



OPTIMIZED NUTRIENT-CHLORAMPHENICOL AGAR MEDIUM FOR SELECTIVE ISOLATION OF PSEUDOMONAS FLUORESCENS

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Abstract: Commercially available medium for selective isolation of *Pseudomonas* are relatively expensive. Incorporation of chloramphenicol into nutrient agar has been used in the selective isolation of *Pseudomonas fluorescens*. The aim of this study was to apply optimization in modification of nutrient-chloramphenicol agar medium (NCAM) so as to optimize it for selective isolation of *Pseudomonas* species. Water sample determined to contain fluorescent greenish-pigment producing bacteria (FGPB) was used for the optimization study. Medium component selected for the optimization process were pH, NaCl concentration (NC), and chloramphenicol concentration (CC), with value ranges of 7.0 – 8.2, 0.5 – 1.0 %, and 40 – 60 µg/ml, respectively. Fifteen media variations were derived from combination of these ranges using Box-Behnken design matrix, and were used in culturing the water sample for isolation of FGPB. Relationship between obtained proportions of bacterial populations that are FGPB and chosen media components were calculated using polynomial equation for three factors design. Prediction profiles derived from the equation showed that the highest proportion of bacterial populations that are FGPB was achievable at pH, NC, and CC combination values of 7.0, 0.2 %, and 60 µg/ml, respectively. NCAM with this optimized combination increased the efficiency of selectively isolating FGPB from the water sample from 0.008 to 0.011 %. Physicochemical/biochemical testing showed that the FGPB isolates were *Pseudomonas fluorescens*. It is concluded that NCAM with pH 7.0, NaCl concentration of 0.2 %, and chloramphenicol concentration of 60 µg/ml can be used for selective isolation of *Pseudomonas fluorescens* with optimum efficiency.

Keywords: Box-Behnken design; pH; NaCl; Chloramphenicol; *Pseudomonas*

1. Introduction

Some species of *Pseudomonas* including *P. aeruginosa*, *P. fluorescens*, and *P. putida* are relevant in bioremediation of polluted environments, control of fungal pathogens of plants, and production of certain substances for domestic and industrial application. *P. aeruginosa* is able to produce bio-active substances such as biosurfactants (Mukherjee & Das, 2010; Bustamante et al., 2012), and can degrade various organic substances including hydrocarbons (Ningthoujam & Shovarani, 2008; Singh & Walker, 2006; Ikhimiukor & Nneji, 2013); making it useful in some industries and in bioremediation of polluted environments. *P. fluorescens* is able to degrade various pollutants

including herbicides, hydrocarbons, and phenol (Garbiet al., 2006; Mahiuddinet al., 2012; Monekeet al., 2010; Vasudevanet al., 2007), and produce active compounds that portray antagonistic activity against some pathogens of plants

(Anbuselviet al., 2010; Haas et al., 1991). Thus *P. fluorescens* is a potential candidate in bioremediation of polluted environment, and in control of pathogens of plants. *P. fluorescens* also produce biosurfactants and antibiotics including Mupirocin, Pyrrolnitrin, and Pyoluteorin (Abouseoudet al., 2007; Sarniguet et al., 1995; Gao et al., 2014). *P. putida* has the ability to degrade many aliphatic and aromatic hydrocarbons, toxic pollutants such as styrene, herbicides, toxins, and organic solvents (Marqués & Ramos, 1993; Otenio et al., 2005; Tan et al., 2015), and is therefore also relevant in bioremediation of polluted environments.

An attribute of the principal species of *Pseudomonas*, which include *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. syringae*, is the production of diffusible greenish pigments in culture media containing certain substances (Boopathi & Rao, 1999; Daly et al., 1984; Scales et al., 2014; Stanier et al., 1977). The pigments usually fluoresce under ultraviolet (UV) light. Therefore, colonies of the principal species of *Pseudomonas* growing in the midst of colonies of other bacteria on translucent agar plate can be detected in daylight and in the dark under UV light. Production of greenish pigment that fluoresces under UV light can thus be relied upon as a guide in the isolation of any of the principal species of *Pseudomonas*.

Different culture media are available in the market for the isolation of greenish pigment producing *Pseudomonas* spp. They include cetrimide agar, King's B medium, *Pseudomonas* agar P, *Pseudomonas* agar F, asparagine broth enriched with K_2HPO_4 and $MgSO_4 \cdot 7H_2O$, and *Pseudomonas* CN selective agar (Al-Hina et al., 2010; Laine et al., 2009; Oliveira et al., 2008; Peekate et al., 2018). These media are relatively expensive and not readily available to researchers working in underfunded research institutions.

A substance which appears to instigate pigment production in *Pseudomonas* is the antibiotic chloramphenicol (Peekate & Abu, 2015; Peekate & Abu, 2017a). *P. aeruginosa*, *P. fluorescens*, and some strains of *P. putida* are resistant to some antibiotics including chloramphenicol; and are able to grow in the presence of chloramphenicol, nalidixic acid, and tetracycline (Blanco et al., 2016; Darak & Barde, 2015; Fernández et al., 2012; Livermore, 2002; Morita et al., 2014; Peekate & Abu, 2015; Strateva & Yordanov, 2009). Incorporation of chloramphenicol into nutrient agar, a relatively inexpensive general purpose culture medium, has been used in the selective isolation of *P. fluorescens* (Peekate & Abu, 2017a). There is however a need to elucidate the precise chloramphenicol concentration, and values of other growth medium parameters so as to optimize such medium for selective isolation of *Pseudomonas* species. Therefore, the aim of this research was to apply optimization models in modification of nutrient-chloramphenicol agar medium so as to optimize it for the selective isolation of *Pseudomonas* species.

2. Materials and Methods

2.1 Sample collection and analysis

Water sample (500 ml) was collected from a temporary stagnant pool of water near the Microbiology laboratory in the Rivers State University, Port Harcourt, Nigeria. Some quantity of the sample was subjected to analysis for bacterial population, while the remaining was stored at 4 °C in a refrigerator for subsequent use in optimization modelling. Bacterial population analyzed for was total heterotrophic bacteria (THB) and fluorescent greenish pigment producing *Pseudomonas* species (FGPs). Determination of THB and FGPs populations were achieved through 10-fold serial dilution and inoculation on plates of nutrient agar and nutrient agar incorporated with chloramphenicol (50 µg/ml), respectively. Inoculated plates were incubated at ambient temperatures (27 – 32 °C) for 2 days. After incubation, colonies on the nutrient agar plates were counted and used to calculate the THB

population. Greenish-pigment producing colonies on the plates of nutrient agar incorporated with chloramphenicol, which fluoresced under UV light in the dark, were suspected as FGPs and their counts used to calculate the FGPs population. UV illumination was provided using a handheld UV torch in a dark room.

2.2 Medium component selected for the optimization process

Medium components chosen for the optimization process include pH and NaCl concentration, in addition to chloramphenicol concentration which served as the pigment-production instigation agent. The value ranges of the medium parameters chosen for the optimization process, based on Box-Behnken design are presented in Table 1 (Design adapted from Zhang & Dequan, 2013). Different combinations of the ranges of the medium parameters using the Box-Behnken experimental design matrix are presented in Table 2 (Design adapted from Kumar *et al.*, 2015).

Table 1: Value range selection of medium parameters for the optimization experiment

Medium parameters	Coded Levels		
	-1	0	+1
pH	7.0	7.6	8.2
NC (%)	0.5	0.75	1.0
CC (µg/ml)	40	50	60

NC: NaCl concentration, CC: chloramphenicol concentration.

Table 2: Combination of ranges of medium parameters for experimentation

CN	pH (X ₁)	NC (X ₂)	CC (X ₃)
- 1	(7.0)	- 1 (0.5)	0 (50)
- 1	(7.0)	+ 1 (1.0)	0 (50)
+ 1	(8.2)	- 1 (0.5)	0 (50)
+ 1	(8.2)	+ 1 (1.0)	0 (50)
- 1	(7.0)	0 (0.75)	- 1 (40)
- 1	(7.0)	0 (0.75)	+ 1 (60)
+ 1	(8.2)	0 (0.75)	- 1 (40)
+ 1	(8.2)	0 (0.75)	+ 1 (60)
0	(7.6)	- 1 (0.5)	- 1 (40)
0	(7.6)	- 1 (0.5)	+ 1 (60)
0	(7.6)	+ 1 (1.0)	- 1 (40)
0	(7.6)	+ 1 (1.0)	+ 1 (60)
0	(7.6)	0 (0.75)	0 (50)
0	(7.6)	0 (0.75)	0 (50)
0	(7.6)	0 (0.75)	0 (50)

CN: Combination number, NC: NaCl concentration (%), CC: chloramphenicol concentration (µg/ml).

2.3 Agar medium preparation for the combination numbers

Chloramphenicol stock solution of 1000 µg/ml was prepared by transferring 0.2g of powdered chloramphenicol into sterile 200 ml sterile distilled water in a sterile conical flask. The volumes of the chloramphenicol stock solution to be added to the different medium of the various combination numbers (Table 2) so as to achieved the specified chloramphenicol concentrations were calculated using equation 1 (Eq. 1). The equation is derived from the equation $M_1V_1 = M_2V_2$ (Manillaet *al.*, 2001).

$$1000 \mu\text{g/ml} \times VCH_x = CC_x \times 75 \text{ ml} \dots \dots \dots \text{(Eq. 1)}$$

Where VCH_x is the volume of the chloramphenicol stock solution required for combination number x , CC_x the chloramphenicol concentration specified for combination number x , x is any of the combination numbers, and 75 ml is the targeted agar medium volume. Therefore, the volumes of the chloramphenicol stock solution required for combination numbers specified for 40, 50, and 60 µg/ml chloramphenicol concentrations were calculated to be 3, 3.75, and 4.5 ml respectively.

Four agar plates of modified nutrient agar medium were prepared for each combination number. Agar medium volume of 75 ml was targeted for each combination number based on the observation that about 18 ml medium is required to completely cover the bottom plate of the size of Petri dish used (18 ml per plate $\times$ 4 plates = 72 ml). For each combination number, 2.1 g nutrient agar which is required for preparation of 75 ml of nutrient agar medium was added to 60 ml distilled water in a beaker. Next, NaCl was added. However, NaCl was not added to the medium for combination numbers specified for 0.5 % NaCl because the Nutrient agar (Himedia, India) used contained 0.5% NaCl. The quantities of NaCl added to agar media for the other combination numbers were worked out using equation 2 (Eq. 2).

$$QNC_x \text{ (g)} = \frac{NC_x - 0.5 \text{ g}}{100 \text{ ml}} \times 75 \text{ ml} \dots \dots \dots \text{(Eq. 2)}$$

Where QNC_x is the quantity of NaCl to be added to medium for combination number x , NC_x is the specified NaCl concentration for combination number x , and x is any of the following combination numbers: 2, 4, 5 – 8, and 11 – 15.

Next the pH of the resulting media for the combination numbers were adjusted to the specified pH using 0.4 M NaOH and 0.01 M H_2SO_4 . The media were then transferred into

100ml measuring cylinders, and distilled water was added so as to achieve media volumes of 72, 71.25, and 70.5 ml for combination numbers specified for 40, 50, and 60 µg/ml chloramphenicol concentrations respectively. The media were transferred into appropriately labelled 150 ml conical flask, and sterilized in an Autoclave at 121 °C for 15 minutes. After sterilization, the flasks were allowed to cool to about 50 °C then the calculated volumes of the chloramphenicol stock solution to be added to the agar medium of the different combination numbers were added. After addition of the stock solution, the media were poured into appropriately labelled sterile Petri plates. The agar media were allowed to harden, and then dried in a hot air oven set at 50 °C.

2.4 Optimization experiment

The water sample collected earlier was used for the optimization experiment. About 0.1 ml of the sample and its 10^{-1} dilution were spread plated separately on the agar medium plates for the different combination numbers in duplicates. The plates were incubated at ambient temperatures (27 – 32 °C) for 2 days. After incubation, ensuing colonies including greenish pigment producing colonies were counted. Greenish-pigment producing colonies that fluoresced under UV light in the dark were also counted. The total colonial counts and counts of greenish-pigment producing colonies that fluoresced under UV light were used to obtain the proportions of THB suspected to be

$$\beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{12}$$

FGP. The proportions for the different combination numbers were then fitted into a regression model corresponding to the generalized polynomial equation (Eq. 3) for three factors design (Kumar *et al.*, 2015).

$$Y = \beta_0 + \beta_1 X_1 + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2 + \dots \quad (\text{Eq. 3})$$

Where Y is the predicted response; X_1 , X_2 , and X_3 represent the values for the three medium parameters (pH, NaCl concentration, and chloramphenicol concentration); β_0 is the value of fitted response at the center point of the design; β_1 , β_2 , and β_3 are the linear coefficients; $\beta_{1,2}$, $\beta_{1,3}$, and $\beta_{2,3}$ are the interaction coefficients; and $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ are the quadratic coefficients.

Matrices were applied, with the aid of Microsoft excel®, in resolving the regression model generated from Eq. 3. The polynomial equation derived from the resolved regression model was used in generating prediction profiles. From the prediction profiles the combination of values of the medium parameters that will lead to the highest proportion of THB that are FGPs was determined and used in preparation of the optimized agar medium.

2.5 Statistical analysis of generated polynomial equation

The analysis of variance (ANOVA) was used in determining if one or more of the coefficients in the generated polynomial equation is/are significant; therefore, indicating the probability of a relationship existing between the responses and one or more of the medium parameters that were modified.

2.6 Enumeration of bacterial population using the optimized agar medium

Agar plates of the optimized medium were used in enumeration of bacterial population in the water sample collected from the stagnant pool near the Microbiology laboratory. The result obtained was compared with results obtained using un-optimized medium.

2.7 Identification of fluorescent greenish-pigment-producing bacteria

Some fluorescent greenish-pigment-producing colonies on culture plates of the optimized medium were isolated onto sterile nutrient agar plates, and their stock cultures prepared. The isolates were subjected to Gram staining & microscopic examination, and the following physicochemical/biochemical tests as described in Peekate (2022): catalase test, oxidase test, motility test, citrate utilization test, indole production test, Methyl red tests, Vogues Proskauer test, haemolysis test, casein hydrolysis test, lecithinase production test, lipase production test, and fermentation tests using glucose, lactose, mannitol, xylose, and glycerol.

3. Results

3.1 Bacterial populations in collected water sample

The populations of total heterotrophic bacteria (THB) and suspected fluorescent greenish pigment-producing *Pseudomonas* species (FGPs) in the collected water sample were $2.2 \pm 0.6 \times 10^6$ and $1.7 \pm 0.3 \times 10^2$ CFU/ml respectively. This culminates into a proportion of 0.008 % THB that are suspected FGPs.

3.2 Bacterial populations as assessed using media of different combination numbers The bacterial populations in the water sample as assessed using media of the different combination numbers are presented in Table 3. In the Table, it can be observed that the proportions of THB that are suspected to be FGPs in the water sample as assessed using media of the different combination numbers range from 0 – 0.0068 %.

3.3 Polynomial equation generated from the optimization experiment

Fitting of the proportions of THB that were suspected to be FGPs as assessed using media of the different combination numbers and the value ranges of the chosen medium parameters into the regression model (Eq. 3) is presented in Table 4. Resolving the values in Table 4 using the matrix equation $\hat{\beta} = (X^T X)^{-1} X^T Y$ (Draper & Smith,

1998), the coefficients $\beta_0, \beta_1, \beta_2, \beta_3, \beta_{1,2}, \beta_{1,3}, \beta_{2,3}, \beta_{1,1}, \beta_{2,2},$ and $\beta_{3,3}$ in Eq. 3 for the combinations of value ranges of

the chosen parameters were deduced to be 0.1802, - 0.0425, - 0.0776, 0.0008, 0.0113, - 0.00004, - 0.0002, 0.0023, - 0.0018, and - 0.000004, respectively. Therefore, the polynomial equation from the resolved model is as follows:

$$Y = 0.1802 - 0.0425 X_1 - 0.0776 X_2 + 0.0008 X_3 + 0.0113 X_1 X_2 - 0.00004 X_1 X_3 - 0.0002 X_2 X_3 + 0.0023 X_1^2 - 0.0018 X_2^2 - 0.000004 X_3^2 \dots \dots \dots \text{(Eq. 4)}$$

Eq. 4 was used in generating prediction profiles which were used in determining the optimized combination of pH, NaCl concentration, and Chloramphenicol concentration for instigating pigment production in FGPs.

3.4 Statistical significance of the generated polynomial equation

A summary of the ANOVA of the polynomial equation derived from the regression model is presented in Table 5. In the Table, it can be seen that calculated F statistics is greater than tabulated F statistics. This indicates that at least one of the coefficients ($\beta_0, \beta_1, \beta_2, \beta_3,$

$\beta_{1,2}, \beta_{1,3}, \beta_{2,3}, \beta_{1,1}, \beta_{2,2},$ and $\beta_{3,3}$) Of the derived polynomial equation is significant. This

means that a regression model exists between proportion of THB that are FGPs and at least one of pH, NaCl concentration, and chloramphenicol concentration.

Table 3: Bacterial populations as assessed using media of the different combination numbers

CN	THB CFU/ml	CRB CFU/ml	FGB CFU/ml	PTF (%)
1	$2.2 \pm 0.6 \times 10^6$	$1.75 \pm 0.08 \times 10^3$	$1.50 \pm 0.35 \times 10^2$	0.0068
2	$2.2 \pm 0.6 \times 10^6$	$8.75 \pm 0.74 \times 10^3$	0	0
3	$2.2 \pm 0.6 \times 10^6$	$3.35 \pm 0.32 \times 10^3$	0	0
4	$2.2 \pm 0.6 \times 10^6$	$3.75 \pm 0.25 \times 10^3$	0	0
5	$2.2 \pm 0.6 \times 10^6$	$2.19 \pm 0.18 \times 10^4$	$5.0 \pm 1.4 \times 10$	0.0023
6	$2.2 \pm 0.6 \times 10^6$	$2.00 \pm 0.13 \times 10^2$	$2.0 \pm 0.7 \times 10$	0.0009
7	$2.2 \pm 0.6 \times 10^6$	$1.66 \pm 0.07 \times 10^4$	$5.0 \pm 0.7 \times 10$	0.0023
8	$2.2 \pm 0.6 \times 10^6$	$6.50 \pm 0.28 \times 10^2$	0	0
9	$2.2 \pm 0.6 \times 10^6$	$1.23 \pm 0.06 \times 10^4$	0	0
10	$2.2 \pm 0.6 \times 10^6$	$1.98 \pm 0.08 \times 10^2$	$1.5 \pm 0.4 \times 10$	0.0007
11	$2.2 \pm 0.6 \times 10^6$	$3.35 \pm 0.32 \times 10^4$	$2.5 \pm 0.4 \times 10$	0.0011
12	$2.2 \pm 0.6 \times 10^6$	$1.40 \pm 0.03 \times 10^2$	0	0
13	$2.2 \pm 0.6 \times 10^6$	$4.30 \pm 0.35 \times 10^3$	0	0
14	$2.2 \pm 0.6 \times 10^6$	$3.45 \pm 0.39 \times 10^3$	$5.0 \pm 0.7 \times 10$	0.0023

15 $2.2 \pm 0.6 \times 10^6$ $2.00 \pm 0.35 \times 10^3$ $1.5 \pm 0.4 \times 10$ 0.0007

CN: Combination number, THB: Population of total heterotrophic bacteria, CRB: Population of chloramphenicol resistant bacteria, FGB: Population of fluorescent greenish-pigment producing bacteria, PTF: proportion of THB suspected to be fluorescent greenish-pigment producing *Pseudomonas* species ($\frac{\text{FGB}}{\text{THB}} \times 100$)

Table 4: Responses from combinations of value ranges of chosen medium parameters

N	pH	NC	CC							PTF (%)
	X_1	X_2	X_3	$X_1 X_2$	$X_1 X_3$	$X_2 X_3$	X_{12}	X_{22}	X_{32}	Y
1	7	0.5	50	3.5	350	25	49	0.25	2500	0.0068
1	7	1	50	7	350	50	49	1	2500	0
1	8.2	0.5	50	4.1	410	25	67.24	0.25	2500	0
1	8.2	1	50	8.2	410	50	67.24	1	2500	0
1	7	0.75	40	5.25	280	30	49	0.5625	1600	0.0023
1	7	0.75	60	5.25	420	45	49	0.5625	3600	0.0009
1	8.2	0.75	40	6.15	328	30	67.24	0.5625	1600	0.0023
1	8.2	0.75	60	6.15	492	45	67.24	0.5625	3600	0
1	7.6	0.5	40	3.8	304	20	57.76	0.25	1600	0
1	7.6	0.5	60	3.8	456	30	57.76	0.25	3600	0.0007
1	7.6	1	40	7.6	304	40	57.76	1	1600	0.0011
1	7.6	1	60	7.6	456	60	57.76	1	3600	0
1	7.6	0.75	50	5.7	380	37.5	57.76	0.5625	2500	0
1	7.6	0.75	50	5.7	380	37.5	57.76	0.5625	2500	0.0023
1	7.6	0.75	50	5.7	380	37.5	57.76	0.5625	2500	0.0007

[X]: design matrix of the polynomial model, Y: responses of the model, PTF (Y): proportion of THB suspected to be fluorescent greenish-pigment producing *Pseudomonas* species.

Table 5: Summary of the ANOVA of the derived polynomial equation

SOV	DOF	MS	F _c	F _t	α	SOS
		0.0000102	7.29	2.66	0.1	Regression $\equiv k$
	3	0.0000306				
Error $\equiv n-(k+1)$	11	0.000015	0.0000014			
Total	14					

SOV: Source of variation, DOF: Degree of freedom, SOS: Sum of squares, MS: Mean squares, F_c: calculated F statistic = MS(Regression)/MS(Error), F_t: tabulated F statistic.

3.5 Prediction profiles and optimized combination

The prediction profiles generated from Eq. 4 for chloramphenicol concentrations of 20 – 100 µg/ml and NaCl concentrations of 0.2 – 1.0 %, at pH 7.0, 7.6, and 8.2 is presented in Table 6. From the prediction profiles (Table 6), it can be seen that the highest proportion (0.01 %) of THB that are FGPs can be achievable with the use of

modified nutrient agar medium having the following combination: pH – 7.0, NaCl concentration – 0.2 %, chloramphenicol concentration – 60 µg/ml.

Table 6: Prediction profiles for % of THB producing fluorescent greenish pigment from combined concentrations of chloramphenicol (CC) and NaCl

X1 (pH)	X2 (NaCl)	X3 (CC)				
		20	40	60	80	100
7.0	0.2	0.0036	0.0084	<u>0.0100</u>	0.0084	0.0036
	0.4	0.0029	0.0069	0.0077	0.0053	-0.0003
	0.6	0.0021	0.0053	0.0053	0.0021	-0.0044
	0.8	0.0010	0.0034	0.0026	-0.0014	-0.0086
	1.0	-0.0001	0.0015	-0.0001	-0.0049	-0.0129
7.6	0.2	-0.0009	0.0035	<u>0.0046</u>	0.0025	-0.0028
	0.4	-0.0002	0.0033	0.0036	0.0008	-0.0053
	0.6	0.0003	0.0030	0.0025	-0.0012	-0.0080
	0.8	0.0006	0.0026	0.0013	-0.0032	-0.0109
	1.0	0.0008	0.0020	-0.0001	-0.0054	-0.0139
8.2	0.2	-0.0037	0.0002	0.0008	-0.0018	-0.0075
	0.4	-0.0017	0.0014	0.0012	-0.0022	-0.0087
	0.6	0.0002	0.0024	0.0015	-0.0027	-0.0101
	0.8	0.0019	0.0033	0.0016	-0.0034	-0.0116
	1.0	0.0035	<u>0.0041</u>	0.0015	-0.0042	-0.0132

3.6 Bacterial populations in the water sample as assessed using optimized agar medium

Populations of THB in the water sample from the stagnant pool as assessed using nutrient agar, and fluorescent greenish pigment producing bacteria as assessed using un-optimized nutrient-chloramphenicol agar and the optimized nutrient-chloramphenicol agar are presented in Table 7. In the Table, it can be seen that the proportion (0.011 %) of THB that are fluorescent greenish-pigment producing bacteria as obtained using the optimized medium is greater than the proportion (0.008 %) of THB that are fluorescent greenish-pigment producing bacteria as obtained using the un-optimized medium.

Table 7: Bacterial populations in the water sample as assessed using nutrient agar, unoptimized and optimized nutrient-chloramphenicol agar

THB	FGP(U)	FGP(O)	U.PTF	O.PTF
(CFU/ml)	(CFU/ml)	(CFU/ml)	(%)	(%)
$2.2 \pm 0.6 \times 10^6$	$1.7 \pm 0.3 \times 10^2$	$2.5 \pm 0.3 \times 10^2$	0.008	0.0114

THB: total heterotrophic bacteria, FGP(U): fluorescent greenish-pigment producing colonial count (FGPCC) obtained using un-optimized medium, FGP(O): FGPCC obtained using optimized medium, U.PFF: proportion of THB that are fluorescent greenish-pigment producing bacteria(PTFGB) as obtained using un-optimized medium, O.PFF: PTFGB as obtained using optimized medium.

3.7 Identity of fluorescent greenish-pigment producing bacteria

The isolated fluorescent greenish-pigment-producing bacteria were all Gram negative rods, and reacted alike to the tests used as follows: catalase positive, oxidase positive, positive motility, positive for citrate utilization, negative for indole production, Methyl red negative, Vogues-Proskauer negative, positive for beta-haemolysis, positive for casein hydrolysis, lecithinase positive, lipase positive, acid production from glucose fermentation, negative for lactose fermentation, negative for mannitol fermentation, negative for xylose fermentation, and positive for glycerol fermentation. This results pattern is similar to the physicochemical/biochemical tests results of *Pseudomonas fluorescens* as cited in Peekate (2022). Therefore, all the isolated fluorescent greenish-pigment-producing bacteria are suspected to be *P. fluorescens*.

4. Discussion

Species of *Pseudomonas* are often required by researchers due to their importance in research and application. Media available for selective isolation of *Pseudomonas* are relatively expensive for researchers working in underfunded institutions. The need to modify readily available and relatively inexpensive medium for selective isolation of *Pseudomonas* thus arises. Selective isolation of *Pseudomonas* is made possible by the ability of some of its species to produce fluorescent greenish pigments in the presence of certain substances. Such substances include the antibiotic chloramphenicol (Peekate & Abu, 2015; Peekate & Abu, 2017a). Therefore, modification of nutrient agar, a relatively inexpensive medium, through addition of chloramphenicol presents a cost-effective means for selective isolation of *Pseudomonas*. However, such medium modification needs to be optimized for efficient selective isolation of the target organism, in this case *Pseudomonas*.

In this study, application of optimization models revealed the pH value, NaCl concentration, and chloramphenicol concentration for compounding nutrient-chloramphenicol agar medium for optimum production of pigment by *Pseudomonas fluorescens*, thereby ensuring efficient selective isolation of the organism. In related studies (Biniarzet *et al.*, 2018; Kalaiarasi & Sunitha, 2009; Peekate & Abu, 2017b), application of optimization models have been shown to elucidate medium parameter values, medium components, and culture conditions for efficient production of certain substances including biosurfactants and proteins by *Pseudomonas fluorescens*.

Selective isolation of fluorescent greenish-pigment producing *Pseudomonas* species (FGPs) was achieved in this study, with increase from 0.008 % for the un-optimized medium (nutrient-chloramphenicol agar medium with pH 7.0, 0.5 % NaCl concentration, and 50 µg/ml chloramphenicol concentration) to 0.011 % efficiency for the optimized medium (nutrient-chloramphenicol agar medium with pH 7.0, 0.2 % NaCl concentration, and 60 µg/ml chloramphenicol concentration). In a previous study (Peekate & Abu, 2017a), FGPs was isolated using un-optimized nutrient agar medium incorporated with 50 µg/ml chloramphenicol with efficiency of about 0.0002 %. Application of optimization models therefore improved the modification of nutrient-chloramphenicol agar for efficient selective isolation of FGPs.

5. Conclusion

Fluorescent greenish-pigment production by some species of *Pseudomonas* allows for its selective isolation. The production of the pigment can be enhanced in the presence of chloramphenicol. In this study, optimization models were applied in compounding nutrient chloramphenicol agar medium for efficient selective isolation of *Pseudomonas fluorescens*. Medium parameters selected for the optimization process were pH, NaCl

concentration, and chloramphenicol concentration. Values of these parameters that allowed for efficient selective isolation of *Pseudomonas fluorescens* as determined through optimization models were as follows: pH – 7.0, NaCl concentration – 0.2 %, chloramphenicol concentration – 60 µg/ml. Therefore, nutrient-chloramphenicol agar medium with pH 7.0, NaCl concentration of 0.2 %, and chloramphenicol concentration of 60 µg/ml can be used for selective isolation of *Pseudomonas fluorescens* with optimum efficiency.

Disclosure of conflict of interest There are no conflicts of interest.

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