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Research Article
Comparing the Efficacy of Kings B, Cetrimide and Chloramphenicol-nutrient Agar Medium in the Isolation of *Pseudomonas* Species

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Abstract

Background and Objective: It is sometimes necessary to acquire *Pseudomonas* species from the environment for research and application. In the acquisition of *Pseudomonas* species from samples certain media have been used. Certain components of these media instigate greenish pigment production in selected species of *Pseudomonas*. The efficacy of some of these media in indicating if a sample contains *Pseudomonas* species is unknown. The aim of this study was thus to compare the efficacy of some media used in the selective isolation of *Pseudomonas* species. The objectives include determining the percentage of greenish pigment producing bacterial population that can be obtained using the different media. **Materials and Methods:** Differential enumeration of greenish pigment producing *Pseudomonas* species in selected samples were carried out using Cetrimide agar (CA), Kings B agar (KBA) and Nutrient agar supplemented with 50 μg mL^{-1} Chloramphenicol (NC). One-way analysis of variance at $p \leq 0.05$ was used to determine if there was any significant difference between the proportions of total bacterial population producing greenish pigment obtained through the use of the different media. Selected greenish pigment producing bacterial colonies were identified using selected physicochemical/biochemical tests. **Results:** The proportion of bacterial population producing greenish pigments (PBG) obtained using KBA ranged from 1.37-3.92%, the PBG obtained using CA ranged from 31.71-100%, while the PBG obtained using NC ranged from 0.86-14.29 %. Statistical analysis of the PBG obtained using the three media showed that the difference between the percentages obtained using the different media is significant ($p \leq 0.05$). The different greenish pigment producing bacteria isolates were all identified as *Pseudomonas* species. **Conclusion:** From the obtained results, it is concluded that Cetrimide agar is a better medium for the selective enumeration and isolation of greenish pigment producing *Pseudomonas* species.

Key words: Kings B medium, cetrimide agar, nutrient agar supplemented with chloramphenicol, greenish pigment producing bacteria, *Pseudomonas*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas species are model organisms for the study of certain bacterial mechanisms and are potentially important in agriculture, industries and in the bioremediation of contaminated/polluted environments. For instance, due to the advanced antibiotic resistance mechanisms of *P. aeruginosa*, it is considered a model organism for the study of antibiotic-resistance in bacteria[1]. Production of active extracellular compounds by some *Pseudomonas* species provides them avenues for application in agriculture, industries and bioremediation of contaminated/polluted environments. These compounds include fungicides, biosurfactants and bioemulsifiers. Species of *Pseudomonas* capable of producing biosurfactants include *P. aeruginosa, P. fluorescens*, *P. stutzeri*, *P. putida* and *P. chlororaphis*[2-5]. The *P. fluorescens* and *P. putida* also produces bioemulsifiers[6,7]. The potential application of biosurfactants and bioemulsifiers include clean-up of oil storage tanks, recovery of oil in reservoirs, control/modification/ improvement agents in food industries, control of pathogenic fungi on agricultural crops as antimicrobial agents and as enhancers in bioremediation of oil polluted environments[8]. The *P. aeruginosa*, *P. fluorescens* and *P. putida* can degrade herbicides, petroleum hydrocarbons, phenol, toxins and organic solvents[9-13]. The *P. fluorescens* and *P. putida* produce antifungal and herbicidal compounds and portray antagonistic activity against pathogenic plant fungi[14-18]. Due to their various capabilities, it is thus sometimes necessary to acquire species of *Pseudomonas* for use in agricultural, industrial, environmental endeavours.

In culturing environmental samples or bio-specimen for the acquisition of *Pseudomonas* species certain media have been used. These media include Cetrimide agar (Pseudocel agar), Tech agar (Kings A and B agar), asparagine broth enriched with K_2HPO_4 and $MgSO_4.7H_2O$ and nutrient agar supplemented with chloramphenicol[19-22]. The compositions of these media are tailored to the fact that certain species of *Pseudomonas* produce greenish pigments in the presence or absence of certain substances. For instance, *Pseudomonas aeruginosa* produces its bluish-green pigment in the absence or scarcity of iron[23] and *P. fluorescens* produces its yellowish-green pigment in the presence of Chloramphenicol[21]. The principal species of *Pseudomonas* that produce greenish pigments include *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. syringae*[24-27]. Greenish pigment production makes it possible to easily notice colonies of these *Pseudomonas* species in the midst of colonies of other bacteria growing on selective or differential agar media.

The effectiveness of some of the media used in the selective isolation of *Pseudomonas* species in indicating if a sample contains *Pseudomonas* species is unknown. The aim of this study is thus to determine and compare the efficacy of the use of Cetrimide agar, kings B medium and nutrient agar supplemented with chloramphenicol in the selective isolation of *Pseudomonas* species.

MATERIALS AND METHODS

Sample collection: Samples were collected from the Eagle Island River located near the Rivers State University, Port Harcourt, from a Gutter located within the university and from a crude oil polluted site in Gokana LGA. The water samples were collected with the aid of 500 mL sterile water-sampling bottles. The river and gutter water samples were coded R1 and G1, respectively. The river water sample was collected at a distance of about 1 m away from the shore line and from the surface of the water body with the aid of a sterile water sampling bottle. Two soil samples were collected from the crude oil polluted site with the aid of sterile wide-mouth amber bottles of about 50 mL capacity and a disinfected hand trowel and coded S1 and S2. The samples were taken to the Microbiology laboratory, Rivers State University for differential enumeration of greenish pigment producing *Pseudomonas* species.

Differential enumeration of greenish pigment producing *Pseudomonas*: Differential enumeration of greenish pigment producing *Pseudomonas* species in the collected samples was carried out using Cetrimide agar, Kings B agar and Nutrient agar supplemented with 50 μg mL^{-1} Chloramphenicol. The media were all prepared according to the manufactures instructions. Nutrient agar supplemented with 50 μg mL^{-1} Chloramphenicol was prepared as outlined in Peekate and Abu[21]. After preparation, the media were poured into labelled sterile Petri plates, allowed to harden and then dried in a Hot air oven set at 50°C.

The samples were serially diluted through a tenfold dilution process to 10^{-4}. About 0.1 mL of the different dilutions was then plated separately on plates of Cetrimide agar, Kings B agar and the Nutrient agar supplemented with Chloramphenicol. Inoculated plates were incubated at ambient temperatures (28-32°C) for a minimum of 48 h. After incubation, the total colonies and greenish pigment producing colonies that developed on the plates were counted and used to calculate the total bacterial and greenish pigment producing *Pseudomonas* populations. Selected

greenish pigment producing bacterial colonies were sub-cultured unto sterile nutrient agar plates for onward identification.

Statistical analysis: One-way analysis of variance (One-way-ANOVA) at $p \leq 0.05$ was used to determine if there was any significant difference between the proportions of total bacterial population producing greenish pigment obtained through the use of the different media.

Identification of greenish pigment producing bacteria: The greenish pigment producing bacterial isolates were subjected to Gram-staining and microscopic examination and the following physicochemical/biochemical tests: Catalase, oxidase, motility, citrate utilization, indole production, Methyl Red-Vogues Proskauer (MRVP), blood haemolysis, casein hydrolysis, lecithinase production and fermentation tests using glucose, lactose, maltose, sucrose, mannitol, xylose, starch and glycerol.

RESULTS

Bacterial population: Greenish pigment producing colonies were obtained on the different agar media after 2-3 days of incubation. The bacterial population of the samples as assessed using Cetrimide agar, Kings B medium and Nutrient agar supplemented with chloramphenicol is shown in Table 1, 2 and 3, respectively. In Table 1 it can be seen that the proportion of bacterial population producing greenish pigments (PBG) as obtained using Cetrimide agar ranged from 31.71-100%, in Table 2 the PBG as obtained using Kings agar ranged from 1.37-3.92% and in Table 3 the PBG as obtained using Nutrient agar supplemented with Chloramphenicol (NC) ranged from 0.86-14.29%. The summary of the data used in calculation of one-way ANOVA ($p \leq 0.05$) of the PBG obtained using the different media and the results obtained from the calculation is presented in Table 4. From the Table 4, it can be seen that the F calculated was greater than the F tabulated. This implied that there was a significant difference between the PBG obtained using the different agar media. A comparison of the PBG obtained using the different media was presented in Fig. 1. From the Fig.1 it can be seen that the highest PBG was obtained using Cetrimide agar.

Identity of the greenish pigment producing bacteria: A total of fifteen greenish pigment producing isolates were selected from the three different media used, five from Cetrimide agar, five from Kings B agar and five from Nutrient agar supplemented with chloramphenicol. The isolates were coded according to the media used for their isolation as follows: CA1, CA2, CA3, CA4, CA5, KB1, KB2, KB3, KB4, KB5, NC1, NC2, NC3, NC4 and NC5. All the isolates were Gram-negative rods, motile and reacted positive to catalase, oxidase, citrate utilization, casein hydrolysis and lecithinase production tests (Table 5). They all produced beta-haemolysis on blood agar and were negative for indole, methyl red and Vogues-Proskauer tests. The isolates did not ferment lactose,

Table 1: Bacterial population of the investigated samples as assessed using cetrimide agar

Parameters	TBP	GPB	PBG (%)
R1 (CFU mL^{-1})	5.10×10^4	3.90×10^4	76.47
G1 (CFU mL^{-1})	4.10×10^5	1.30×10^5	31.71
S1 (CFU g^{-1})	1.07×10^4	1.07×10^4	100.00
S2 (CFU g^{-1})	3.60×10^4	3.40×10^4	94.44

TBP: Total bacterial population, GPB: Greenish pigment producing bacterial population, PBG: Proportion of bacterial population producing greenish pigment $\left(\frac{GPB\times100}{TBP}\right)$

Table 2: Bacterial population of the investigated samples as assessed using kings B medium

Parameters	TBP	GPB	PBG (%)
R1 (CFU mL^{-1})	7.30×10^5	1.00×10^4	1.37
G1 (CFU mL^{-1})	1.02×10^5	4.00×10^3	3.92
S1 (CFU g^{-1})	1.33×10^9	3.00×10^7	2.26
S2 (CFU g^{-1})	1.15×10^9	3.22×10^7	2.80

TBP: Total bacterial population, GPB: Greenish pigment producing bacterial population, PBG: Proportion of bacterial population producing greenish pigment $\left(\frac{GPB\times100}{TBP}\right)$

Table 3: Bacterial population of the investigated samples as assessed using nutrient agar supplemented with chloramphenicol (NC)

Parameters	TRP	GPB	PBG (%)
R1 (CFU mL^{-1})	1.40×10^3	2.0×10^2	14.29
G1 (CFU mL^{-1})	1.75×10^4	1.5×10^2	0.86
S1 (CFU g^{-1})	4.44×10^4	5.0×10^3	11.26
S2 (CFU g^{-1})	1.91×10^4	1.5×10^3	7.85

TRP: Total chloramphenicol resistant bacteria, GPB: Greenish pigment producing bacterial population, PBG: Proportion of bacterial population producing greenish pigment $\left(\frac{GPB\times100}{TBP}\right)$

Table 4: Summary of data and results obtained from the calculation of one-way ANOVA of the PBG obtained using the different media

Groups	Count	Sum	Average	Variance
Cetrimide	4	302.62	75.6550	959.127500
Kings B	4	10.45	2.6125	1.153425
NC	4	34.26	8.5650	33.305630

Results obtained from ANOVA

Source of variation	SS	df	MS	F calculated	p-value	F tabulated
Between groups	13162.28	2	6581.1380	19.8709	0.0005	4.2565
Within groups	2980.76	9	331.1955			
Total	16143.04	11				

PBG: Proportion of bacterial population producing greenish pigment

Table 5: Identification test results of the selected greenish pigment producing bacteria isolated using Cetrimide agar and Kings B medium, Nutrient agar supplemented with chloramphenicol

	CA1	CA2	CA3	CA4	CA5	KB1	KB2	KB3	KB4	KB5
Gm	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods	- rods
Ctl	+	+	+	+	+	+	+	+	+	+
Oxd	+	+	+	+	+	+	+	+	+	+
Mtl	+	+	+	+	+	+	+	+	+	+
CtU	+	+	+	+	+	+	+	+	+	+
Ind	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-	-
HBA	β-H	β-H	β-H	β-H	β-H	β-H	β-H	β-H	β-H	β-H
CsH	+	+	+	+	+	+	+	+	+	+
LcP	+	+	+	+	+	+	+	+	+	+
GluF	A	A	A	A	A	A	A	A	A	A
LtF	0	0	0	0	0	0	0	0	0	0
XsF	A	A	A	A	A	A	A	A	A	A
MalF	0	0	0	0	0	0	0	0	0	0
MntF	A	A	A	A	A	A	A	A	A	A
SucF	0	0	0	0	0	0	0	0	0	0
StaF	0	0	0	0	0	0	0	0	0	0
GlyF	A	A	A	A	A	A	A	A	A	A

For isolates obtained using Nutrient agar supplemented with chloramphenicol

	NC1	NC2	NC3	NC4	NC5
Gm	- rods	- rods	- rods	- rods	- rods
Ctl	+	+	+	+	+
Oxd	+	+	+	+	+
Mtl	+	+	+	+	+
CtU	+	+	+	+	+
Ind	-	-	-	-	-
VP	-	-	-	-	-
MR	-	-	-	-	-
HBA	β-H	β-H	β-H	β-H	β-H
CsH	+	+	+	+	+
LcP	+	+	+	+	+
GluF	A	0	A	A	A
LtF	0	0	0	0	0
XsF	A	0	0	0	0
MalF	0	0	0	0	0
MntF	A	0	A	A	A
SucF	0	0	0	0	0
StaF	A	0	0	A	0
GlyF	A	0	A	A	A

Gm: Gram staining reaction, Ctl: Catalase, Oxd: Oxidase, Mtl: Motility, CtU: Citrate utilization, Ind: Indole, VP: Vogues Proskauer, MR: Methyl red, HBA: Haemolysis on blood agar, β-H: Beta haemolysis, CsH: Casein hydrolysis, LcP: Lecithinase production, GluF: Glucose fermentation, LtF: Lactose fermentation, XsF: Xylose fermentation, MalF: Maltose fermentation, MntF: Mannitol fermentation, SucF: Sucrose fermentation, StaF: Starch fermentation, GlyF: Glycerol fermentation, A: Only acid produced, 0: No change

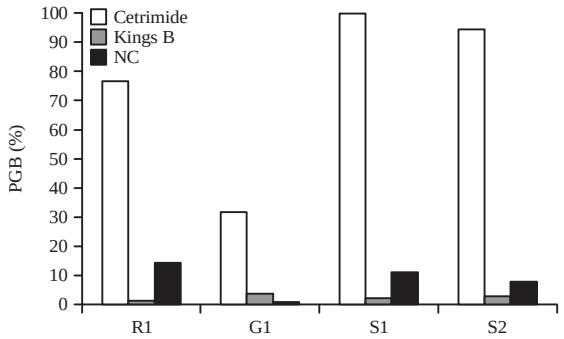

Fig. 1: Comparison of the PBG obtained using the different media

maltose and sucrose. However, 2 of the isolates produced only acid from starch fermentation, while 11-14 of the isolates produced only acid from fermentation of glucose, xylose, mannitol and glycerol.

DISCUSSION

The capacity of chemically defined media used in the acquisition of *Pseudomonas* species from samples in indicating if investigated samples contain greenish pigment producing *Pseudomonas* species is largely unknown. In this research, the potency of three media (Cetrimide agar, Kings B

agar and Nutrient agar supplemented with 50 μg mL^{-1} Chloramphenicol) that can be used in the selective isolation of greenish pigment producing *Pseudomonas* species was compared. The media were used to culture the same set of samples so as to determine which of the media had the capacity to highly encourage the growth of greenish pigment producing *Pseudomonas* present in the samples.

The data obtained from the study showed that the highest proportion of bacterial population producing greenish pigments (PBG) was obtained using Cetrimide agar, while the lowest PBG was obtained using Kings B agar. The PBG obtained using Nutrient agar supplemented with Chloramphenicol ranged from 0.86-14.29%. This range does not differ much from that obtained in the study of Peekate and Abu[21], who reported a PBG of 11.11-30.77%. The relatively low value obtained using Nutrient agar supplemented with Chloramphenicol could be attributed to the broad inhibitory nature of Chloramphenicol. Although a species of *Pseudomonas* (*P. aeruginosa*) has been shown to be intrinsically resistant to Chloramphenicol[28], obviously, a large population of the greenish pigment producing *Pseudomonas* population in the samples could not resist the inhibitory activity of the antibiotic against them. The few ones that survived were thus responding by producing their greenish pigment which may be tied to an antibiotic resistance mechanism.

In a related study, Cetrimide agar supplemented with Nalidixic acid has been shown to have a high sensitivity of about 87% in the detection of *P. aeruginosa* in freshwater, seawater and sewage samples[29]. This is in agreement with the high proportion (31.71-100%) of bacterial population producing greenish pigments obtained in this study using Cetrimide agar.

Greenish pigment producing bacteria are generally accepted to belong to the *Pseudomonas* genus, specifically it has been recorded that *P. aeruginosa* is the only organism of the glucose non-fermenting Gram-negative bacilli capable of producing bluish-green pigment[24]. It is also recorded that *P. aeruginosa* is the only known organism capable of producing pyocyanin (bluish-green pigment), other than a species of *Streptomyces* which produces a pigment that appears to be identical to pyocyanin. It should be noted that bacteria belonging to the *Streptomyces* genus are Gram-positive bacteria[30]. Thus, Gram-negative bacteria that produce greenish pigments can be initially identified to be species of *Pseudomonas*. The greenish pigment producing bacteria isolated in this study were all Gram-negative rods and reacted the same way that species of *Pseudomonas* react to the physicochemical/biochemical tests used in this study[31-34]. It can thus be established that all the greenish pigment producing bacteria isolated in this study belongs to the *Pseudomonas* genus.

In this research it has been shown that Cetrimide agar is a better medium than Kings B agar and Nutrient agar supplemented with 50 μg mL^{-1} Chloramphenicol in the selective enumeration and isolation of greenish pigment producing *Pseudomonas* species from environmental samples. Cetrimide agar is thus recommended for use in sourcing for *Pseudomonas* species from the environment.

CONCLUSION

Special media are used in culturing environmental samples or bio-specimen for the acquisition of *Pseudomonas*. The efficacy of these media in indicating if investigated samples contain *Pseudomonas* species has not been specified. In this research work it is shown that Cetrimide agar is a better medium than Kings B agar and Nutrient agar supplemented with Chloramphenicol in the selective enumeration and isolation of greenish pigment producing *Pseudomonas* species from environmental samples.

The spread plate method was used in this study in the enumeration of greenish pigment producing bacteria in the investigated samples. Small volumes (0.1 mL) of the diluted samples were thus plated and the probability of obtaining the required isolates, especially with the use of media containing Chloramphenicol, was thus low. It is suggested that the pour plate method, where a fairly large inoculum volume of 1 mL is required, be used in future researches related to this kind of study so as to increase the probability of obtaining the isolates been sourced for.

SIGNIFICANCE STATEMENT

Cultures of *Pseudomonas* species are required for research and application in agriculture, industries and bioremediation. Special media are used for obtaining these bacteria from environmental and biological samples. Some of these media, Cetrimide agar has been shown in this research study to be a better medium for isolation of *Pseudomonas* species.

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