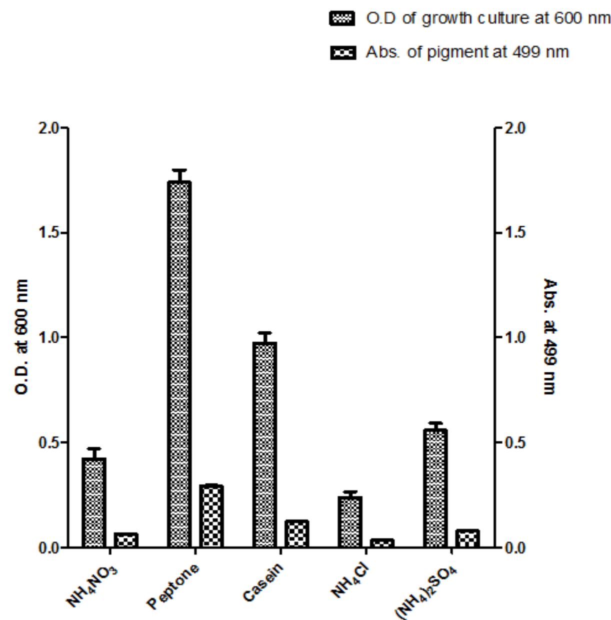

Fig.11: Effect of Different Nitrogen Sources on Cellular Growth and Pigment Production of Bacterial Isolate G153

Fourier Transport Infrared Radiation (FTIR) analysis

The Infrared spectroscopy was used to detect the presence of functional groups in GB152 and G153 pigments (Figures 12 and 13). The infrared (IR) spectral analyses of G152 and G153 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. In view of this, the IR spectrum accounts for the presence of hydroxyl group and alkene i.e. the samples contain alcohol and alkene (olefinic) functionalities

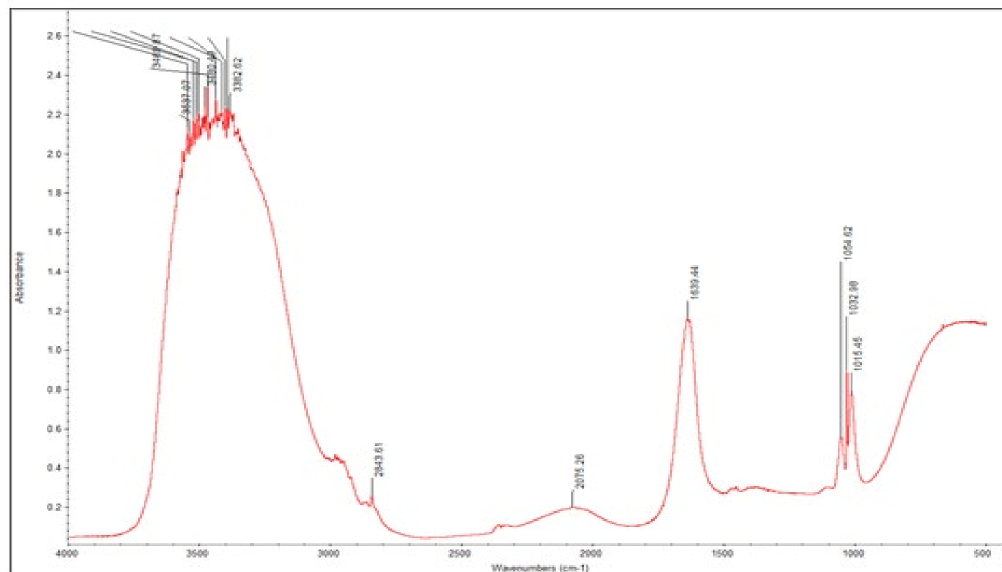

Figure 12: FTIR Spectrum of Yellow Pigment Produced by Isolate G152

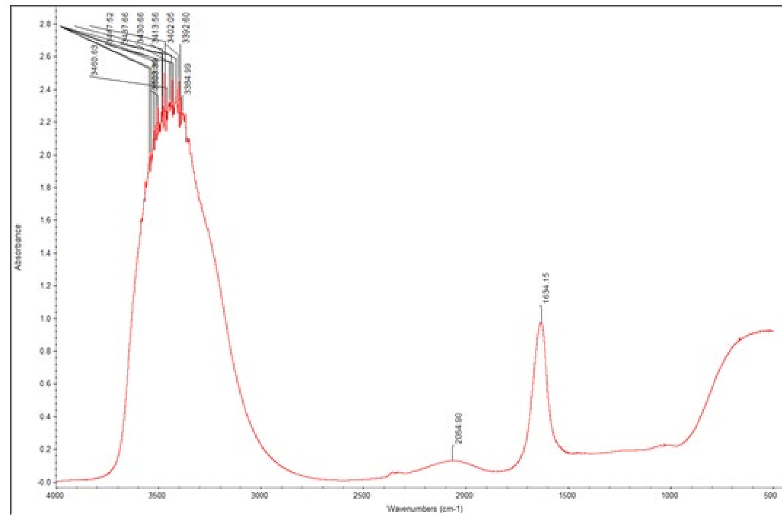

Figure 13: FTIR Spectrum of Green Pigment Produced by Isolate G153.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

A total of ten compounds were identified in the yellow pigment of G152 via GC – MS. The GBB152 chromatogram (Figure 14) shows four prominent compounds identified as octadecanoic acid (20.40%), n-hexadecanoic acid (20.03%), cis- vaccenic acid and pyrrolo (1,2-a) pyrazine-1,4-dione (14.02%). Eugenol and phenol appeared on the chromatogram with the same percentage (4.32%) in high purity of 98 and 96% respectively. Also, the green pigment of G153 isolate was composed of 18 compounds as detected via GC-MS. The chromatogram (Figure 15) shows three prominent compounds 2-Furancarboxaldehyde (26.41 and 23.59%) and octadecanoic acid (11.98%). 1-Hydroxyphenazine was detected at 20.170 min retention time with high purity of 95%. The synthesis of this metabolite by *Pseudomonas aeruginosa* was corroborated by Sudhakar *et al.*[12] who detected pyocyanin synthesis in the GC-MS analysis carried out on the pigment produced by the organism. In conclusion, the various pigments produced by the isolated organisms could find application in food, pharmaceutical, and textile industries to replace the synthetic dyes that are not safe for the environment and human consumption.

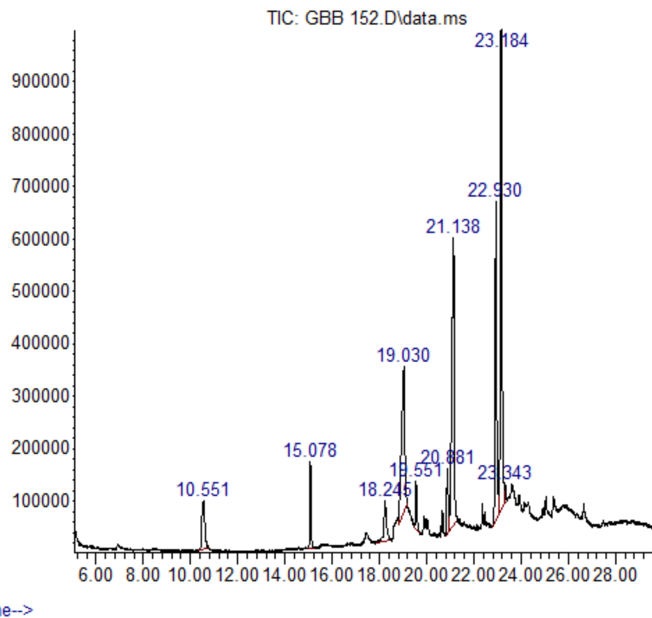

Figure 14: Chromatogram of Yellow Pigment Produced by Bacterial Isolate G152

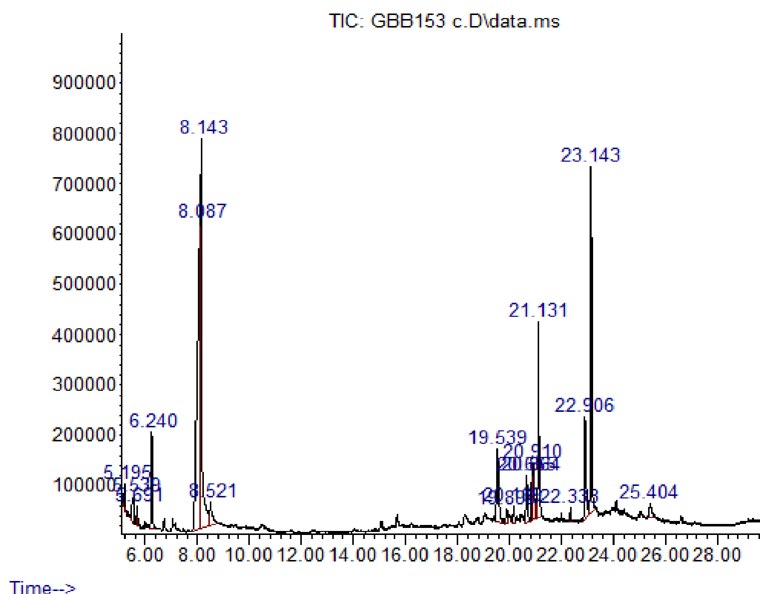

Figure 15: Chromatogram of Green pigment Produced by Bacterial Isolate G153

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