BRJ BASIC RESEARCH JOURNALS

Basic Research Journal of Microbiology ISSN 2354-4082 Vol. 4(4) pp. 33-41 July 2017
Available online http//www.basicresearchjournals.org

Full Length Research Paper

Use of chloramphenicol in the differential enumeration of greenish pigment producing *Pseudomonas*

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Accepted 11 July, 2017

ABSTRACT

Cultures of *Pseudomonas* are often required for research, biotechnology, industrial application, and environmental application. Sourcing for species of *Pseudomonas* from environmental samples can be made easy by using a procedure that promotes production of greenish pigments in selected species of *Pseudomonas*. Differential enumeration of greenish pigment producing bacteria in river water, samples was carried out using separate nutrient agar plates containing 50µg.ml^{-1}, and 100µg.ml^{-1} Chloramphenicol. Greenish pigment producing colonies were obtained on nutrient agar plates containing 50µg.ml^{-1} Chloramphenicol after 48 hours of incubation at ambient temperature. The proportion of the bacterial population producing greenish pigments on the plates ranged from 11.11% - 30.77%. Results generated from Gram staining and physicochemical/biochemical tests carried out on selected isolated colonies which produced green pigments revealed that the isolates are all *P. fluorescens*. Also, selected results generated for one of the isolate which was submitted to an internet based bacterial identification software showed that the isolate is 89% related to *P. fluorescens*. Biomolecular identification via 16S rRNA gene sequencing suggested that the isolate is *P. fluorescens*. The procedure used in this research provides a means of identifying environmental samples containing *Pseudomonas* and the subsequent isolation of the bacterium.

Keywords: *Pseudomonas, P. fluorescens*, Greenish pigment, Chloramphenicol, Differential enumeration

INTRODUCTION

Pseudomonas comprises of a group of aerobic, Gram-negative, rod shaped bacteria that can degrade an exceptionally wide variety of organic compounds (Prescott *et al*., 1999). Selected species of *Pseudomonas* are bacteria of an environmental, industrial, and medical importance. For instance, the ability of *P. aeruginosa*, *P. fluorescens*, and *P. putida* to degrade herbicides, hydrocarbons, phenols, and xenobiotics, and produce extracellular compounds makes them relevant in the bioremediation of certain polluted environments, and in some agricultural and industrial application (Ningthoujam and Shovarani, 2008; Singh and Walker, 2006; Bustamante *et al*., 2012; Vasudevan *et al*., 2007; Moneke *et al*., 2010; Mahiuddin *et al*., 2012; Haas *et al*., 1991; Persson *et al*., 1998; Otenio *et al*., 2005; Botelho and Mendonça-Hagler, 2006).

Greenish pigment producing *Pseudomonas* include *P. aeruginosa*, *P. fluorescens*, *P. putida,* and *P. syringae* (Rhodes, 1959; Yabuuchi and Ohyama, 1972; Stanier *et al*., 1977; Reyes *et al*., 1981; Daly *et al*., 1984; Scales *et al*., 2014). These *Pseudomonas* species are listed by Stanier *et al.* (1977) as the principal species of

fluorescent *Pseudomonas*. This implies that the pigments produced by these species of *Pseudomonas* will fluoresce under ultraviolet (UV) light. Greenish pigment production can make it easy to notice colonies of *P. aeruginosa*, *P. fluorescens*, *P. putida, or P. syringae* in the mist of colonies of other bacteria growing on translucent agar plate. Colonies of these *Pseudomonas* species will however produce their pigment under certain conditions.

For an environmental microbiologist, the aquatic or terrestrial environment would be the preferred place of choice for isolation of species of *Pseudomonas*. Usually, the bacterial population in samples collected from the environment will first be enumerated. The enumeration may be done using selective or differential media which can select or differentiate colonies of *Pseudomonas* from other bacteria genera. Some complex media that have been developed to select or differentiate colonies of *Pseudomonas* from other bacteria genera include cetrimide broth, Pseudocel agar, Pseudomonas agar P, Pseudomonas agar F, Pseudomonas CN selective agar, and asparagine broth enriched with K_2HPO_4 and $MgSO_4.7H_2O$ (Trust and Bartlett, 1976; Oliveira *et al*., 2008; Laine *et al*., 2009; Al-Hinai *et al*., 2010). These complex media can be categorized under relatively expensive media as a result of their advanced composition.

In a study carried out previously by the authors (Peekate and Abu, 2015), agar media containing 62.5µg.ml^{-1} Chloramphenicol and 100µg.ml^{-1} Nystatin allowed for the growth of greenish pigment producing *Pseudomonas*. Chloramphenicol could be responsible for eliciting the pigment production, as has been asserted that growth in media containing antibiotics is one of the conditions for pigment production by *P. aeruginosa* (Sweedan, 2010). Incorporating Chloramphenicol in plate count media thus presents a means for the differential enumeration of *Pseudomonas* species in environmental media, and their subsequent isolation. The aim of this study is thus to provide an inexpensive means that relies on greenish pigment production by certain *Pseudomonas* species for the acquisition of *Pseudomonas* from environmental media for use in research, biotechnology, and environmental application.

MATERIALS AND METHODS

Sourcing for *Pseudomonas* from river water samples

Water samples were collected at five different points from a river located near the Rivers State University of Science and Technology, Nigeria. The GPS (Global Positioning System) location of a point within the sampling area captured using the GPS available on Google Maps app on an android enabled phone is given as 4° 47' 33.4" N, 6° 58' 24.1" E. The water samples were collected from the surface of the water bodies (top 0.0 – 0.3m) with the aid of sterile bottles. The five selected points from which the samples were collected were chosen at random, and were about 1m apart.

Physicochemical and Microbiological analysis of the river water

A fraction of the river water samples were sent for pH measurement, turbidity measurement, and salinity determination. The remainder were refrigerated prior to enumeration and differential enumeration of total heterotrophic bacterial population and greenish pigment producing bacterial population, respectively.

The total heterotrophic bacterial population of the river water samples were enumerated using the standard plate count method. In the method, about 1ml of the river water samples were serially diluted, via ten-fold serial dilution, to 10^{-3}. About 0.1ml of the original samples and the different dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were then plated, in triplicates, on nutrient agar plates using the spread plate technique. The plates were incubated at 37°C for about 24 hours, after which the ensuing colonies were counted and the total heterotrophic bacterial population calculated.

Differential enumeration of greenish pigment producing *Pseudomonas*

Differential enumeration of greenish pigment producing bacterial population in the river water samples was carried out using separate nutrient agar plates containing 50µg.ml^{-1}, and 100µg.ml^{-1} Chloramphenicol. The standard plate count method was also applied in the enumeration. The river water samples were however serially diluted to a dilution that previously gave colony counts of hundreds, based on results obtained from the enumeration of the total heterotrophic bacterial populations. The dilutions were then plated separately, in triplicates, on nutrient agar plates containing 50µg.ml^{-1}, and 100µg.ml^{-1} Chloramphenicol. The plates were incubated at ambient temperature (27 - 31^{0}C) for 48 hours. However, they were checked at 24 hours for growth.

Preparation of separate nutrient agar plates containing 50µg.ml^{-1} and 100 µg.ml^{-1} Chloramphenicol

A stock solution of 1000µg.ml^{-1} Chloramphenicol was first prepared by transferring about 0.1g of powdered Chloramphenicol, retrieved from Chloramphenicol

capsules, into a sterile 150ml capacity conical flask containing 100ml of distilled water. The flask and its content (100ml distilled water) were previously sterilized in an Autoclave and allowed to cool to ambient temperature before addition of the antibiotic.

The volume of nutrient agar media required to plate the 5 river water samples (using 3 plates per water sample, and specifying a minimum volume of 20ml for each plate) was worked out to be 300ml. However, a nutrient agar media volume of 350ml was targeted. The volume of the Chloramphenicol stock solution (V_1) required to be added to 350ml of nutrient agar media to obtained nutrient agar media having a concentration of 50µg.ml^{-1} Chloramphenicol was determined using the equation $M_1V_1 = M_2V_2$ (Manilla *et al*., 2001), i.e. 1000µg.ml^{-1} × V_1 = 50µg.ml^{-1} × 350ml. Thus, V_1 = 17.5ml. The nutrient agar media volume used was thus 332.5ml, i.e. 350 – 17.5ml. However, the quantity of nutrient agar required for preparing nutrient agar media volume of 350ml was used, i.e 9.8 g (which is required for preparing a nutrient agar media volume of 350ml) of nutrient agar was added to 332.5ml distilled water. [28g of nutrient agar is usually specified for a volume of 1000ml]. In the preparation of nutrient agar plates containing 100µg.ml^{-1} Chloramphenicol, the volume of the Chloramphenicol stock solution (V_1) required to be added to 350ml of nutrient agar media was also determined using the equation $M_1V_1 = M_2V_2$, i.e. 1000µg.ml^{-1} × V_1 = 100µg.ml^{-1} × 350ml. Thus, V_1 = 35ml. The nutrient agar media volume used was thus 315ml, i.e. 350 – 35ml. Also, the quantity of nutrient agar required for preparing nutrient agar media volume of 350ml was used, i.e 9.8g of nutrient agar was added to 315ml distilled water. Both media were prepared separately in 500ml conical flasks, sterilized in an Autoclave, and allowed to cool to about 50°C before adding the required volume of the Chloramphenicol stock solution (17.5ml to the one for 50µg.ml^{-1}, and 35ml to the one for 100µg.ml^{-1}). After the addition, each agar medium was swirled gently, and poured into 15 labelled sterile Petri plates. The agar media in the plates were allowed to harden, and then the plates were dried in a hot air oven set at 45°C.

Screening of greenish pigment producing bacterial isolates

Colonies producing greenish pigmentation on nutrient agar plates containing Chloramphenicol were enumerated, and their fluorescence was checked for using a UV LED (ultra violet light emitting diode) torch. Selected fluorescent greenish pigment producing colonies were isolated unto sterile nutrient agar plates from whence their stock cultures were prepared. The isolates were subjected to Gram staining and microscopic examination, followed by selected physicochemical and biochemical tests. The biochemical and physicochemical tests used include catalase, oxidase, motility, citrate utilization, indole production, sugar fermentation, MRVP (Methyl Red-Vogues Proskauer), lipase production, blood haemolysis, starch hydrolysis, casein hydrolysis, lecithinase production, and growth on MacConkey agar.

The results obtained for a selected isolate from the lot having similar physicochemical and biochemical test results attributed to members of the *Pseudomonas* genus were submitted to ABIS (Advanced Bacterial Identification Software) online (http://www.tgw1916.net/bacteria_logare.html), an online laboratory tool for bacterial identification based on morpho-biochemical characters, growth conditions, etc. Biomolecular identification of the selected isolate was also carried out via DNA extraction, 16S rRNA gene amplification, and sequencing of the 16S rRNA gene. The 16S rRNA gene sequence obtained from the isolate was edited using Trace edit, a bioinformatics algorithm, and compared with sequences deposited in the National Center for Biotechnology Information (NCBI) data base using BLASTN (a bioinformatics software).

RESULTS

The pH, turbidity, and salinity of the river water from whence *Pseudomonas* was sourced for are 6.8, 23.2 NTU, and 9.5‰ respectively. The heterotrophic bacterial population of the water samples from the five randomly selected points as estimated using nutrient agar plates without and with Chloramphenicol is shown in Table 1 below. From the Table it can be seen that the population estimated using nutrient agar plates containing Chloramphenicol is low compared to that estimated using nutrient agar plates without Chloramphenicol.

The fraction of bacterial population, estimated using nutrient agar plates containing Chloramphenicol, presenting greenish pigmentation is presented in Table 2 below. In Table 2 below, it can be seen that the proportion of the bacterial population producing greenish pigments on nutrient agar plates containing 50µg.ml^{-1} Chloramphenicol ranged from 11.11% - 30.77%. Also, it can be seen in Table 2 below that Chloramphenicol at a concentration of 100µg.ml^{-1} inhibited the growth of the total heterotrophic bacterial population. Yellowish green pigment exhibited by some colonies growing on nutrient agar plates containing 50µg.ml^{-1} Chloramphenicol, alongside their fluorescence is presented in Figure 1 below.

Selected isolated colonies presenting with greenish pigmentation were coded as follows; BC3, P1, P7, P10, and X1. Greenish pigmentation exhibited by isolate BC3 and P1 is shown in Figure 2 and Figure 3 below respectively. Fluorescence exhibited by P1 under UV light is also shown in Figure 3 below. On careful

Table 1. Bacterial population of the river water samples estimated from plates incubated for 24 hours

SC	THB (cfu/ml)	BC50 (cfu/ml)	BC100 (cfu/ml)
Rp1	2.02×10^{7}	1.10×10^{2}	0
Rp2	8.00×10^{6}	1.00×10	0
Rp3	1.92×10^{7}	1.00×10	0
Rp4	9.95×10^{6}	1.50×10	0
Rp5	1.31×10^{6}	0	0

SC – sample code; THB – total heterotrophic bacterial population; BC50 – bacterial population enumerated using nutrient agar plates containing 50 µg.ml^{-1} Chloramphenicol; BC100 – bacterial population enumerated using nutrient agar plates containing 100 µg.ml^{-1} Chloramphenicol.

Table 2. Bacterial population estimated from plates containing Chloramphenicol incubated for 48 hours

SC	BC50 (cfu/ml)	BC100 (cfu/ml)	APG (cfu/ml)	PPA (%)
Rp1	3.15×10^{2}	0	3.50×10	11.11
Rp2	1.10×10^{2}	0	0	0
Rp3	1.30×10^{2}	0	4.00×10	30.77
Rp4	1.20×10^{2}	0	0	0
Rp5	6.00×10	0	0	0

SC – sample code; APG – average population producing greenish pigments; PPA – proportion of colonies producing greenish pigments ($\frac{APG \times 100}{BC50}$).

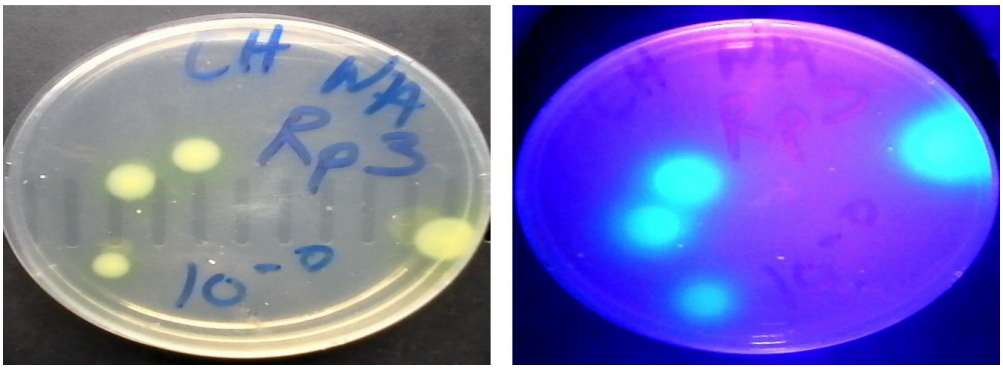

Figure 1. Yellowish green pigment produced by some colonies growing on nutrient agar plates containing 50 µg.ml^{-1} Chloramphenicol, alongside fluorescence of the pigments under UV light.

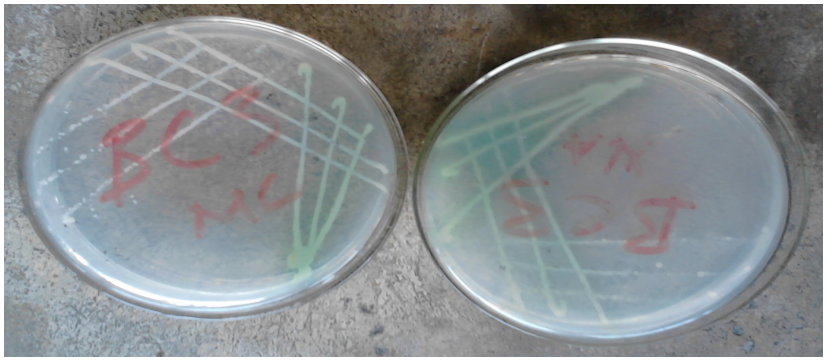

Figure 2. Greenish pigmentation exhibited by isolate BC3.

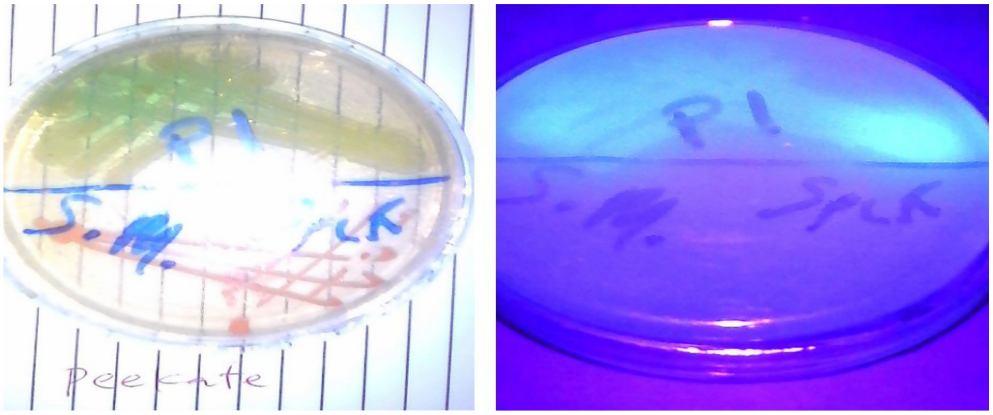

Figure 3. Greenish pigment produced by P1 and its fluorescence (NOTE: the reddish pigment produced by the control isolate, *Serratia marcescens*, on the other half of the plate did not fluoresce)

Table 3. Identification test results of selected green pigment producing isolates

	BC3	P1	P7	P10	X1
Gm	- ve rods	- ve rods	- ve rods	- ve rods	- ve rods
Ctl	+ ve	+ ve	+ ve	+ ve	+ ve
Oxd	+ ve	+ ve	+ ve	+ ve	+ ve
Mtl	+ ve	+ ve	+ ve	+ ve	+ ve
CtU	+ ve	+ ve	+ ve	+ ve	+ ve
Ind	+ ve	- ve	- ve	- ve	+ ve
VP	- ve	- ve	- ve	- ve	- ve
MR	- ve	- ve	- ve	- ve	- ve
LPs	+ ve	+ ve	+ ve	+ ve	+ ve
HBA	β-H	β-H	β-H	β-H	β-H
SH	- ve	- ve	- ve	- ve	- ve
CsH	+ ve	+ ve	+ ve	+ ve	+ ve
LcP	+ ve	+ ve	+ ve	+ ve	+ ve
MC	+ ve	+ ve	+ ve	+ ve	+ ve
GcF	A	0	0	A	A
LtF	0	0	0	0	0
XsF	A	0	0	A	A
MnF	A	0	0	A	A

Gm – Gram stain, Ctl – catalase; Oxd – oxidase; Mtl – motility; CtU – citrate utilization; Ind – indole; VP – Vogues Proskauer; MR – Methyl red; LPs – Lipase production; HBA – haemolysis on blood agar; β-H – beta haemolysis (clear transparent zone around colonies); SH – starch hydrolysis; CsH – Casein hydrolysis; LcP - lecithinase production; MC – growth on MacConkey agar; GcF – Glucose fermentation; LtF – Lactose fermentation; XsF – Xylose fermentation; MnF – Mannitol fermentation; A – only acid produced; - ve – negative; + ve – positive.

observation of Plate 2, it can be seen that the pigment diffused into the medium.Results generated from Gram staining, and the physicochemical/biochemical tests carried out on the isolates is presented in Table 3. From Table, it can be seen that all the isolates exhibited the same reaction pattern, with exception to the indole test, and the fermentation test with glucose, xylose, and mannitol. The reaction pattern of the different isolates are similar to the reaction pattern usually exhibited by *P. fluorescens* to the physicochemical/biochemical tests used as deciphered from selected literature and texts (Lysenko, 1961; Reynolds *et al*., 1979; Prescott *et al*., 1999). Also, selected results generated for one of the isolate, P1, which was submitted to ABIS (Advanced Bacterial Identification Software) online (http://www.tgw1916.net/bacteria_logare.html) showed

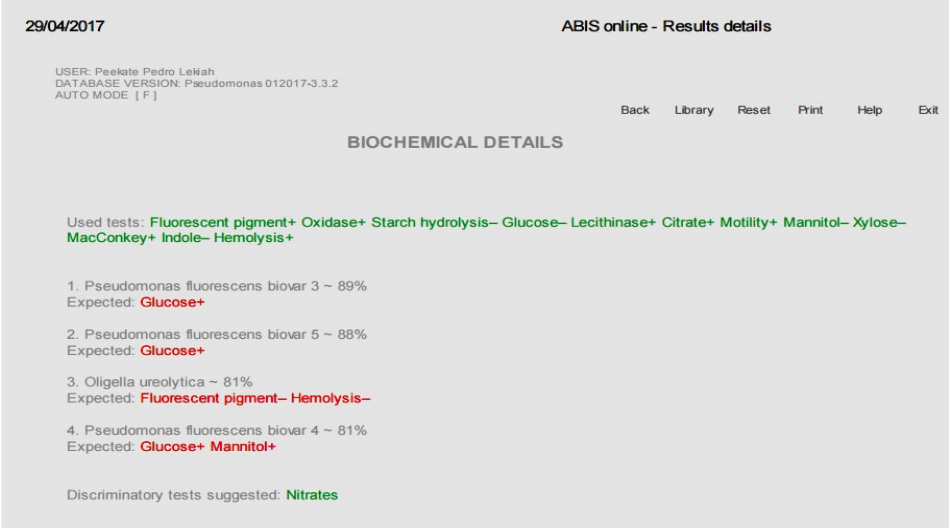

Figure 4. ABIS online result generated for P1

Table 4. Yield and purity of extracted DNA of P1

Isolate ID	Yield (ng/µl)	A260	A280 ($A_{260/280}$)	Purity
P1	479.46	9.589	5.294	1.81

that the isolate is 89% related to *P. fluorescens* biovar 3 (Figure 4). The probably of identity P1 as obtained by comparing its edited 16S rRNA gene sequence with sequences deposited in the NCBI data base using BLASTN suggested that the isolate is EU543578.1 *P. fluorescens*. The concentration of the extracted DNA from P1 is 479.46 ng/µl (Table 4).

DISCUSSION

Cultures of selected species of *Pseudomonas* are often required for research, biotechnology, and environmental application. Where it is not feasible to acquire species of *Pseudomonas* from culture collection centres due to distance, location, paper work, financial constraint, bio-security issues, etc., the aquatic or the terrestrial environment becomes the preferred place of choice for isolation of the bacteria. Identification of environmental samples containing *Pseudomonas* and the subsequent isolation of the bacteria using a simple procedure that promotes the production of fluorescent greenish pigments, which is a unique characteristic of selected *Pseudomonas* species, presents a cost effective means for acquisition of *Pseudomonas*.

Bluish greenish pigment production is regarded as a unique feature of *P. aeruginosa* among Gram negative bacteria (Reyes *et al*., 1981; Kumar, 2012), while yellowish green pigments are known to be produced by *P. fluorescens, P. putida*, and *P. syringae* (Stanier *et al*., 1977) In this study, the use of nutrient agar plates containing 50µg.ml^{-1} Chloramphenicol in the enumeration of river water samples led to the production of greenish pigments by some colonies. The plates where incubated at ambient temperature based on reasoning that pigmentation may not occur at elevated temperatures, just as is the case with *Serratia marcescens*; *Serratia marcescens* produces its red pigment at ambient temperature (24 – 30°C) but not at 32°C and above (Syzdek, 1985; Tanaka *et al*., 2004). The production of greenish pigment provides a possibility of enumerating species of *Pseudomonas* in environmental media, and subsequent isolation of the bacteria. This phenomenon also provides a means of identifying samples containing *Pseudomonas*. Colonies exhibiting greenish pigmentation on nutrient agar plates containing 50µg.ml^{-1} Chloramphenicol were achieved from water samples having relatively high heterotrophic bacterial population. Environmental media having a relatively high heterotrophic bacterial population may thus be a reliable place to source for *Pseudomonas*.

The high bacterial population of the river water (1.31 × 10^6 – 2.02 × 10^7) can be attributed to the high turbidity (23.3 NTU) and low salinity (9.5‰) of the river water.

High turbidity has been correlated with maximum bacterial density (Bilgrami *et al*., 1986), and it has been observed that total and active bacterial numbers in brackish waters are higher than in marine waters (Almedia *et al*., 2001). Based on the salinity of the river water, the river habitat where the samples were collected can be said to be brackish water. Brackish waters are regarded as river habitats having water with salinity values of 0.5 ‰ – 30‰ (Snoeijs, 1999). On the other hand, a turbidity value of 23.3 NTU can be regarded as a high turbidity value based on WHO's guidelines for water quality. In WHO's guidelines, a turbidity value of and greater than 5 NTU is inferred as a high turbidity value (WHO, 2011). The pH (6.8) of the river water is also within the range for optimum growth by some bacteria. Optimum growth of bacteria species such as *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Clostridium sporogenes* has been established to occur within a pH range of 6.0 – 7.6 (Prescott *et al*., 1999). In general, the physicochemical parameters indicate that a wide variety of bacteria species can be found in the river water.

The reduced bacterial population (Table 1) as estimated using nutrient agar plates containing Chloramphenicol is evident that the antibiotic inhibited a fraction of the bacterial population at a concentration of 50µg.ml^{-1}, and at a concentration of 100µg.ml^{-1} inhibited the total bacterial population. The continued inhibition of bacterial growth on nutrient agar plates containing 100µg.ml^{-1} Chloramphenicol after another 24 hours (Table 2) suggests that Chloramphenicol at a concentration of 100µg.ml^{-1} is lethal to a bacterial population size of and below 10^7. On the other hand, there was a slight increase in the bacterial population on nutrient agar plates containing 50µg.ml^{-1} Chloramphenicol after another 24 hours. This indicates the development of resistance to the antibiotic by some bacteria in the sample. *P. aeruginosa* is intrinsically resistant to Chloramphenicol (Morita *et al.,* 2014). Being related in many aspects to *P. aeruginosa*, *P. fluorescens* can also be intrinsically resistant to Chloramphenicol. There is thus a possibility of finding *P. fluorescens* among the bacteria which have developed resistance to the antibiotic. The possibility is further enhanced by the production of greenish pigments by some of the Chloramphenicol resistant bacteria. Growth in the presence of antibiotics has been cited as one of the conditions necessary for the production of pigments by *P. aeruginosa* (Sweedan, 2010). Again, this could also apply to *P. fluorescens* and other greenish pigment producing *Pseudomonas*. There is thus a high likelihood that Chloramphenicol resistant bacteria producing greenish pigments are members of the fluorescent *Pseudomonas* which include *P. aeruginosa, P. fluorescence, P. putida*, and *P. syringae.* There was no pigment production on nutrient agar plates which did not contain the antibiotic. This indirectly indicates that Chloramphenicol elicited the pigment production by *P. fluorescens* colonies.

Species of greenish pigment producing *Pseudomonas* can be differentiated by the colour variation of their pigment, their growth range, and utilizable substrates. For instance, though *P. aeruginosa* produces yellowish green pigments it is the only species of *Pseudomonas* that can produce bluish green pigment (Stanier *et al*., 1977; Daly *et al*., 1984), it is able to grow at 42^{0}C within 24hrs, and produce acid from the oxidation of ethanol (Lysenko, 1961; Reyes *et al*., 1981). These characteristics differentiate it from *P. fluorescens, P. putida,* and other *Pseudomonas* species. *P. fluorescens* can grow at 4°C (Scales *et al*., 2014) and produces lecithinase (Lysenko, 1961; Younes *et al*., 2015). These characteristics differentiate it from *P. aeruginosa* and *P. putida*. The ability of *P. putida* to produce acid from utilization of xylose and glycerol, and its inability to hydrolize gelatine and casein, differentiates it from *P. fluorescens*, while it's ability to grow at 5 and 10°C differentiates it from *P. aeruginosa* (Lysenko, 1961). The growth rates of *P. syringae* both in synthetic and in complex media are much lower; and they are less versatile nutritionally (Stanier *et al*., 1977). The presence of *P. syringae* in the midst of other bacteria which have relatively high growth rates may thus be obscured. The pigment producing isolates acquired in this study via differential enumeration using Chloramphenicol all exhibited yellowish green pigmentation which fluoresced under UV light. The biochemical tests used in the study involved two tests, the lecithinase production test and the casein hydrolysis test, which can in part differentiate between the principal fluorescent greenish pigment producing species of *Pseudomonas*. Species of *Pseudomonas* utilize citrate and can hydrolyze lipids (Lysenko, 1961). They are oxidase positive (with the exception of *P. syringae*), catalase positive, Methyl red-Voges Proskauer negative, and are motile by one or several polar flagella (Prescott *et al*., 1999; Stanier *et al*., 1977; Lysenko, 1961). Some species of *Pseudomonas* produces beta-haemolysis when grown on blood agar (Egwuatu *et al*., 2014). However, for *P. aeruginosa*, it is recorded that beta-haemolysis is achieved when the blood used is obtained from cow, sheep, or humans.

The reaction pattern of the different selected isolates, which produced greenish pigments, to the physicochemical/biochemical tests used was similar, except for the indole test, and fermentation test with glucose, xylose and mannitol. Many biochemical reaction pattern deciphered for the *Pseudomonas* genus were achieved through the study of *P. aeruginosa*, and it is agreed that *P. aeruginosa* is indole negative (Ndip *et al*., 2005). *P. fluorescens* isolates positive for indole production could be as a result of certain unusual properties of selected biovars, false positive results, or could be attributed to the presence of an indole positive

contaminating bacterium in the test culture broth.

Generally, it is agreed that *Pseudomonas* species do not ferment sugars but can occasionally ferment glucose with no gas production (Stanier *et al*., 1977). However, in Figure 4 it can be seen that *P. fluorescens* biovar 4 is expected to ferment mannitol. Thus isolate BC3, P10, and X1 can be regarded as biovars of *P. fluorescens* since they fermented mannitol and glucose. The ability of *P. fluorescens* to hydrolyze a protein found in egg yolk which is assessed through the lecithinase production test differentiates it from *P. aeruginosa* and *P. putida* (Lysenko, 1961; Younes *et al*., 2015). In Table 3 it can be seen that all the isolates were positive for lecithinase production. In general, the patterns exhibited by the isolates are similar to the reaction pattern usually exhibited by *P. fluorescens* as deciphered using selected literature and texts.

The concentration of the extracted DNA (479.46ng/µl) from the isolate, P1, which was sent for biomolecular identification fall within the range of the concentration of DNA that have been extracted from *P. fluorescens* isolated from soil and humans (Hamod, 2015). The concentration of DNA extracted from *P. fluorescens* by Hamod (2015) ranged from 338ng/µl – 1091ng/µl. Three of his *P. fluorescens* isolates actually had values of 488ng/µl, 489ng/µl, and 498ng/µl. The concentration obtained for our isolate closed in to these values. This, in addition to the identity obtained from the sequencing and BLASTN, and the biochemical characterization, is a confirmation that the isolate P1 is *P. fluorescens*. Thus all isolates exhibiting the yellowish green pigmentation on the differential enumeration media (nutrient agar containing 50µg.ml^{-1} Chloramphenicol) and reacted similarly to most of the biochemical tests used are highly suspected to be *P. fluorescens*.

In the selective isolation of *Pseudomonas* based on pigment production, procedures which sometimes are cumbersome and involve the use of more than one media have been used. In the procedure carried out by Oliveira *et al.* (2008), for samples taken from materials and equipments, collection swabs were first moistened with brain-heart infusion, while filtration was first carried out for water samples. Swabs and filters were then used to inoculate plates of two media (Pseudomonas agar P and Pseudomonas agar F) specially used for the isolation of *Pseudomonas*. In the procedure carried out by Al-Hinai *et al.* (2010), soil samples were first inoculated in asparagine broth enriched with K_2HPO_4 and $MgSO_4.7H_2O$ so as to increase the population of *Pseudomonas* in the soil samples. Inoculated broths were incubated at 37^{0}C with shaking at 200rpm for 48hrs. The resulting growth from the broth cultures were then streaked on agar plates containing asparagines and incubated again at 37^{0}C until colonies developed. The different bacterial colonies that developed were then isolated on separate agar plates containing asparagines so as to obtain yellowish green pigment producing *Pseudomonas*. In our procedure, we used a relatively cheap media and an inexpensive antibiotic to achieve the isolation of *Pseudomonas* based on greenish pigment production. Our simple procedure was uncomplicated and, more importantly, relatively cheap. Based on the inexpensive nature and less laborious protocol of our procedure, we recommend it for the differential enumeration and subsequent isolation of *Pseudomonas* from environmental media.

The issue of development of antibiotic resistance as a result of this procedure may be a thing of concern. However, adhering strictly to the protocols in biosafety and laboratory safety will go a long way in curtailing the escape of such bacteria from the laboratory and the subsequent spread of antibiotic resistance.

CONCLUSION

Greenish pigment production avails us a means of identifying colonies of *Pseudomonas* growing on agar media. However, the pigment is only produced under certain conditions. One of such conditions has been cited as growth in the presence of antibiotics. Chloramphenicol was shown in this research as an antibiotic that can elicit pigment production by *P. fluorescens.*

Differential enumeration of the bacterial population present in an environmental media using nutrient agar containing 50µg.ml^{-1} Chloramphenicol can lead to the identification of samples containing *Pseudomonas* and thus subsequent isolation. This provides a cost effective means for acquisition of *Pseudomonas* for research, biotechnology, industrial application, and environmental application.

As a result of growth in media containing an antibiotic, bacteria can develop resistance to other antibiotics. Determining the potency of selected antibiotics on such bacteria may be necessary so as to be prepared for laboratory acquired infections that may occur due to work with the bacteria. Determining the potency of selected antibiotics on the bacteria will also make known the antibiotics that can be use against the bacteria in case of an outbreak of infection resulting from escape of the bacteria from the laboratory.

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