FACTORIAL ANALYSIS OF GROWTH PARAMETERS FOR MESOPHILIC BACTERIUM, *Pseudomonas putida (*ATCC 49128)

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ABSTRACT: **Studies on bacterial growth pattern from the conventional approach are defective due to their failure to explain the interactions or simply the complementary effects of the factors influencing the bacterial growth. In this study, the individual and collaborative effects of *Pseudomonas putida* growth variables were evaluated using a 2-level fractional factorial design of experiment (FFDOE). The growth of the organism was found to respond remarkably to different concentrations of nutrient media (carbon source) and the other independent variables. Factorial models were developed from the experimental design to study the individual and interactive effects of the studied parameters on the response. The studied parameters and their levels were as follows: nutrient concentration (4-16 g/L), acclimatization time (24-72 hrs), agitation (140-200 rpm), and temperature (30-40^{o}C). These parameters were statistically validated using analysis of variance (ANOVA) and the results revealed that the model terms were statistically significant with an F-value of 415.17 at *P* <0.002. The growth factor with the most influence (positive) on the response was the nutrient concentration. The level of the parameters influence on the response was in the order of nutrient concentration > temperature > nutrient concentration versus temperature > agitation > nutrient concentration versus agitation. Based on the R^2 and the adjusted R^2 values of >95%, the estimated variables showed a high degree of relationship between the observed and the predicted values; thus, the predictive ability of the models was suggested. It could, therefore, be concluded that nutrient concentration, temperature, and agitation can greatly influence the growth of *P. putida* within a specific range.**

KEYWORDS: *Screening; Growth; Markers; Bacteria; Factorial design.*

1. INTRODUCTION

The growth of bacterial via cell division and biomass production (biosynthesis) is a function of many prospective factors which may include but not limited to the age of the inoculum, substrates composition and availability, temperature, pH, and exposure to toxic metabolites. Changes in such growth influencing factors (universally known as stress phenomenon) could affect the rate of bacterial growth (Munna *et al*., 2014). Various studies have been conducted to ascertain the relative linear effects of these growth factors on the growth of microbes. Such approaches are notorious for certain drawbacks which are probably due to the inability of the system to explain the interactions or the complementary effects of these factors on the response. The conventional approach which involves varying one variable at a time while fixing others at a certain level (known as one-variable-at-a-time (OVAT) or one-factor-at-a-time (OFAT)) has been presumed unsatisfactory (Mandenius & Brundin, 2008; Mosquera *et al.*, 2014; Navaneeth *et al*., 2009; Singh *et al.*, 2011). Additionally, it is time-consuming as it involves several experimental runs and full of bias. Comparatively, the fractional factorial design of experiment (DOE) offers an alternative approach to the identification of both linear and interactive effects of variables on the dependent variable (Onsekizoglu *et al.*, 2010). A factorial design serves as a baseline data for future response surface optimization studies which facilitate the determination of the optimum model conditions for any process (Hooshyar & Abbas, 2014; Ridzuan *et al*., 2016).

The relationship between the response and the process variables is expressed in Eq. 1;

$$\eta = f(x_1, x_2, ..., x_n) + \varepsilon \quad (1)$$

where η is the response, *f* is the unknown function of the response, x_1, $x_{2,...,}xn$ denotes the independent variables, *n* is the number of independent variables, and ε is the statistical error (noise) that represents other sources of variability not accounted for by *f*.

Generally, the relationship of these parametric factors is depicted by a polynomial model of a full quadratic equation as:

$$Y = \beta_0 + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where *Y* is the predicted response, β_0, βi, βii, *and* βij are the regression coefficients for intercept, linear, quadratic, and interaction coefficients respectively, while *xi* and *xj* are the coded independent variables.

The determination of the actual bacterial growth pattern and the limiting factors involved are obviously unpredictable owing to the complexity of the mechanisms involved. Consequently, the present study envisaged to highlight an approach of applying factorial experimental statistical design to screen the individual and interaction effects of different concentrations of growth media (nutrient broth), acclimatization time, and other physical parameters (such as agitation speed, and temperature) on the growth of *P. putida* based on cell density (optical density) and biomass accumulation in batch mode of orbital shake flasks. The outcome of the parameters screening process and their optimum values would be marked for further optimization of a model process, reciprocating each other on the dependent variable.

2. MATERIALS AND METHODS

2.1 Strain and cultivation

The isolate (*P. putida* ATCC 49128) and its growth media were obtained from Microbiologic (217 Osseo Ave. North, St. Cloud, USA). An enriched culture media was prepared in accordance with the manufacturer's guidelines with some modifications. Briefly, 8 g of nutrient broth was dissolved in de-ionized water (DI) to a final volume of 1 L in Schott bottles and shaken vigorously until dissolved. The solution was heated on a hot plate and sterilized in an autoclave (H+P Varioklav Steam Sterilizer ESCO) at 121°C for 15 mins. The broth was cooled in a water bath to 47°C before pouring into various sampling bottles of 20 mL volume. A pre-culture of the bacterial strain was done by suspending a loopful from the stock culture into a 20 mL freshly prepared nutrient broth 10% (wv^{-1}). The seeded culture was incubated in a microbiological incubator (M Emmert-Germany/BE 600) at ambient temperature for 24 h. Thereafter, the inoculum was transferred into a 500 mL Erlenmeyer flask containing 150 mL of nutrient broth (30% vv^{-1} of the original volume of the shake flask) (Standbury *et al.*, 1984). The experiments were carried out by placing the flasks in an orbital shaker (B. Braun, German model) under the predetermined temperatures and agitation for 24 hrs.

2.2 Cell biomass determination

The bacterial biomass synthesis was determined using cell dry weight measurement. In brief, the sample was centrifuged at 12,000 rpm for 15-20 mins in a pre-weighed tube. The supernatant was disposed and the pellets were resuspended in 0.15 M saline solution and centrifuged again as earlier described (François *et al.*, 2012; Momen *et al.*, 2016). The supernatant was discarded and each tube containing the cell mass was dried at 100°C for 1 h and weighed to get the dry cell weight. The mass was repeatedly dried and measured until a stable weight was obtained.

2.3 Analytical procedure

For the growth determination based on cell density and biomass accumulation, 2.5 mL aliquots were withdrawn at defined intervals for 24 hrs. The growth of the organism was monitored turbidimetrically by measuring the optical density (absorbance) of the withdrawn aliquots at 600 nm in a UV-VIS spectrophotometer (Hitachi, U-1800, Japan) after appropriate dilution to obtain an OD value of less than 0.5 (Roebuck *et al.*, 1995).

2.4 Screening of parameters that influence the growth of the bacterial cell

In this study, four factors including nutrient concentration, temperature, acclimatization time, and agitation speed were investigated and screened for their effects on the growth of *P. putida* using a two-level (2^{4-1}) fractional factorial design of experiment (FFDOE). The levels of the independent variables (nutrient concentration, temperature, acclimatization time, and agitation speed) were based on the results obtained in previous OFAT studies reported by Azoddein *et al.*, (2015). Each variable was studied at two-coded level: low-level (-1) and high-level (+1). Tables 1 and 2 showed a design matrix of the factors and the levels employed for the experiment. A total of eight runs (2^3) were conducted in replicates to minimize presumed experimental errors. The effect of each variable, as well as their interactions on the

dependent variable, was statistically determined. A first-order model with interaction terms proposed for each response variable (*Yi*) based on the multiple linear regression was employed. A polynomial model in coded terms (Eq. 3) was used to predict the response (bacterial growth) to the studied variables.

3. RESULTS AND DISCUSSION

3.1 Model Fitness

Table 2, showed the effects of the four variables (nutrient concentration, temperature, agitation, and acclimatization time) on the bacterial growth using a fractional factorial design. It can be seen that the two maximum growth values of 2.87 and 3.00 cell density at OD_{600} nm which corresponds to 1.12 g/L and 1.17 g/L biomass accumulation were recorded at 16 g/L nutrient concentration under varying operational parameters. From the results, it can be suggested that temperature and agitation were the most significant factors, while acclimatization time was observed to be insignificant. The independent and dependent variables were found to have fitted to the first-order polynomial model equation with interaction terms, and for each response, the variable was examined for the goodness of fit. Tables 3 and 4 showed the screened results via student's t-test of ANOVA with the regression relationships for each response monitored. The results showed that the *P*-values for both linear and interactive effects were lower than 0.05, casting a notable impact of the variables on the response at 95% confidence level (refer to Table 4).

The model depicted a high coefficient of determination (R^2 = 0.9990), showing its capability to explain 99.90% of the variability in bacterial growth. An R^2 value of close to 1 is desirable for a good model and should not be less than 0.8 for biological processes (Olmez, 2009). It was however argued that a large value of R^2 is not an indication of model goodness; thus, it is preferred to adopt the adjusted-R^2 for the evaluation of model fitness since it is adjusted for the number of terms in the model (Mani *et al.*, 2017). An adjusted-R^2 of over 90% spelled a high degree of relationship between the observed and predicted values. Table 4 showed that the R^2 and adjusted-R^2 values for the models did not differ significantly, indicating that non-significant terms have were not included in the model. The predicted and actual values demonstrated a distribution of the predicted values near the straight-line, showing a reasonable agreement with the experimental data (Adj. R^2 of 99.66%). Indeed, this further confirmed the good predictive ability of the models. The *P*-values were employed to check the significance of each of the coefficients which in turn, may show the pattern of interactions between the variables, with smaller values indicating highly significant effects (Heo *et al.*, 2009). An empirical relationship between the response and the independent variables was expressed by the following response surface reduced polynomial model equations (Eq. 4).

$$\gamma\,(Cell\ growth) = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} * A * B + \beta_{13} A * C + \beta_{23} B * C \quad (3)$$

$$Growth = 2.0413 + 0.4613 * Nutrient - 0.1787 * Agitation - 0.2337 * Temperature + 0.1212 * Nutrient * Agitation - 0.1987 * Nutrient * Temperature \quad (4)$$

Table 1: The actual value range of variables used in the two-level fractional factorial design of experiment (FFDOE).

Variables	**Units**	**Range**	
		low (-)	**high (+)**
Nutrient Conc. (A)	g/L	4	16
Acclim. time (B)	h	24	72
Agitation (C)	rpm	140	200
Temperature (D)	^{o}C	30	40

Table 2: Two-level fractional factorial design matrix of factors influencing *P. putida* growth.

Run	**Factors**				**Response (growth)**	
	code/actual A	**code/actual B**	**code/actual C**	**code/actual D**	**Average OD 600 nm**	**cell biomass (g/L)**
1	(-1) 4	(+1) 72	(+1) 200	(-1) 30	1.34±0.03	0.52
2	(+1) 16	(-1) 24	(+1) 200	(-1) 30	2.87±0.10	1.12
3	(-1) 4	(-1) 24	(-1) 140	(-1) 30	1.89±0.01	0.74
4	(+1) 16	(-1) 24	(-1) 140	(+1) 40	2.12±0.04	0.83
5	(-1) 4	(-1) 24	(+1) 200	(+1) 40	1.22±0.06	0.48
6	(+1) 16	(+1) 72	(+1) 200	(+1) 40	2.02±0.02	0.79
7	(+1) 16	(+1) 72	(-1) 140	(-1) 30	3.00±0.03	1.17
8	(-1) 4	(+1) 72	(-1) 140	(+1) 40	1.87±0.01	0.73

3.2 Main variables effect analysis on the dependent variable

Table 3 showed the analysis of variance (ANOVA) of the experimental factors and their percentage contribution, coupled with their interactive effects on the bacterial growth. Figure 1 showed the trend of the main effect plots when the factors are varied in their positive and negative levels. For the overall individual effect, these graphs depicted that factors A-, D+, and C+ had the least significant effect on the cell growth compared to A+, C-, and D-. The factors with steeper slopes demonstrated the major

effects, and thus, contributed significantly to the response. Comparatively, Figure 2 (a perturbation plot) depicted the main individual effect of signals on the dependent variable. It can be used to compare the effects of factors by default in their corresponding center levels in the design space. The response is plotted by changing only one factor over its range while keeping other factors constant. A steep slope or curvature in a variable indicates that the response is sensitive to the factor. A relatively flat line shows insensitivity to changes in the related factor (Ahmad *et al.*, 2017). It is clear that the impact of nutrient concentration was much at a higher concentration (positive deviation) over all the other factors. On the other hand, temperature and agitation were less steep and contributed minimally to the response.

Figure 3 showed the individual and interactive effects of the variables. The bar lengths of a Pareto chart are proportional to the absolute value of the estimated effects at 95% confidence level. This indicates the order of significance of each linear and interactive effect of the variables. Nutrient concentration demonstrated the most significant effect on the response (bacterial growth). The interactive effect of nutrient concentration and temperature was less significant compared to the linear effects. This observation agreed with the report of Onsekizoglu *et al.*, (2010). Table 5 showed the linear effect of the independent variables based on their weighted signs (+ or -). The positive and negative signs indicated parameter effects at either low or high levels of each variable on the response. Nutrient concentration and acclimatization time were observed to influence the bacterial growth at a higher rate while temperature and agitation had minimal effects at their low levels. However, all the model terms were significant at $P < 0.05$ except for acclimatization time ($P > 0.05$); hence, it has no notable impact on the response. The model term with the most significant effect on the response was A (F-value = 1249.18, $P < 0.05$). The effects were in the following order: A > D > *A*D > C > AC.

Table 3: ANOVA result for the growth response.

Source	**df**	**Adj. SS**	**Adj. MS**	**F-Value**	**p-Value**	**%Contributio**
Model	5	2.82836	0.56567	415.17	0.002	
Linear	3	2.39474	0.79825	585.87	0.002	
Nutrient	1	1.70201	1.70201	1249.18	0.001	60.12
Agitation	1	0.25561	0.25561	187.61	0.005	9.03
Temperature	1	0.43711	0.43711	320.82	0.003	15.44
Interactions	2	0.43362	0.21681	159.13	0.006	
Nutrient versus Agitation	1	0.11761	0.11761	86.32	0.011	4.15
Nutrient versus Temp.	1	0.31601	0.31601	231.94	0.004	11.16
Residual Error	2	0.00273	0.00136			
Total	7	2.83109				

Table 4: Statistics used to test the goodness of fit of the model.

R-Squared	0.9990	Std. Dev	0.037
Adj. R-Squared	0.9966	Mean	2.04
Pred. R-Squared	0.9846	CV (%)	1.81
Adeq. Precision	54.666	PRESS	0.044

Table 5: Explicative analysis of signal factors on *P. putida* growth.

Variables	Terms	Main effect	t-value	p-value	Confidence level (%)
A	Nutrient Conc.	0.46	35.34	**0.001	99.90%
B	Acclim. time	0.03	1.85	†0.314	68.60%
C	Agitation	-0.18	-13.7	*0.005	99.50%
D	Temperature	-0.23	-17.91	* 0.003	99.70%

**Significant factor at higher (+) range *Significant factors at low (-) range † Not significant factor.

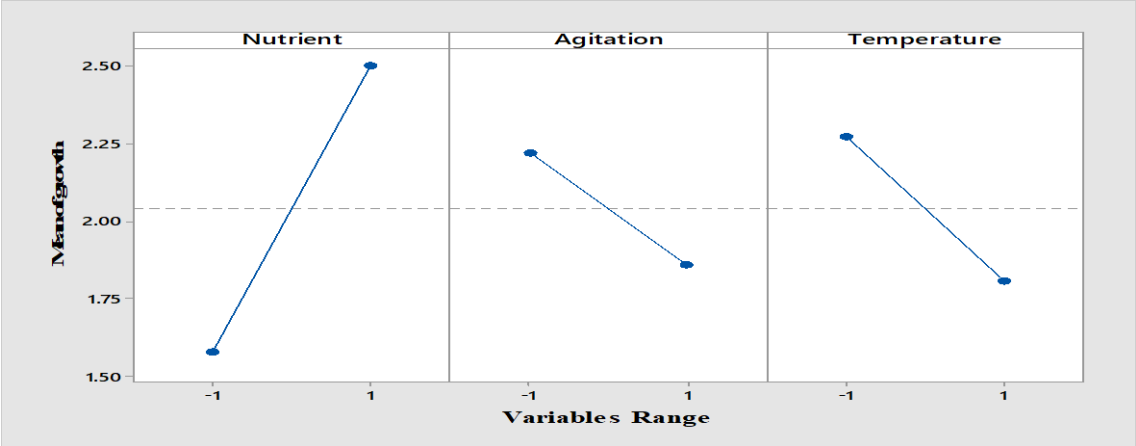


Figure 1: Main effects plot for the screening of growth markers (signal factors) using FFD.

3.2.1 Effect of nutrient concentration

The nutrient concentration had the highest percentage contribution of 60.12%, t-value of 35.34, and a main effect of 0.46. It was observed as the main and most important factor affecting *P. putida* growth. Nutrient, being the main constituent of cell biomass, is required for bacterial growth and biosynthesis under optimum physical parameters of temperature and acclimatization time. For microbes, growth is their most essential response to their physiochemical environment (Franklin *et al.*, 2011) and was, therefore, found to rely

heavily on different compositions ranging from single to multiple substrates although screening and optimization approaches of medium constituents are not much popular (Mosquera *et al.*, 2014). In addition, the growth rate is a function of nutrient composition, uptake, and utilization.

3.2.2 Effect of temperature

The environmental temperature range was the next factor that contributed more (15.44%) to the growth of *P. putida* in terms of main effects. This isolate, being a mesophile and non-spore forming, was observed to thrive better at an optimum temperature of 31.8ºC although a temperature range of 36-38^{o}C could speed up the rate of substrate uptake, utilization, and subsequent incorporation for cell biomass synthesis. Srivastava *et al.*, (2008) observed a rare growth of *Pseudomonas sp* at a temperature of 40^{o}C using multiple substrates compositions. However, this finding was slightly higher than the report of Munna, (2015). Enzymatic activities are progressive with temperature until a certain temperature threshold where enzymes are denatured and decelerates enzyme activity. A similar trend of bacterial growth was reported by Azoddein *et al.*, (2015) for *P. putida* growth in mercury-contaminated petroleum refinery wastewater.

3.2.3 Effect of agitation speed

Agitation or shaking was found to trail temperature in terms of impact on the response, with a percentage contribution of 9.03%. It is an important marker, especially when related to the oxygen transfer rate (OTR) which requires moderate shaking between a period of 24-36 hrs during the peak exponential growth. It can be seen that *P. putida* growth was higher at an agitation speed of 140 rpm. It was argued that the effect of agitation speed on aeration could invariably influence growth as it is a function of the flask diameter, culture volume, and flask size. The function of these parameters is to create enough surface area for optimum homogenous aeration. Munna *et al.*, (2014) recorded a maximum growth at the optimum agitation of 170 rpm in 36 hrs.

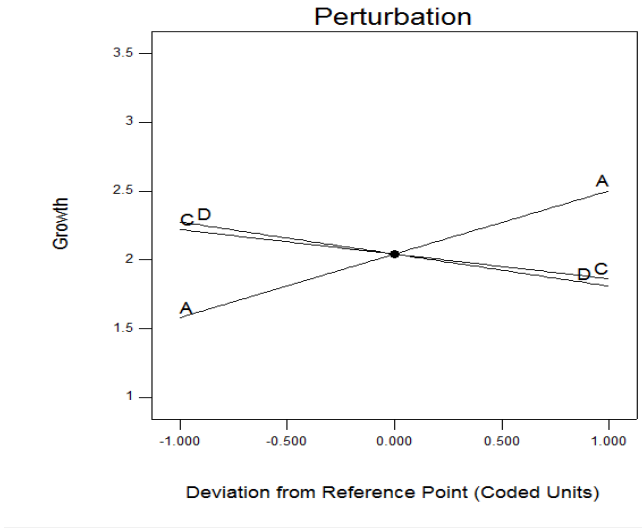


Figure 2: Perturbation plot of main signal effects on *P. putida* growth.

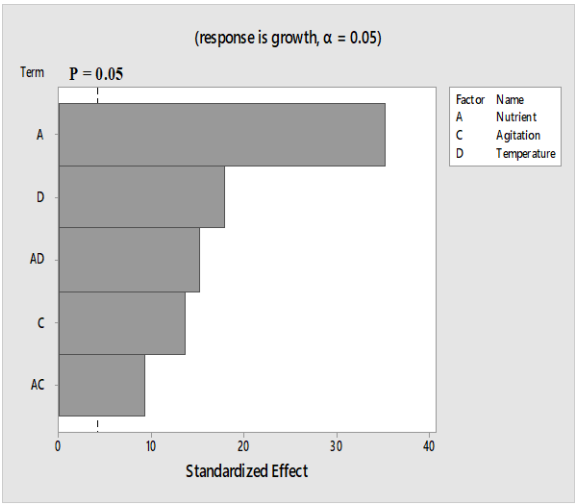


Figure 3: Pareto chart of factors main and interactive effects.

3.3 Interactive effects of the variables on the bacterial growth

This aspect examined the interactive effects of the variables on the response. Figures 4a and 4b showed the interaction (two-way interaction effects 2FI) of the factors at 95% significant level. Figure 4a depicted the interaction of nutrient concentration and temperature difference (AD) on the bacterial growth. This interaction was statistically significant, presenting an F-value of 231.9, *P*-value of 0.004, and a percentage contribution of 11.16%. This was observed to be the most important interactive effect on the response. It can be observed that the bacterial growth was at the lowest level and insensitive to temperature differences at a nutrient concentration of 4 g/L. However, the interaction was much significant at a nutrient concentration range of 10-16 g/L and temperature of 30ºC compared to a temperature of 40ºC at a fixed acclimatization time of 48 hrs and agitation speed of 170 rpm. The steeper the contour plot, the more significant the interaction effect on the dependent variable. The results were in agreement with previous findings (Dorn *et al.*, 2003). It can be suggested that the activities of metabolic enzymes were activated and sustained within a specific temperature threshold, and this enhanced the rate of substrate utilization.

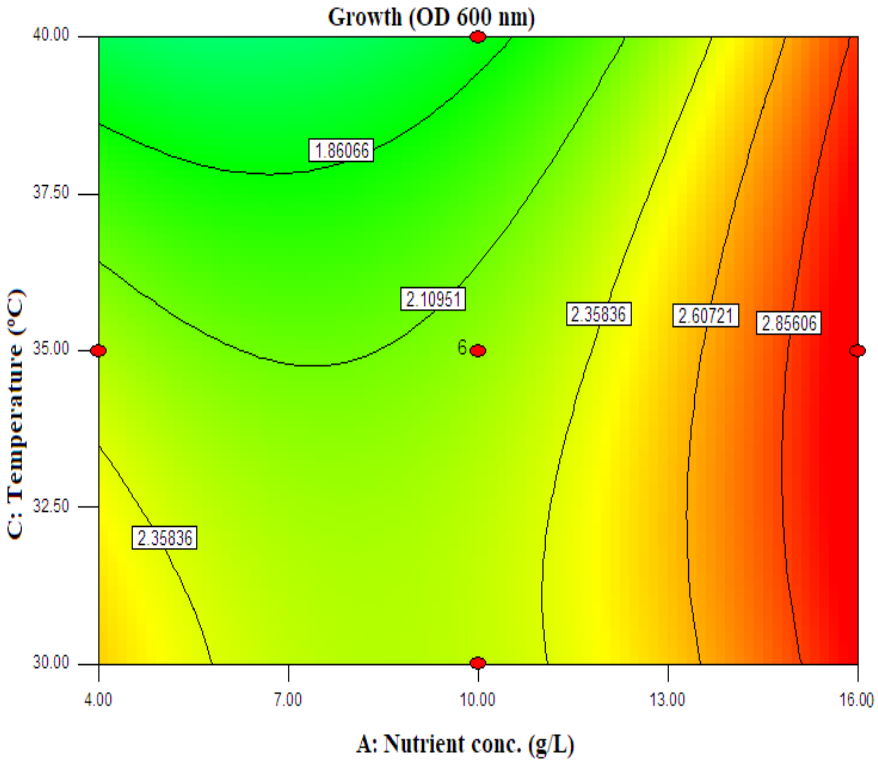
Figure 4b on the other hand showed the interactive effect of nutrient and agitation (AC) which was found to be less significant on the response at a fixed temperature of 36ºC and acclimatization time of 48 hrs compared to AD. Unlike the former interactive effect, the bacterial growth was found to vary with agitation speed at a lower nutrient concentration. In addition, there was an insignificant difference between low and high agitation speeds at a high nutrient concentration even though growth was high. The relative impact of agitation speed on nutrient metabolism and subsequent cell growth and biomass production is related to the effective distribution of nutrient, oxygen and the inoculum. This trend was also supported in many texts, among which were Munna *et al.*, (2014) and Caroline *et al.*, (2000). However, excess agitation was found to cast a shearing effect on the cells which subsequently cause cell death.

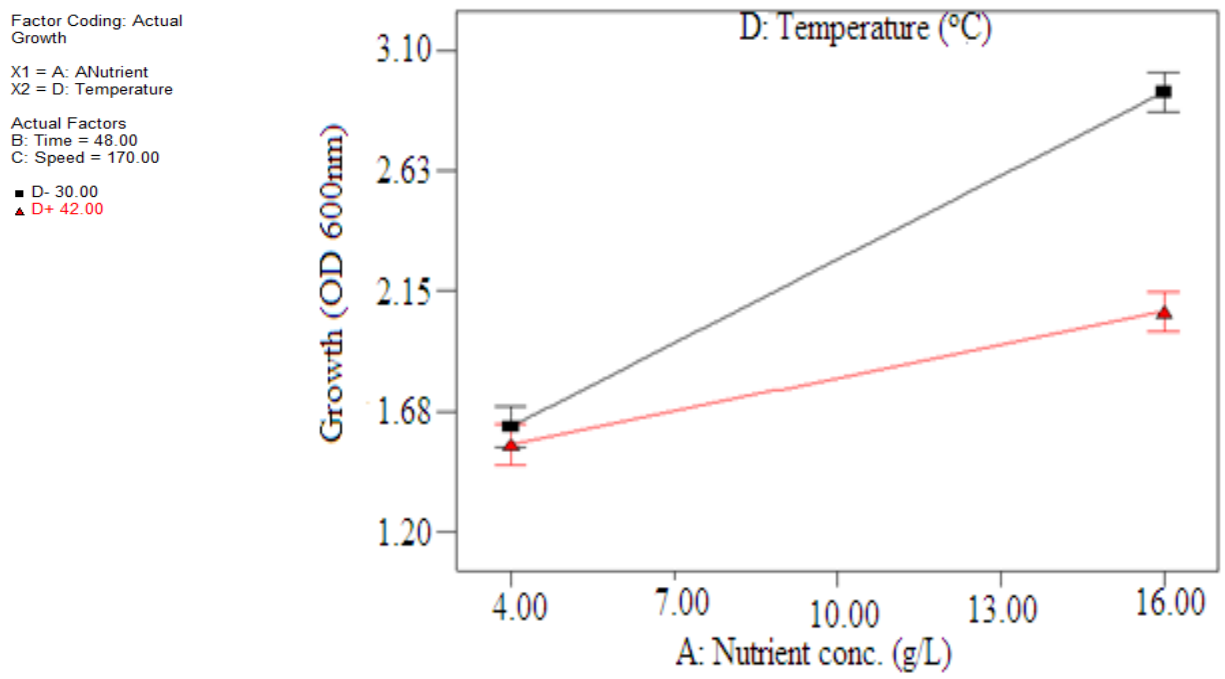
3.4 Model validation

The suggested best optimal conditions by the software and their corresponding observed values for *P. putida* growth are shown in Table 6. The experiments were conducted to validate the suggested conditions efficacy on the response, and the observed values are reported in Table 6. Based on Eq. 5, the error from the experiment was calculated and the results for the triplicate runs were 4.71 %, 1.14 %, and 7.55 %, respectively. Therefore, the models' adequacy was validated since the errors were all less than 10 % (Alara *et al.*, 2017).

$$\boldsymbol{Error = 100\ \%\ X \left|\frac{observed - predicted}{observed}\right|} \quad (5)$$

(a)


Growth (OD 600 nm)
40.00
37.50
35.00
32.50
30.00
C: Temperature (°C)
1.86066
2.10951
2.35836
2.60721
2.85606
6
2.35836
4.00
7.00
10.00
13.00
16.00
A: Nutrient conc. (g/L)


Factor Coding: Actual
Growth
X1 = A: ANutrient
X2 = D: Temperature
Actual Factors
B: Time = 48.00
C: Speed = 170.00
D- 30.00
D+ 42.00
D: Temperature (°C)
3.10
2.63
2.15
1.68
1.20
Growth (OD 600nm)
4.00
7.00
10.00
13.00
16.00
A: Nutrient conc. (g/L)

(b)

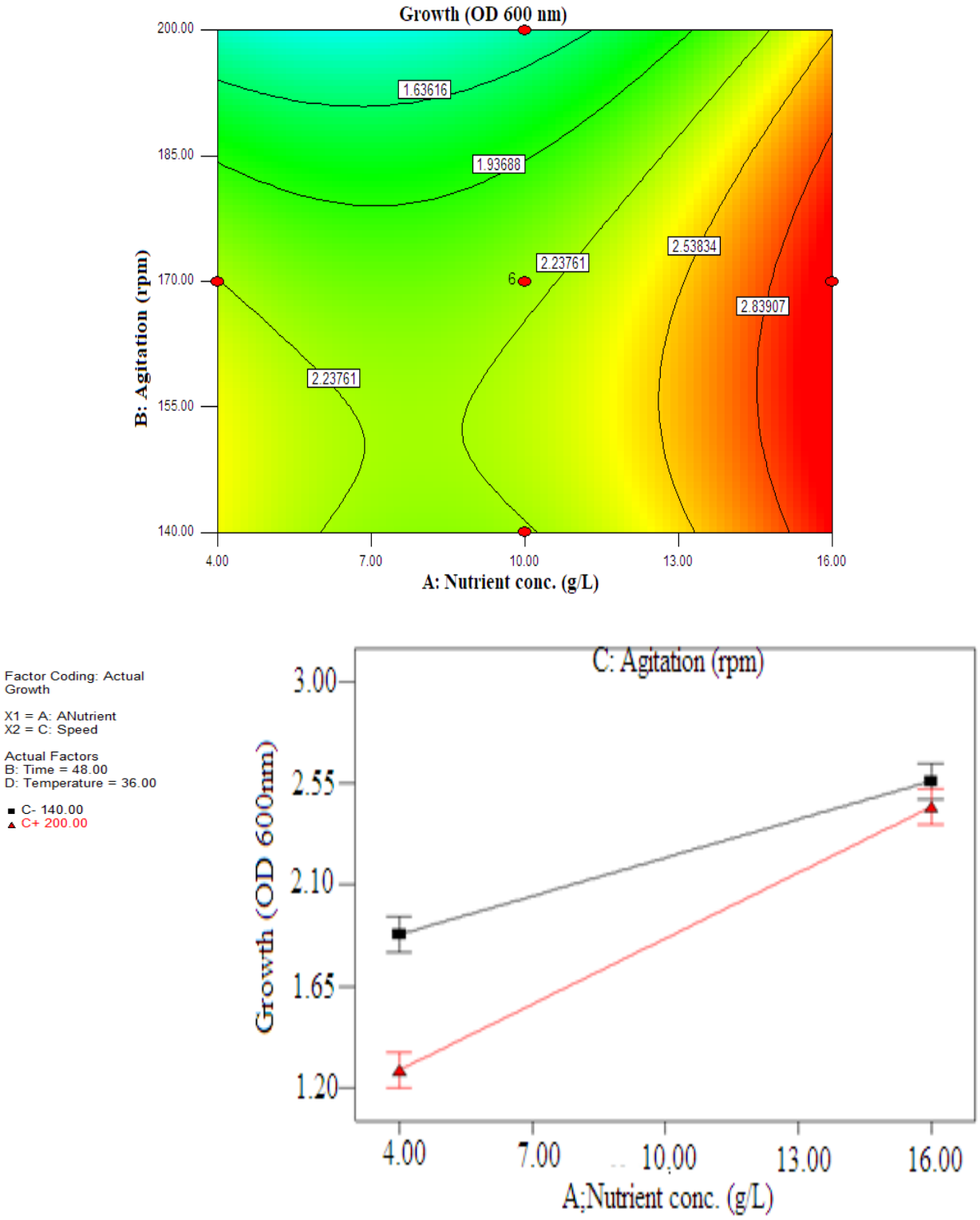


Figure 4: 3D surface plots: (a) nutrient concentration (A) and temperature (D) at a fixed acclimation time (C) of 48 hrs and agitation speed (D) of 170 rpm; (b) nutrient concentration

(A) and agitation (C) at a fixed temperature (D) of 36^{o}C and acclimatization time (B) of 48 hrs.

Table 6: Predicted model terms for response optimization.

Run	Factors			Response (growth OD)		
	Nutrient (g/L)	Temperature (^{o}C)	Speed (rpm)	Predicted	Observed	Error (%)
1	15	32	199	2.67	2.55	4.71
2	14	30	160	2.69	2.64	1.14
3	16	30	140	2.99	2.78	7.55

4. CONCLUSION

A two-level (2^3) fractional factorial design with two center points was used to investigate the linear and interactive effects of varying nutrient concentrations under different shake flask operational parameters on *P. putida* growth and biosynthesis. All the variables except the acclimatization time showed significant effects on the dependent variable. The results indicated that nutrient concentration was more significant in terms of both linear and interactive effects on the response. Based on the adequacy testing tables, the estimated model terms showed a high degree of relationship between the observed and predicted values; thus, further confirming the predictive ability of the developed models. Conclusively, the estimated and predicted model terms could further be used to optimize the process conditions of *P. putida* growth and biosynthesis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Ahmad, M. M., Azoddein, A. A. M., Zahari, M. A. K. M., Seman, M. N. bin A., Saedi, M. J., Olalere, O. A., & Alara, O. R. (2017). Optimization of Process Parameters in Mixed Sulfide Oxidation Bacterial Culture Using Response Surface Methodology as a Tool. Journal of King Saud University - Science, 1–8. https://doi.org/10.1016/j.jksus.2017.11.001

[2] Alara, O. R., Abdul Mudalip, S. K., & Olalere, O. A. (2017). Optimization of mangiferin extracted

from Phaleria macrocarpa fruits using response surface methodology. Journal of Applied Research on Medicinal and Aromatic Plants, 5, 82–87. https://doi.org/10.1016/j.jarmap.2017.02.002

[3] Azoddein, A. A. M., Yunus, R. M., Sulaiman, N. M., Bustary, A. B., & Sabar, K. (2015). Mercury Removal Using Pseudomonas putida (ATTC 49128): Effect of Acclimatization Time, Speed and Temperature of Incubator Shaker. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering, 9(2), 204–209.

[4] Caroline, C., Philippe, S., Soumille, H., Nguyen-the, C., & Schmitt, P. (2000). Effect of temperature on growth characteristics of Bacillus cereus TZ415, (August 2016), 1–6. https://doi.org/10.1016/S0168-1605(00)00197-5

[5] Dorn, J. G., Frye, R. J., & Maier, R. M. (2003). Effect of Temperature, pH, and Initial Cell Number on luxCDABE and nah Gene Expression during Naphthalene and Salicylate Catabolism in the Bioreporter Organism Pseudomonas putida RB1353. Applied and Environmental Microbiology, 69(4), 2209–2216. https://doi.org/10.1128/AEM.69.4.2209

[6] François, F., Lombard, C., Guigner, J. M., Soreau, P., Brian-Jaisson, F., Martino, G., … Rebuffat, S. (2012). Isolation and characterization of environmental bacteria capable of extracellular biosorption of mercury. Applied and Environmental Microbiology, 78(4), 1097–1106. https://doi.org/10.1128/AEM.06522-11

[7] Franklin, O., Hall, E. K., Kaiser, C., Battin, T. J., Franklin, O., Hall, E. K., Richter, A. (2011). Optimization of Biomass Composition Explains Microbial Growth-Stoichiometry Relationships. The American Naturalist, 177(2), 29–42. https://doi.org/10.1086/657684

[8] Heo, S., Lee, H., & Ha, S. (2009). A Predictive Model for the Growth Rate of Bacillus cereus in Broth by Response Surface Methodology. Biotechnology and Bioprocess Engineering, 202–206. https://doi.org/10.1007/s12257-008-

0187-0

[9] Hooshyar Hossini, Abbas Rezaee, S. R. (2014). Statistical screening of hexavalent chromium biosorption by Sargassum. Iranian Journal of Health, Safety, and Environment, 1(1), 36-42.

[10] Mandenius, C.-F., & Brundin, A. (2008). Bioprocess Optimization Using Design-of-experiments Methodology. Biotechnol Progr, 24, 1191–1203. https://doi.org/10.1021/bp.67

[11] Mani, M. A., Abd. Aziz, M. A., Mior Ahmad Khusairi, bin M. Z., Mazrul Nizam, bin A. S., & Mohammed, S. J. (2017). Screening of Effective Markers for Mesophilic Bacterium Growth Using Factorial Experimental Design. International Journal of Bio-Science and Bio-Technology, 9(3), 59–74. https://doi.org/10.14257/ijbsbt.2017.9.3.06

[12] Md. Sakil Munna, Z. Z. and R. N. (2015). Influence of temperature on the growth of Drosophila melanogaster. Stamford Journal of Microbiology, 5(1), 9–12.

[13] Momen, S. B., Siadat, S. D., Akbari, N., Ranjbar, B., & Khajeh, K. (2016). Applying central composite design and response surface methodology to optimize growth and biomass production of Haemophilus influenzae type b. Jundishapur Journal of Microbiology, 9(6). https://doi.org/10.5812/jjm.25246

[14] Mosquera, S., González-Jaramillo, L. M., Orduz, S., & Villegas-Escobar, V. (2014). Multiple response optimization of Bacillus subtilis EA-CB0015 culture and identification of antifungal metabolites. Biocatalysis and Agricultural Biotechnology, 3(4), 378–385. https://doi.org/10.1016/j.bcab.2014.09.004

[15] Munna, M. S., Tamanna, S., Afrin, M. R., Sharif, G. A., Mazumder, C., Kana, K. S., Noor, R. (2014). Influence of Aeration Speed on Bacterial Colony Forming Unit (CFU) Formation Capacity. American Journal of Microbiological Research, 2(1), 47–51. https://doi.org/10.12691/ajmr-2-1-7

[16] Navaneeth, S., Bhuvanesh, S., Bhaskar, V., P, V. K., & Kandaswamy, S. K. J. (2009). Optimization of medium for the production of subtilisin from

Bacillus subtilis MTCC 441. Journal of Microbiol. Biotechnology, 8(22), 6327–6331.

[17] Olmez, T. (2009). The optimization of Cr(VI) reduction and removal by electrocoagulation using response surface methodology. Journal of Hazardous Materials, 162(2–3), 1371–1378. https://doi.org/10.1016/j.jhazmat.2008.06.017

[18] Onsekizoglu, P., Bahceci, K. S., & Acar, J. (2010). The use of factorial design for modeling membrane distillation. Journal of Membrane Science, 349, 225–230. https://doi.org/10.1016/j.memsci.2009.11.049

[19] Ridzuan, N., Adam, F., & Yaacob, Z. (2016). Screening of factor influencing wax deposition using full factorial experimental design. Petroleum Science and Technology, 34(1), 84–90. https://doi.org/10.1080/10916466.2015.1122625

[20] Roebuck, K., Brundin, A., & Johns, M. (1995). Response surface optimization of temperature and pH for the growth of Pachysolen tannophilus. Enzyme and Microbial Technology, 17(1), 75–78. https://doi.org/10.1016/0141-0229(94)00046-T

[21] Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K., & Garg, S. K. (2011). Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic Pseudomonas putida SKG-1 isolate. Microbial Cell Factories, 10(1), 114. https://doi.org/10.1186/1475-2859-10-114

[22] Srivastava, S., Yadav, A., Seem, K., Mishra, S., Chaudhary, V., & Nautiyal, C. S. (2008). Effect of high temperature on Pseudomonas putida NBRI0987 biofilm formation and expression of stress sigma factor RpoS. Current Microbiology, 56(5), 453–457. https://doi.org/10.1007/s00284-008-9105-0

[23] Standbury, P.F., Whitaker, A. and Hall, S. J. (1984). P. of F. T. O. B. H. (1984). Principle of Fermentation. Standbury, P.F., Whitaker, A. and Hall, S. J. (1984). Principles of Fermentation Technology. Oxford:

Butterworth Heinemann (2nd ed., Vol. 53). Oxford: Butterworth Heinemann.

https://doi.org/10.1017/CBO9781107415324.004.