Application of Optimization Models in Modification of Nutrient-Chloramphenicol Agar Medium for Use in Selective Isolation of *Pseudomonasfluorescens*

Atemie, Wisdom Dawoye and Peekate, Lekiah Pedro*
Rivers State University, Faculty of Science, Department of Microbiology, P.M.B. 5080, Port Harcourt, Nigeria
*Corresponding author E-mail: lekia.peekate@ust.edu.ng peekatepedro@gmail.com

DOI: 10.56201/jbgr.v9.no1.2023.pg54.66

Abstract
Commercially available medium for selective isolation of Pseudomonas are relatively expensive. Incorporation of chloramphenicol into nutrient agarhas been used in the selective isolation of Pseudomonasfluorescens. Theaim 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 samplefrom 0.008 to 0.011 %. Physicochemical/biochemical testing showed that the FGPBisolates 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 (Garbi*et al.*, 2006; Mahiuddin*et al.*, 2012; Moneke*et al.*, 2010;Vasudevan*et al.*, 2007), and produce active compounds that portray antagonistic activity against some pathogens of plants (Anbuselvi*et 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(Abouseoud*et 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 pigmentsin 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* growingin the midst of colonies of other bacteria on translucent agar plate can be detected in daylight andin 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 $MgSO_4.7H_2O$, and Pseudomonas CN selective agar (Al-Hinai*et al.*, 2010; Laine*et al*, 2009; Oliveira *et al.*, 2008; Peekate*et al.*, 2018). These media are relatively expensiveand 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-pigmentproducing *Pseudomonas* species (FGPs). Determination of THB and FGPs populations were achievedthrough 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 FGPsand 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 ofmediumparameters 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	- 1 (7.0)	- 1 (0.5)	0 (50)
2	- 1 (7.0)	+ 1 (1.0)	0 (50)
3	+ 1 (8.2)	- 1 (0.5)	0 (50)
4	+ 1 (8.2)	+ 1 (1.0)	0 (50)
5	- 1 (7.0)	0 (0.75)	- 1 (40)
6	- 1 (7.0)	0 (0.75)	+ 1 (60)
7	+ 1 (8.2)	0 (0.75)	- 1 (40)
8	+ 1 (8.2)	0 (0.75)	+ 1 (60)
9	0 (7.6)	- 1 (0.5)	- 1 (40)
10	0 (7.6)	- 1 (0.5)	+ 1 (60)
11	0 (7.6)	+ 1 (1.0)	- 1 (40)
12	0 (7.6)	+ 1 (1.0)	+ 1 (60)
13	0 (7.6)	0 (0.75)	0 (50)
14	0 (7.6)	0 (0.75)	0 (50)
15	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 thecombination 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 $M_1V_2 = M_2V_2$ (Manilla*et al.*, 2001).

$1000\ \mu g/ml \times VCH_x = CC_x \times 75\ ml$ … … … … … … … (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 numberbased on the observation thatabout 18 ml medium is required to completely cover the bottom plate of the size of Petri dish used (18 ml per plate × 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 % NaClbecause the Nutrient agar (Himedia, India) used contained 0.5% NaCl. The quantities of NaCl added to agar media forthe other combination numbers were worked out using equation 2 (Eq. 2).

$QNC_x\ (g) = \frac{NC_x - 0.5\ g}{100\ ml} \times 75\ ml$ … … … … … … … (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 differentcombination 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 FGPs. 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).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{1,2} X_1 X_2 + \beta_{1,3} X_1 X_3 + \beta_{2,3} X_2 X_3 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2 \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \text{(Eq. 3)}$$

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 centre 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 FGPswas 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

Somefluorescent greenish-pigment-producing colonies on culture plates of the optimized medium were isolated unto 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±0.6 × 10^6 and 1.7±0.3 × 10^2CFU/ml respectively. This culminates into a proportion of 0.008 % THB that are suspected FGPs.

3.2 Bacterial populationsas 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 numbersrange 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 β_0 , β_1 , β_2 , β_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:

$$Y = 0.1802 - 0.0425X_1 - 0.0776X_2 + 0.0008X_3 + 0.0113X_1X_2 - 0.00004X_1X_3 - 0.0002X_2X_3 + 0.0023X_1^2 - 0.0018X_2^2 - 0.000004X_3^2 \quad \ldots \quad \ldots \quad \ldots \text{(Eq. 4)}$$

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 ANOVAof 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 thecoefficients(β_0 , β_1 , β_2 , β_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	$2.2\pm0.6 \times 10^6$	$1.75\pm0.08 \times 10^3$	$1.50\pm0.35 \times 10^2$	0.0068
2	$2.2\pm0.6 \times 10^6$	$8.75\pm0.74 \times 10^3$	0	0
3	$2.2\pm0.6 \times 10^6$	$3.35\pm0.32 \times 10^3$	0	0
4	$2.2\pm0.6 \times 10^6$	$3.75\pm0.25 \times 10^3$	0	0
5	$2.2\pm0.6 \times 10^6$	$2.19\pm0.18 \times 10^4$	$5.0\pm1.4 \times 10$	0.0023
6	$2.2\pm0.6 \times 10^6$	$2.00\pm0.13 \times 10^2$	$2.0\pm0.7 \times 10$	0.0009
7	$2.2\pm0.6 \times 10^6$	$1.66\pm0.07 \times 10^4$	$5.0\pm0.7 \times 10$	0.0023
8	$2.2\pm0.6 \times 10^6$	$6.50\pm0.28 \times 10^2$	0	0
9	$2.2\pm0.6 \times 10^6$	$1.23\pm0.06 \times 10^4$	0	0
10	$2.2\pm0.6 \times 10^6$	$1.98\pm0.08 \times 10^2$	$1.5\pm0.4 \times 10$	0.0007
11	$2.2\pm0.6 \times 10^6$	$3.35\pm0.32 \times 10^4$	$2.5\pm0.4 \times 10$	0.0011
12	$2.2\pm0.6 \times 10^6$	$1.40\pm0.03 \times 10^2$	0	0
13	$2.2\pm0.6 \times 10^6$	$4.30\pm0.35 \times 10^3$	0	0
14	$2.2\pm0.6 \times 10^6$	$3.45\pm0.39 \times 10^3$	$5.0\pm0.7 \times 10$	0.0023
15	$2.2\pm0.6 \times 10^6$	$2.00\pm0.35 \times 10^3$	$1.5\pm0.4 \times 10$	0.0007

CN: Combination number, THB: Population of total heterotrophic bacteria, CRB: Population of chloramphenicolresistant 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

	.pH	**NC**	**CC**							**PTF (%)**
N	X_1	X_2	X_3	X_1X_2	X_1X_3	X_2X_3	X_1^2	X_2^2	X_3^2	
[X]										***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**	**SOS**	**MS**	**F_c**	**F_t**	α
Regression ≡ *k*	3	0.0000306	0.0000102	7.29	2.66	0.1
Error ≡ 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	0.0100	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	0.0046	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	0.0041	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 bacteriaas 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, un-optimized and optimized nutrient-chloramphenicol agar

THB (CFU/ml)	**FGP(U)** (CFU/ml)	**FGP(O)** (CFU/ml)	**U.PTF** (%)	**O.PTF** (%)
$2.2\pm0.6 \times 10^6$	$1.7\pm0.3 \times 10^2$	$2.5\pm0.3 \times 10^2$	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-Proskauernegative, positive for beta-haemolysis, positive for casein hydrolysis, lecithinasepositive, lipase positive, acid production from glucose fermentation, negative for lactose fermentation, negative for mannitol fermentation,negative for xylose fermentation, andpositive 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 meansfor 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 (Biniarz*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), FGPswas isolated using un-optimized nutrient agar medium incorporated with 50µg/ml chloramphenicol with efficiency ofabout 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 *Pseudomonasfluorescens*. 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 *Pseudomonasfluorescens*as determined through optimization modelswere 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/mlcan be used for selective isolation of *Pseudomonasfluorescens*with optimum efficiency.

Disclosure of conflict of interest

There are no conflicts of interest.

References

Abouseoud, M., Maachi., R., and Amrane, A. (2007). Biosurfactant production from olive oil by *Pseudomonas fluorescens.*InA. Méndez-Vilas (Ed.), *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* (pp. 340-347). Retrieved from http://www.formatex.org/microbio/pdf/Pages340-347.pdf

Al-Hinai, A. H., Al-Sadi, A. M., Al-Bahry, S. N., Mothershaw, A. S., Al-Said, F. A., Al-Harthi, S. A., &Deadman, M. L. (2010). Isolation and characterization of*Pseudomonas aeruginosa*with antagonistic activity against *Pythiumaphanidermatum*.*Journal of Plant Pathology*, 92 (3): 653-660.

Anbuselvi, S., Jeyanthi, R., and Karunakaran, C. M. (2010). Antifungal activity of *Pseudomonas fluorescens* and its biopesticide effect on plant pathogens. *National Journal of ChemBiosis,* 1 (1): 15-18.

Biniarz, P., Coutte, F., Gancel, F., &Łukaszewicz, M. (2018).High-throughput optimization of medium components and culture conditions for the efficient production of a lipopeptidepseudofactin by *Pseudomonas fluorescens* BD5. *Microbial Cell Factories*, 17, article no. 121. https://doi.org/10.1186/s12934-018-0968-x

Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., ...Martinez, J. L. (2016). Bacterial multidrug efflux pumps: Much more than antibiotic resistant determinants. *Microorganisms,* 4 (14): 1-19. Doi:10.3390/microorganisms4010014

Boopathi, E. &Rao, K. S. (1999). A siderophore from *Pseudomonas putida* type A1: structural and biological characterization. *BiochimicaetBiophysicaActa,* 1435 (1-2): 30-40.

Bustamante, M., Durán, N., &Diez, M. C. (2012).Biosurfactants are useful tools for the bioremediation of contaminated soil: A review. *Journal of Soil Science and Plant Nutrition*, 12(4): 667- 687.

Daly, J. A., Boshard, R., &Matsen, J. M. (1984).Differential primary plating medium for enhancement of pigment production by *Pseudomonas aeruginosa*.*Journal of Clinical Microbiology*, 19 (6): 742-743.

Darak, O. &Barde, R. D. (2015).*Pseudomonas fluorescens* associated with bacterial disease in Catlacatla in Marathwada Region of Maharashtra. *International Journal of Advanced Biotechnology and Research*, 6 (2): 189-195.

Draper, N. R. & Smith, H. (1998).*Applied Regression Analysis* (3rded.). John Wiley & Sons, Inc. pp. 115-134.

Fernández, M., Conde, S., de la Torre, J., Molina-Santiago, C., Ramos, J., and Duque, E. (2012). Mechanisms of Resistance to Chloramphenicol in *Pseudomonas putida* KT2440. *Antimicrobial Agents and Chemotherapy*, 56(2): 1001-1009. Doi:10.1128/AAC.05398-11

Gao, S. S., Hothersall, J., Wu, J., Murphy, A. C., Song, Z., Stephens, E. R., ...Willis, C. L. (2014). Biosynthesis of mupirocin by *Pseudomonas fluorescens* NCIMB 10586 involves parallel pathways. *Journal of the American Chemical Society*, 136 (14): 5501-5507. Doi:10.1021/ja501731p

Garbi, C., Casasús, L., Martinez-Álvarez, R., Robla, J. I., & Martín, M. (2006).Biodegradation of oxadiazon by a soil isolated *Pseudomonas fluorescens* strain CG5: Implementation in an herbicide removal reactor and modelling. *Water Research*, 40 (6): 1217-1223. Doi:10.1016/j.watres.2006.01.010

Haas, D., Keel, C., Laville, J., Maurhofer, M., Oberhansli, T., Schnider, U., ...Defago, G. (1991). Secondary metabolites of *Pseudomonas fluorescens* strain CHA0 involved in the suppression of root diseases. In H. Hennecke, and D. P. S. Verma (Eds.), *Advances in molecular genetics of plant-microbe interactions* (pp. 450-456).Springer Netherlands.

Ikhimiukor, O. O. &Nneji, L. M. (2013).The Review of the use of microorganisms in biodegradation of crude oil spill: Challenges and prospects. *Researcher*, 5 (12): 155-163.

Kalaiarasi, K., &Sunitha, P. U. (2009).Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. African Journal of Biotechnology, 8 (24), 7035-7041.

Kumar, A. P., Janardhan, A., Radha, S., Viswanath, B., &Narasimha, G. (2015).Statistical approach to optimize production of biosurfactant by *Pseudomonas aeruginosa* 2297.*3 Biotech*, 5: 71-79. Doi:10.1007/s13205-014-0203-3

Laine, L., Perry, J. D., Lee, J., Oliver, M., James, A. L., De la Foata, C., ...Gould, F. K. (2009). A novel chromogenic medium for the isolation of *Pseudomonas aeruginosa* from sputa of cystic fibrosis patients.*Journal of Cystic Fibrosis*, 8: 143-149. Doi:10.1016/j.jcf.2008.11.003

Livermore, D. M. (2002).Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*:Our Worst Nightmare? *Clinical Infectious Diseases*, 34: 634-640.

Mahiuddin, M., Fakhruddin, A. N. M., and Al-Mahin, A. (2012). Degradation of phenol via meta cleavage pathway by *Pseudomonas fluorescens* PU1. *ISRN Microbiology*, 2012 (Article ID 741820): 1-6. Doi:10.5402/2012/741820

Manilla, P. N., Ogali, R. E., &Uzoukwu, B. A. (2001).*Undergraduate chemistry: Fundamental principles*. Timi Hyacinth Enterprises. pp. 55-56.

Marqués, S., & Ramos, J. L. (1993). Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. *Molecular Microbiology*, 9 (5): 923-929. Doi:10.1111/j.1365-2958.1993.tb01222.x

Moneke, A. N., Okpala, G. N., and Anyanwu, C. U. (2010).Biodegradation of glyphosate herbicide *in vitro* using bacterial isolates from four rice fields.*African Journal of Biotechnology*, 9 (26): 4067-4074.

Morita, Y., Tomida, J., & Kawamura, Y. (2014).Responses of *Pseudomonas aeruginosa* to antimicrobials.*Frontiers in Microbiology*, 4 (Article 422): 1-8. Doi:10.3389/fmicb.2013.00422

Mukherjee, A. K. & Das, K. (2010). Microbial surfactants and their potential applications: An overview. In R. Sen (Ed.), *Biosurfactants* (pp. 54-64).Springer Science and Business Media.

Ningthoujam, D. S. &Shovarani, N. (2008). Isolation and characterization of a *Pseudomonas aeruginosa* strain DN1 degrading p-nitrophenol. *Research Journal of Microbiology*, 3 (5): 345-351.

Oliveira, A. C., Maluta, R. P., Stella, A. E., Rigobelo, E. C., Marin, J. M., & Avila, F. A. (2008). Isolation of *Pseudomonas aeruginosa* strains from dental office environments and units in Barretos, State of Sao Paulo, Brazil, and analysis of their susceptibility to antimicrobial drugs. *Brazilian Journal of Microbiology*, 39: 575-584.

Otenio, M. H., da Silva, M. T. L., Marques, M. L. O., Roseiro, J. C., &Bidoia, E. D. (2005). Benzene, Toluene and Xylene biodegradation by *Pseudomonas putida* CCMI 852.*Brazilian Journal of Microbiology*, 36(3): 258-261. Doi:10.1590/S1517-83822005000300010

Peekate, L. P. (2022). *Deciphering the identity of bacterial isolates through conventional means: A practical guide*. Port Harcourt, Nigeria: Edese Printing & Publishing Co.

Peekate, L. P.& Abu, G. O. (2015).A preliminary investigation on the emergence of antibiotic resistant bacteria resulting from inappropriate use of antibiotics in the purification of Algal cultures.*Nigerian Journal of Microbiology*, 29, 2993-3001.

Peekate, L. P.& Abu, G. O. (2017a).Use of chloramphenicol in the differential enumeration of greenish pigment producing *Pseudomonas*.*Basic Research Journal of Microbiology*, 4 (4), 33-41.

Peekate, P. L.& Abu, G. O. (2017b). Optimizing C:N ratio, C:P ratio, and pH for biosurfactant production by *Pseudomonas fluorescens*. *Journal of Advances in Microbiology*,7(2), 1-14. https://doi.org/10.9734/JAMB/2017/38199

Peekate, L. P.,Sigalo, B., & Basil, N. P. (2018).Comparing the efficacy of Kings B, Cetrimide and Chloramphenicol-Nutrient agar medium in the isolation of *Pseudomonas* species.*Asian Journal of Biological Sciences*, 11 (3), 145-151. https://doi.org/10.3923/ajbs.2018.145.151

Sarniguet, Kraus, J, Henkels, M. D., Muehlchen, A. M., and Loper, J. E. (1995). The sigma factor s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proceedings of the National Academy of Science of the United States of America*, 92 (26): 12255-12259. Doi:10.1073/pnas.92.26.12255

Scales, B. S., Dickson, R. P., LiPuma, J. J., &Huffnagle, G. B. (2014). Microbiology, Genomics, and clinical significance of the *Pseudomonas fluorescens* species complex, an unappreciated colonizer of humans.*Clinical Microbiology Review*, 27 (4): 927-948.

Singh, B. K. & Walker, A. (2006). Microbial degradation of organophosphorus compounds. *FEMS Microbiology Reviews*, 30: 428-471. Doi:10.1111/j.1574-6976.2006.00018.x

Stanier, R. Y., Adelberg, E. A., and Ingraham, J. L. (1977).*General Microbiology* (4th ed.). London: The Macmillan press Ltd. pp. 593-595.

Strateva, T., and Yordanov, D. (2009).*Pseudomonas aeruginosa* – a phenomenon of bacterial resistance.*Journal of Medical Microbiology*, 58: 1133-1148. Doi:10.1099/jmm.0.009142-0

Tan, G.-Y.A., Chen, C.-L., Ge, L., Li, L., Tan, S. N., & Wang, J.-Y. (2015). Bioconversion of styrene to poly(hydroxyalkanoate) (PHA) by the new bacterial strain *Pseudomonas putida* NBUS12. *Microbes and Environments*, 30(1): 76-85. Doi:10.1264/jsme2.ME14138

Vasudevan, N., Bharathi, S., and Arulazhagan, P. (2007).Role of plasmid in the degradation of petroleum hydrocarbon by *Pseudomonas fluorescens* NS1.*Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances and Environmental Engineering*, 42 (8): 1141-1146.

Zhang, X. &Dequan, L. (2013).Response surface analyses of rhamnolipid production by *Pseudomonasaeruginosa* strain with two response values.*African Journal of Microbiological Research,* 7 (22): 2757-2763.