

## Characterization of polycyclic aromatic hydrocarbon (PAH) degrading bacteria and optimization of physicochemical conditions for the production of catechol 1, 2-dioxygenase

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### ABSTRACT

Biodegradation of recalcitrant polycyclic aromatic hydrocarbons (PAHs) is highly dependent on the activities of catabolic enzyme and the conditions for metabolism. The most significant studies on process optimization of conditions for catechol 1, 2 dioxygenase (C12O) metabolism of PAHs used “one variable at a time” (OVAT) method, however, with its limitations. In this study, optimization of conditions for optimal C12O metabolism of PAH pollutants using the central composite design (CCD) of response surface methodology (RSM) was used. Enrichment technique using mineral salt medium (MSM) was used to isolate bacteria from oil-polluted water and soil milieu from Awoye community in Nigeria. Thereafter, the bacterial isolates were primarily screened through growth on mineral salt medium plates supplemented with (100 - 200) mM catechol and were subjected to secondary screening based on their initial catechol 1, 2-dioxygenase activity. Molecular tools were used to identify the isolate. Amongst the five (5) bacterial isolates acquired from primary screening, it was found that the cell free extract from isolate FEP B16a displayed the highest enzyme activity. Additionally, isolate FEP B16a was able to grow on MSM plate with 200 mM catechol. Based on CCD of RSM, the C12O produced from isolate FEP B16a had maximum activity at 35 °C, pH 8.0, and 80µM of catechol. Molecular analysis confirmed it as a strain of *Microbacterium hydrothermale* FEP\_B16a. This study concluded that CCD of RSM may be an efficient method to optimize C12O activity over the conventional approach of using single variable at a time.

**Keywords:** Catechol 1, 2-Dioxygenase; Bacteria; Polycyclic Aromatic Hydrocarbon (PAH); Central composite design (CCD) and Response Surface Methodology (RSM)

### Introduction

Organic substances belonging to the group, polycyclic aromatic hydrocarbons (PAHs) is a major environmental problem, due to their ubiquity, persistency in different environments, and capabilities of posing several human and animal health risks such as cancer, liver, and

kidney damages. PAHs are a group of hydrophobic and recalcitrant organic compounds composed of two or more fused aromatic rings in their molecular structure. Although PAHs are released into the environment in aberrant amounts, they can bioaccumulate in the atmosphere, lithosphere, and hydrosphere (Amenu, 2014). PAHs may exist as lower or higher molecular weight depending on the

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number of fused aromatic rings in their molecular structures. Regardless of the forms in which they exist, studies have identified that their occurrence in the environments is associated with various toxic attributes such as: carcinogenicity, genotoxicity, mutagenicity, and teratogenicity (Nwanna *et al.*, 2006; McCarrick *et al.*, 2019; Wang *et al.*, 2019). Thus, there is a need to develop safer degradative paradigms to prevent current and future problems. To remediate PAHs, physical and chemical methods have been utilized (Eldos *et al.*, 2022). The drawbacks of these approaches urge the use of biological methods more of which microorganisms especially bacteria have been employed to degrade PAHs. Microbes solve these limitations as they are safer, more effective, and cost effective. Thus, they serve as promising agents to decontaminate these organic health threats. Microbial degradation of highly recalcitrant aromatic molecules including; PAHs and heterocyclic aromatic compounds are dependent on the activities of catabolic enzymes, and the conditions for metabolism thus, a proper knowledge of these factors is relevant to understand the mechanistic for effective degradation. So far, the ring-cleavage dioxygenase metalloenzymes have been a participant in PAHs degradation (Dhote *et al.*, 2010). They disrupt chemical bonds by adding molecular oxygen into the docile aromatic ring nucleus (Whiteley and Lee, 2006), subsequently initiating the metabolism of PAHs by more precise dioxygenases into catechol through the orthometa-dioxygenase cleavage pathways (Habe and Omori, 2003). The initial enzyme (catechol 1, 2-dioxygenase) cleaves catechol as intradiol (or ortho-cleavage) producing cis, cis-muconic acid (MacLeod and Daugulis, 2005), while the later (catechol-2, 3-dioxygenase) cleaves catechol as extradiol (or meta-cleavage) producing 2-hydroxymuconic semialdehyde. While several studies have explored the conditions for the maximal capability to biodegrade PAHs using C23O (Silva *et al.*, 2012; Hassan *et al.*, 2014; Zeng *et al.*, 2020), little is still known about the

conditions affecting the C12O degradation of PAHs. One limitation for effectuation may be attributed to the extreme catalytic characteristics of C12O (Guzik *et al.*, 2011). The reports on factors influencing PAH degradation have been documented by (Guzik *et al.*, 2009; Giedraityte and Kalėdienė 2009; Guzik, *et al.*, 2011; Guzik *et al.*, 2014; Setlhare, 2019; Briganti *et al.*, 2000; ) Hence, we aim to isolate and screen efficient bacterial strains capable of utilizing PAH as sole carbon and energy source; identify and characterize the bacteria using 16S rRNA molecular techniques and optimize significant factors influencing C12O production using the central composite design (CCD) of response surface methodology (RSM).

## Materials and Methods

### *Reagents and Chemicals*

All chemicals used in this study were of high-purity analytical grade and obtained from commercial sources (Sigma-Aldrich and Merck, Germany). MSM containing crude oil was used as the inducer of the enzyme. The pH of the culture medium was maintained at  $7.0 \pm 0.2$  using 0.1 N ( $H_2SO_4$  and NaOH) and sterilization was carried out at  $121^\circ C$  for 15 min and 15 psi.

### *Description of the Sampling Site*

Water and soil samples contaminated with crude oil were collected from the riverine area of Awoye, in Ilaje Local Government of Ondo State, Nigeria (Sahara Reporters (2020)). The sampling site is located at (longitude  $6^\circ 58'$  N and latitude  $4^\circ 50'$  E).

### *Collection of Samples*

Water samples contaminated with crude oil were collected using sterile bottles of about 50 ml while crude oil contaminated soil samples (100g) were aseptically collected in sterile zip-lock bags. Samples were transported on ice and stored at  $4^\circ C$  until usage.

### **Preparation of Culture Media**

Mineral salt medium (MSM), used for isolation of bacteria and enrichment medium was prepared according to Sowani *et al.*, 2019). The final pH of the MSM medium was adjusted to  $7.0 \pm 0.2$  and sterilized by autoclave at  $121\text{ }^{\circ}\text{C}$  for 15 min.

### **Isolation of PAHs Degrading Bacteria**

Summarily, isolation of PAHs degrading bacteria was done using enrichment technique. The enrichment of bacterial species was carried out in a 250 ml flask containing MSM and naphthalene (25 mg/L) as the carbon source and incubated in a rotary incubator at  $37\text{ }^{\circ}\text{C}$  and 150 rpm for one week. A similar experiment was repeated thrice with a gradual increase in the concentration of naphthalene (50 mg/L-200 mg/L) for microbial adaptation in the PAHs environment. By the end of the 4<sup>th</sup> week, cultures of PAHs-degrading bacteria were obtained by serially diluting 1 ml of culture by dilution factor ( $10^{-3}$ ) and pour plating 200  $\mu\text{L}$  of aliquot on MSM-agar plates supplemented with naphthalene (Sowani *et al.* (2018).

### **Identification and Phylogenetic Analysis of Bacterial Isolate**

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S rRNA target region was amplified from the purified genomic DNA of the bacterial isolate using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the universal primer pairs: 27F-5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R-5'-CGGTTACCTTGTTACGACTT-3'. The Zymoclean™ Gel DNA Recovery Kit was used to extract the gel after the PCR products were run on it (Zymo Research, Catalogue No. D4001). The extracted fragments were purified using Zymo Research's ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050, and then sequenced both forward and backward using Nimagen's

Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3- 100/1000. The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as listed in Section 1. CLC Bio Main Workbench v7.6 was used to analyze the ab1 files generated by the ABI 3500XL/ABI 3730XL Genetic Analyzer and results were obtained by a BLAST search (NCBI). To determine the taxonomic position of the isolate, a phylogenetic tree was developed using the partial 16S rRNA of the isolate, and 10 related sequences to the reference strains from GenBank. CLUSTL omega was used for multiple sequence alignment, and the phylogenetic tree was constructed by maximum approach using MEGA v. 11.0 using bootstrap method with Juke-Cantor algorithm (Hesham *et al.*, 2012).

### **Preparation of Cell-Free Extracts and C12O Activity**

#### **Cell free extract preparation**

The crude extract was prepared by growing the bacterial cells for 36 h in a mineral salt medium supplemented with 100 mg/L naphthalene as an enzyme inducer. The cells were harvested at the late exponential phase of growth by centrifugation at  $10,000 \times g$  for 15 min at  $4\text{ }^{\circ}\text{C}$ . Thereafter, the cells were washed twice with 50 mM phosphate buffer, pH 7.5 (containing 1mM EDTA, and 1mM  $\beta$ -mercaptoethanol to halt protease activity). The cell extract was prepared by sonication with 8 cycles each with a pulse of 30 s on/off for 4 min. The cell extract was centrifuged at  $20,000 \times g$  for 30 min at  $4\text{ }^{\circ}\text{C}$  (Sowani *et al.*, 2019). The clear supernatant was used as a crude extract for enzyme assays, while the remaining extract was stored at  $-4\text{ }^{\circ}\text{C}$  for further investigation.

#### **C12O activity assay**

With a few minor modifications, the descriptions of how to measure C12O activity in a 1 mL

reaction mixture were used (Mahiuddin *et al.*, 2012; Setlhare *et al.*, 2019). The reaction mixture contains 50 mM sodium phosphate buffer and 10 mM catechol (pH 8.0). The reaction was commenced by adding 100  $\mu$ L of crude enzyme into the reaction mixture and incubated for 10 min at 35°C. Buffer plus substrate without the enzyme was used as control. The formation of *cis*, *cis*-muconic acid was measured at 260 nm using UV spectrophotometer (A & E LAB-UK, AE-560-20). One unit of enzyme activity is defined as the amount of the enzyme that produced 1  $\mu$ M of *cis*, *cis*-muconic acid at 260 nm (catechol 1, 2-dioxygenase) under standard assay conditions. Enzyme activity was calculated using the equation:

$$\text{enzyme activity } (\mu\text{m of product formed/min}) = [(\epsilon \times L/V) (\Delta\text{OD/min})] \quad (1)$$

where  $\Delta\text{OD}$  is the change in optical density at 260 nm;  $\epsilon$  is the molar extinction coefficient of the product; “V” is the reaction volume, and “L” is the path length (mm). The molar extinction coefficients 16,800  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  (muconic acid) was used to determine the activity for catechol 1,2-dioxygenase (Mahiuddin *et al.*, 2012; Setlhare *et al.*, 2019). The protein concentration was determined according to the procedure of Bradford, (Bradford, 1976) using bovine serum albumin (BSA) as standard.

### Design of Experiments (DOE)

#### Statistical optimization of independent variables

The central composite design (CCD) of response surface methodology (RSM) was employed for optimizing temperature, pH and substrate concentration for optimal enzyme activity. Subsequently, Design Expert 13.0 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to design and analyze the experiments.

#### Details of experimental design

The optimization of conditions for C12O activity was performed using Design-Expert 13.0 (Stat-

Ease, Minneapolis, MN, USA). The full-factorial central composite design consisted of a complete  $2^k$  factorial design, where k is the number of test variables;  $n_0$  is ascribable to the number of repetition at the center points ( $n_0 \geq 1$ ) and two axial points on the axis of each design variable at a distance of:  $\alpha = 2^{k/4}$  from the design center. In this study, production of C12O was optimized using a  $2^3$ -full factorial central composite design (CCD) with six star-( $\alpha$ )-points (at  $\alpha = \pm 1.682$ ), six replicates at the center points and eight factorial points. The independent variables in this study, were selected based on the perusal of literatures and were optimized by assessing each factor at 5 different levels as presented in Table 1. The selected factors include; temperature (A), pH (B), substrate concentration (C). In the designed experiments, a total of 20 different experimental runs were carried out (Table 2). The maximum and minimum ranges of each variable were determined from literatures. The average C12O activity (U/mg) for two independent determination was taken as the dependent variable for each experimental run.

**Table 1:** Experimental range and levels of independent variables used in central composite design (CCD) for C12O catalytic potential

Independent Variables	Levels				
	$-\alpha$ (-1.682)	-1	0	+1	$+\alpha$ (+1.682)
Temperature (°C): (A)	26.48	35.00	47.50	60.00	68.52
pH: (B)	2.64	4.00	6.00	8.00	9.36
Substrate concentration ( $\mu$ M): (C)	12.96	30.00	55.00	80.00	97.04

All the experiments for C12O activity based on the 20 experimental runs of CCD were assayed. The activity of C12O (Y) was predicted by the accompanying mathematical model in equation (2):

$$Y \text{ (U/mg)} = 5.39 - 0.9790A + 0.4703B + 0.0551C - 0.8024AB - 0.1194AC + 0.2239BC - 0.6815A^2 - 0.2983B^2 - 0.0603C^2 \quad (2)$$

The quadratic model above represents C12O specific activity (Y). A, B, and C are the coded values of temperature, pH, and catechol concentration as described in Table 1. Generally, the F-test from the analysis of variance (ANOVA) determines the statistical significance of a second-order polynomial model from RSM.

### Statistical analysis and modeling

The results obtained in the experimental runs were subjected to analysis of variance (ANOVA) in CCD. It is worth mentioning that a second-order polynomial equation (3), was used to represent the function of the interacting factors and to predict C12O activity.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_{21} + \beta_{22}X_{22} + \beta_{33}X_{23} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (3)$$

where  $Y$  is the measured response,  $\beta_0$  is the intercept term, and  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$  are linear coefficients,  $\beta_{11}$ ,  $\beta_{22}$ , and  $\beta_{33}$  are quadratic coefficients,  $\beta_{12}$ ,  $\beta_{13}$ , and  $\beta_{23}$  are interaction coefficients, and  $X_1$ ,  $X_2$  and  $X_3$  are coded independent variables.

## Results and Discussion

### Screening of PAH degrading bacteria

Bacterial strains having naphthalene degradation potential were isolated from crude oil contaminated water. Initially, fifteen (15) strains were isolated, among which five (5) of the strains showed numerous growths on mineral salt medium plates fortified with 100 mM and 200 Mm catechol and were selected for further study. These strains were observed to have different colonies, shapes, colours, and growths on nutrient agar plates. Among these isolates, one of the strains FEP\_B16a, was believed to effectively utilize catechol as a carbon source and energy. The merit of its growth on mineral salt medium plates fortified with 200 mM catechol was revealed.

### Catechol 1, 2-dioxygenase activity

The strain FEP\_B16a showed the highest initial catechol 1, 2-dioxygenase activity (Table 2). This observation led to the selection of FEP\_B16a in a subsequent study.

**Table 2:** Enzyme activities of cell extracts during the degradation of catechol.

SN	Isolates	Enzyme activity, U/ml
1	FEP_B11	0.027 ± 0.009
2	FEP_B14	0.036 ± 0.012
3	FEP_B15	0.027 ± 0.021
4	FEP_B16	0.042 ± 0.006
5	FEP_B17	0.014 ± 0.004

Data shown represent the mean for two independent determinations ± SD.

**Table 3:** Summary of enzyme activities in cell extract of bacterial strain FEP\_B16a

Isolate	C12O activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)
FEP_B16a	0.140 ± 0.002	0.066 ± 0.003	2.121

Data shown represent the mean for two independent determinations ± SD.

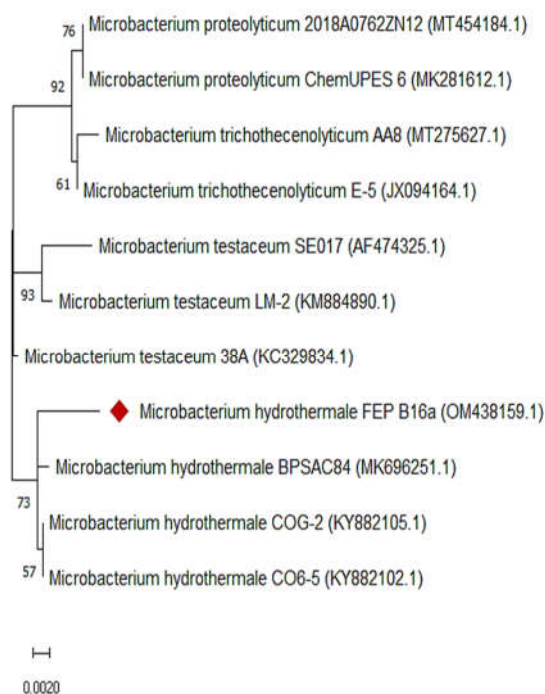
### Molecular characterization

An amplicon of 862 bp size was observed through partial amplification of 16S rRNA and sequencing (Figure 1). Based on the analysis of 16S rRNA sequences, FEP\_B16a had shown the maximum similarity with *Microbacterium hydrothermale* with 99% sequence similarity, and the sequence have been submitted in the GenBank database under the accession number (OM438159.1). To confirm the taxonomic position of the strain FEP\_B16a in phylogeny, a number of sequences representative of some species in the *Microbacterium* genus with relatively high sequence similarity to the identified strain were selected from GenBank and used for the construction of a phylogenetic tree.



**Figure 1:** The visualization of product of partial amplification of 16S rRNA gene. 'A' represents the standard molecular ladder used (1 kb DNA Ladder) while 'B' represents the product of 16S rDNA gene of Bacteria strain FEP\_B16a.

As illustrated in Figure 2, the strain FEP\_B16a and all selected strains of *Microbacterium hydrothermale* are located in the same cluster. Consequently, the strain FEP\_B16a has been identified as *Microbacterium hydrothermale*. Similarly, a number of *Microbacterium* strains have been previously reported for the degradation of PAHs (Sheng *et al.*, 2009; Haleyur *et al.*, 2018). The literature corroborates with our current finding that the isolated *M. hydrothermale* showed the potential for aromatic compound degradation.



**Figure 2:** Phylogenetic analysis of 16S rRNA gene of isolate FEP\_B16a and other related *Microbacterium* spp. by Maximum-Likelihood method. Numbers at the nodes indicate bootstrap support (%) based on 1000 replicates. The scale bar indicates 0.0020 nucleotide substitutions per nucleotide position. GenBank accession numbers are presented in parentheses.

### ***Evaluation of response surface methodology (RSM)***

The CCD, consisting of 20 experiments with five levels of three variables, observed and predicted values of catechol 1, 2-dioxygenase activity are shown in Table 4.

Table 5 exhibits the ANOVA for response surface quadratic model of the titled enzyme. The statistical significance of each of the studied factors were evaluated by observing the p-values (p value < 0.05 = significant) from the analysis of variance table. Based on the observation of Table 3, the model had an F-value of 8180.79. Statistically, this indicates that the model is significant and there was only 0.01% chance that an F-value this large could occur due to noise. In addition, p-values less than

0.05 indicate that a model term is significant and vice-versa. In this case A, B, C, AB, BC, AC, A<sup>2</sup>, B<sup>2</sup>, and C<sup>2</sup> are considered to be significant model terms. The model showed an F-value of 4.62 for “Lack of Fit test,” implying that the lack of fit was insignificant. It is worth noting, that there is only a 5.91 % chance that a Lack of Fit F-value this large could occur due to pure error which is good for the model to fit. To this end, the presented quadratic model in equation 3, is valid and adequate to maximize C12O activity based on the studied parameters. The proximity of the multiple correlation coefficient to 1.0 implies better correlation between the predicted and experimental values. The model showed a relatively high coefficient of determination with an R<sup>2</sup> value of 0.9999 (Table 6). This indicates that the model could explain 99.99 % of the response variability.

Page | 4553

**Table 4:** Full Factorial central composite design (CCD) matrix with their corresponding experimental and predicted responses for C12O production

Run	Coded independent Variables			Observed response (U/mg)	Predicted response (U/mg)
	A	B	C		
17	47.50	6.00	55.00	5.379	5.390
16	47.50	6.00	55.00	5.378	5.390
10	47.50	6.00	55.00	5.405	5.390
20	35.0	4.00	30.00	4.109	4.100
11	68.52	6.00	55.00	1.811	1.820
3	35.00	8.00	80.00	7.003	7.000
13	26.48	6.00	55.00	5.103	5.110
14	47.50	2.64	55.00	3.771	3.750
15	60.00	8.00	30.00	2.900	2.880
9	35.00	8.00	30.00	6.223	6.200
2	35.00	4.00	80.00	3.991	4.010
5	60.00	8.00	80.00	3.200	3.200
6	47.50	6.00	55.00	5.403	5.390
7	47.50	6.00	55.00	5.389	5.390
12	47.50	9.36	55.00	5.311	5.340
4	47.50	6.00	12.96	5.100	5.130
1	47.50	6.00	97.04	5.328	5.310
18	60.00	4.00	80.00	3.400	3.410
8	47.50	6.00	55.00	5.383	5.390
19	60.00	4.00	30.00	3.993	3.990

**Table 5:** Analysis of variance (ANOVA) for catechol 1, 2-dioxygenase activity as a function of independent variables.

Source	Sum of Squares	Df	Mean Square	F-Value	p-value	Inferences
<b>Model</b>	29.29	9	3.25	8180.79	<0.0001	Significant
A-Temperature	13.09	1	13.09	32894.38	<0.0001	
B-pH	3.02	1	3.02	7592.14	<0.0001	
C-Substrate concentration	0.0415	1	0.0415	104.20	<0.0001	
AB						
AC	5.15	1	5.15	12944.62	<0.0001	
BC	0.1140	1	0.1140	286.52	<0.0001	
A <sup>2</sup>	0.4010	1	0.4010	1007.73	<0.0001	
B <sup>2</sup>	6.69	1	6.69	16823.09	<0.0001	
C <sup>2</sup>	1.28	1	1.28	3222.28	<0.0001	
<b>Residual</b>	0.0524	1	0.0524	131.82	<0.0001	
Lack of Fit	0.0040	10	0.0004			
Pure Error	0.0033	5	0.0007	4.62	0.0591	not significant
Cor Total	0.0007	5	0.0001			
	29.30	19				

**NB:** ‘Df’ denotes the degree of freedom of each model terms, ‘A’ denotes temperature, ‘B’ denotes pH, ‘C’ denotes substrate concentration, ‘AB’ denotes interaction between temperature and pH, ‘AC’ denotes interaction between temperature and substrate concentration, ‘BC’ denotes interaction between pH and substrate concentration.

Interestingly, the predicted R<sup>2</sup> of 0.9991 is in reasonable agreement with the adjusted R<sup>2</sup> of 0.9997 since the difference between both R<sup>2</sup> was less than 0.2. The adequacy of the model to

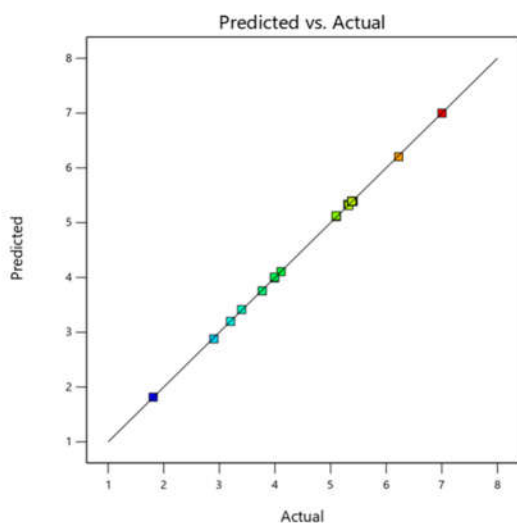
maximize C12O activity of isolate FEP\_B16a is illustrated in Figure 3. The degree of accuracy with which the experiments were associated is represented by the coefficient of variation (CV). A high value of CV indicates a lower reliability of the experiments and vice-versa. More interestingly, this study showed a low CV of 0.4263 % (Table 6).

Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The present study showed a ratio of 367.5312 (Table 6), signifying an adequate signal. Therefore, this model can be used to navigate the design space.

**Table 6. Fit statistic for RSM model.**

Std. Dev. = 0.0199	R <sup>2</sup> = 0.9999
Mean = 4.68	Adjusted R <sup>2</sup> = 0.9997
C.V. % = 0.4263	Predicted R <sup>2</sup> = 0.9991
	Adeq Precision = 367.5312

CV represent coefficient of variation; R<sup>2</sup> represent coefficient of determination.

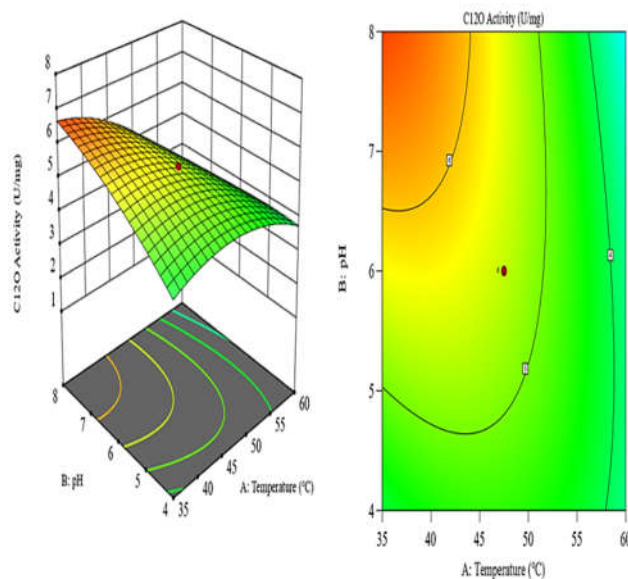


**Figure 3:** Model adequacy plot illustrating the distribution of experimental and predicted values of C120 activity.

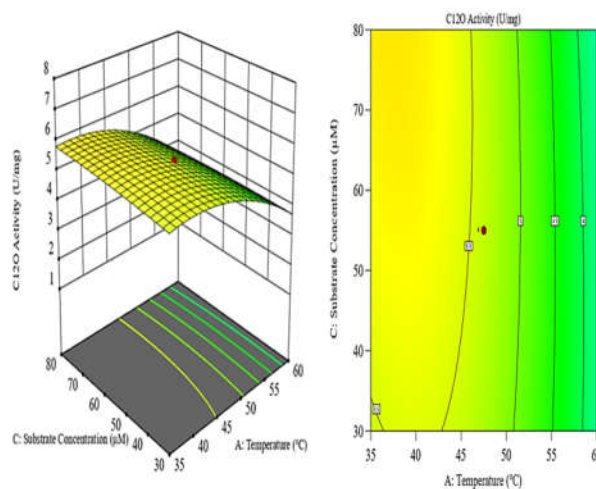
**Effect of interactive process parameters**

**Effect of pH and temperature on C120 activity**

The possible combination of each process variables to maximize C120 activity is illustrated in Figures 4 - 6.

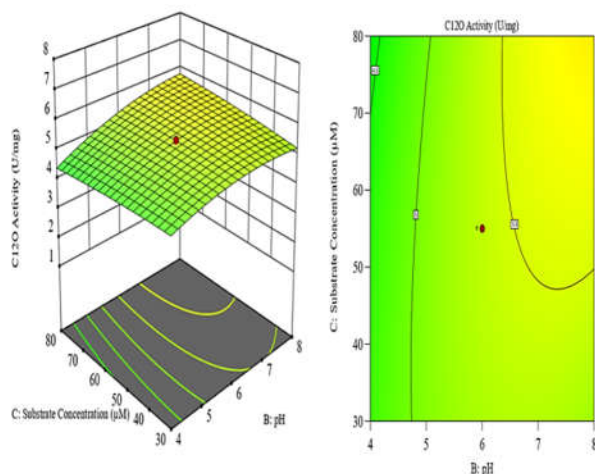


**Figure 4:** Three-dimensional (3D) response surface and contour plot showing the interactive effect of pH and temperature on C120 activity when substrate concentration was kept at its optimal level.



**Figure 5:** Three-dimensional (3D) response surface and contour plot showing the interactive effect of substrate concentration and temperature on C120 activity when pH was kept at its optimal level.





**Figure 6:** Three-dimensional (3D) response surface and contour plot showing how pH and substrate concentration interacted to affect C12O activity when temperature was kept at its ideal level.

Based on the observation of the plots, it was evident that optimal C12O activity were achieved at central levels of each variable. According to Figure 4, maximum C12O activity was achieved when pH and temperature were at their middle to high level. However, there was gradual reduction in C12O activity upon the concomitant decrease or increase of both independent factors. In this context, maximum C12O activity was recorded at pH (6.25 to 8.00). However, temperature had profound influence on C12O activity between 35 °C and 47.5 °C. Similar to the findings from this study, the optimum temperature of 30 °C have been reported for enhanced C12O activity (Silva *et al.*, 2012). Purified C12O retained 81 to 82 % of its activity at 25 °C and 35 °C (Setlhare *et al.*, 2019). The highest activity of C12O from the cell extract of *Pseudomonas putida* N6 was obtained at 35 °C (Guzik *et al.*, 2011). However, further increase in temperature was observed to reduce enzyme activity by 17.4 % (Guzik *et al.*, 2011). Similar observation was also noted when temperature interacted with substrate concentration with increasing level (Figure 5). In line with this study, Miguez *et al.* (1992) have also observed reduction

in enzymatic activity with increasing level of temperature.

In the context of pH, Silva *et al.* (2012) have recorded an optimum pH of 8.0 for highest C12O activity in two different buffers. The result of pH obtained in this study is in agreement with the study by Guzik *et al.* (2013). In their study, they reported that pH-activity and temperature-activity curves showed maximum catechol 1, 2-dioxygenase activity at pH 8.0 and 40 °C using cell extract from *Stenotrophomonas maltophilia* KB2. More also, the report by Setlhare *et al.* (2019) showed that purified C12O retained 84 % and 80 % of its activity at pH 7 and 8 respectively. They further demonstrated that C12O activity reduced significantly at pH 4. The observation of Figures 4 and 5 also showed significant decrease at pH 4 with increasing level of the other interacting variables.

#### ***Effect of substrate concentration and temperature on C12O activity***

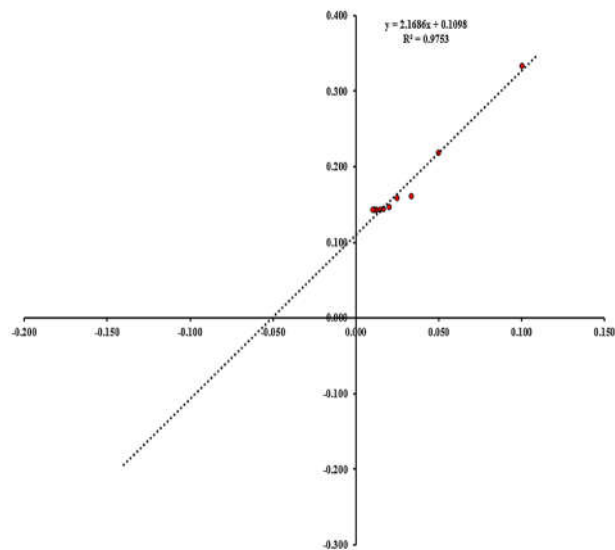
The observation of Figure 6, shows that middle to high levels of substrate concentration recorded elevated C12O activity between 5.675 to 5.807  $\text{Umg}^{-1}$ . According to Figure 6, maximum C12O activity is achievable even at higher level of substrate concentration till about 80  $\mu\text{M}$ . Similarly, Guzik *et al.* (2013) have reported the observation of high C12O activity by crude intracellular enzyme of *Putida strain* N6 at a higher concentration of catechol > 80  $\mu\text{M}$  (Guzik *et al.*, 2011). Nevertheless, lower, or higher level of temperature reduces C12O activity when temperature interacted with substrate concentration (Figure 6).

#### ***Effect of substrate concentration and pH on C12O activity***

In the response surface and contour plot of the interaction between pH and substrate concentration, it is clear that both variables interacted moderately (Figure 7). Nonetheless, C12O activity increased when pH levels are kept at middle to high levels (i.e.,  $\geq 6$ ) with decreasing

concentration of the substrate. We observed a slight decrease in C12O activity when substrate concentration was kept at about 60  $\mu\text{M}$  at middle pH level.

Page | 4556



**Figure 7.** Effect of substrate concentration on catechol 1, 2-dioxygenase activity in isolate FEP\_B16a cell extract.

### Conclusion

The most significant outcome of this study is the optimization of notable physico-chemical conditions influencing C12O activity using CCD of RSM, thereby overcoming the limitations associated with OVAT techniques. This method is efficient and enhanced timeliness in the optimization of C12O activity over the conventional approach of using single variable at a time. In addition, the bacterial strain with the highest degradation ability was identified as *M. hydrothermale* FEP\_B16a. Interesting to note, is that the model satisfies all multi-variable analyses indicating its reliability and adequacy to predict C12O activity for small-large scale utilization from *M. hydrothermale* FEP\_B16a in bioremediation of polluted sites.

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