Optimizing the Bioremediation of Free Cyanide containing Wastewater by *Fusarium oxysporum* grown on Beetroot Waste using Response Surface Methodology

Enoch Akinbiyi Akinpelu, Seteno Karabo Obed Ntwampe, Lukhanyo Mekuto and Elie Fereche Itoba Tombo

***Abstract* -This work reports the use of a cyanide resistant fungus *Fusarium oxysporum* for the bioremediation of wastewater containing free cyanide, its preference for beetroot agro-waste as the primary carbon source, and the optimization of the free cyanide (CN^-) bioremediation conditions using statistical modelling of response surface methodology (RSM). Higher growth rate of *F. oxysporum* was observed on beetroot waste (OD = 3.430) compared to glucose (OD = 1.953). The cultures highest free cyanide biodegraded was 180.9 mg CN^-/L from an initial 300 mg CN^-/L after 72 h at 25°C, pH of 12.70, and substrate concentration of 300 mg/L. The ANOVA of the quadratic model indicated the model obtained is highly significant (R^2 = 0.9240). The response from the central composite design (CCD) indicated that temperature and substrate concentration are significant factors affecting the CN^- biodegradation. The fungus growth on cheap agro-waste would ensure economic sustainability of free cyanide biodegradation system in environmental engineering applications. This study provides a platform for further research on the thermodynamics of CN^- biodegradation.**

***Keywords*- Beetroot, cyanide, *Fusarium oxysporum*, response surface methodology, wastewater**

I. INTRODUCTION

Industrial discharge contains a variety of contaminants from different sources. In most developing countries, more than 70% of industrial wastewater are disposed into usable water bodies which does not only pollute rivers but leach into the water table resulting in groundwater deterioration [1]. This may not be detected for many years as monitoring is non-existent in developing countries. A majority of the toxicants in industrial wastewater are generated in mining, pharmaceutical, refineries industries among others. Poorly treated wastewater discharge into fresh water bodies is common in developing countries due to cost implication of operating wastewater treatment plants.

Cyanide remains the preferred reagent for metal recovery from ores despite its toxicity, thus its presence in process wastewater is unavoidable. Several CN^- chemical treatment methods such as alkaline chlorination, copper catalyzed hydrogen peroxide among others are being used in mining industry [2]. However, due to different challenges associated with poor treatment performance, addition of hazardous chemicals to the wastewater, excess precipitate accumulation, inability to remove ammonia and chlorides, and others, makes microbial remediation of CN^- in wastewater a feasible alternative [3].

There are several reports on biological treatment of cyanide containing wastewater, but very few mineral processing industries have adopted this approach. In addition to the Homestake Mine, (USA), for which this process was determined to be economically viable and sustainable for the treatment of cyanide containing wastewater [4], Outotec has recently designed a biological process known as the Activated Sludge Tailings Effluent Remediation (ASTER^{TM}) for the safe handling of cyanide containing wastewater in South Africa [5, 6]. Some of the microbial remediation methods have focused on the application of bacterial strains such as *Pseudomonas sp*. and *Baccillus sp*. [7, 8] with few studies focusing on *Aspergillus sp*. and *Fusarium sp*. [9, 10]. Although, this process is environmentally friendly, the nutrient requirements for sustenance of microbial growth on a large scale is a challenge. An alternative is the utilization of agricultural waste. When agro-waste is dumped on land, its leachate enters water bodies, causing changes in ecological biodiversity, increasing dead zones which results in eutrophication [11, 12]. With the enormous agro-waste generated annually, some of which contains soluble sugars, trace elements and proteins, it can be used to sustain microbial growth in cyanide biodegradation processes, an important factor to be considered for large scale bioremediation applications [13]. Beetroot has been shown to

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contain more soluble sugars when used raw. *Fusarium oxysporum* has also been shown to produce numerous enzymes when grown on Beetroot [14]. The compatibility of Beetroot and *F. oxysporum* in the degradation of cyanide in wastewater will be significant in the development of suitable biological process.

Therefore, the objective of this study was to (a) isolate and identify a cyanide degrading fungi capable of utilizing agro-waste for cyanide degradation, (b) assess the effect of limiting substrate (Beetroot) on *F. oxysporum* growth, and (c) optimize the operating conditions (pH, temperature and substrate concentration) for free cyanide biodegradation.

II. MATERIALS AND METHODS

A. Isolation and Identification

An isolated fungus identified as *Fusarium oxysporum* from environment containing pesticides was used in this study. The genomic deoxyribonucleic acid (DNA) was extracted using PowerBiofilm DNA kit (MOBIO Lab. Inc., CA- USA) for PCR analysis using the method described earlier with a slight adjustment [15]. The translation elongation factor 1-alpha and internal transcribe spacer (ITS) rDNA sequences were amplified using the primers (EF1 Forward: 'ATGGGTAAGGARGACAAGAC' and EF1 Reverse: 'GGARGTACCAGTSATCATGTT') and (ITS1: ITS 'TCCGTAGGTGAACCTGCGG' and ITS4: ITS 'TCCTCCGCTTATTGATATGC') respectively. The resultant nucleotide sequences was deposited in GenBank database (Accession numbers: KU985430 and KU985431).

B. Substrate Limiting Growth

A volume of 79 mL synthetic wastewater with 1 mL spore concentration of 1.25 % (v/v) was used in a multiport flask of 200mL. The wastewater had similar features with that of Acheampong *et al.* (2013) particularly with regards to metal content. The growth of the isolate was observed on both refined carbon source (glucose) and agro-waste (red beetroot) at a feed rate of 0.05 g/L h. The cultures were incubated in a rotary shaker ZHCHENG (model ZHYWY-1102) at 25°C, pH of 11 and 160rpm. The uninoculated bioreactors served as a control. Samples were taken hourly for optical density measurements in a Jenway 6715 UV/Visible spectrophotometer at a wavelength of 300nm. All experiments were in triplicate. Experimental error was determined using standard deviation obtained from sets of data (n=3).

C. Agrowaste and Inoculum Preparation

Beetroot waste obtained from an agricultural processing facility in Cape Town, South Africa was milled and oven-dried at 60°C for 72 h before being pulverized into a particle size of less than 0.30 mm. Synthetic gold mining wastewater samples containing heavy metals (arsenic, copper, iron, lead and zinc) adapted from Acheampong *et al.* (2013) was used in this experiment [16]. The isolate (1 mL spore solution) was inoculated in 49 mL of wastewater and incubated in an orbital shaking incubator at 70 rpm at various pH, temperature and concentrations for beetroot waste specified in Table 1 for 48 h. Afterwards, free cyanide (as KCN) at concentration of 300 mg CN^-/L was added to the culture subsequent to further incubation for 72 h in the rotary shaker incubator at 70 rpm. An uninoculated culture served as a control at various specified conditions. The pH of the samples was adjusted using 1 M NaOH or 1 M HCl accordingly.

D. Central Composite Design

A Response Surface Methodology (RSM) assesses the influence of parameters in a process that leads to peak performance. The central composite design (CCD) of RSM was used in this study for evaluating three variables; pH, temperature and substrate concentration which gives a minimum number of experimental runs for determining the optimal operating conditions for maximizing free cyanide biodegradation. The range of variables (pH and temperature) was specified based on optimum values reported for most cyanide degrading fungi [9, 17]. The range of the substrate concentration was based on the attainment of an exponential phase in substrate-limiting experiment which was carried out on the isolated *F. oxysporum* –Fig. 1. The Design-Expert® software version 6.0.8 (Stat-Ease Inc., USA) was used to generate the experimental design. A set of 20 runs was carried out consisting of six center points, eight factorial points and six axial points at five different coded levels; -α, -1, 0, +1, and +α. All experiments were in triplicate and the mean of measured values was used to generate the response (*Y*), representing the cyanide biodegraded after 72 h.

$$Y = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \alpha_{ij} X_i X_j + \varepsilon \quad (1)$$

Where $X_1, X_2, X_3 \ldots, X_n$ are the independent coded variables, α_0 is the offset term, α_i, α_{ii} and α_{ij} is linear, squared and interaction effects respectively.

The quantity of free cyanide degraded and volatilized was quantified using the mass balance (2) and (3) respectively.

$$CN_B^- = CN_I^- - CN_R^- - CN_V^- \quad (2)$$

$$CN_V^- = CN_{IC}^- - CN_{FC}^- \quad (3)$$

Where CN_B^- is the bioremediated free cyanide; CN_I^- is the initial free cyanide concentration in the culture broth; CN_V^- is the free cyanide volatilized during incubation; CN_R^- is the residual free cyanide measured after incubation; CN_{IC}^- is the initial free cyanide in the control culture; CN_{FC}^- is the final free cyanide in the control culture.

TABLE I
EXPERIMENTAL DESIGN VARIABLES

Variables	Code	Units	High	Medium	Low
Temperature	A	°C	30	25	20
pH	B	-	11	8.5	6
Substrate concentration	C	mg/L	400	300	200

The control culture was prepared under the same conditions

as other cultures except that it was not inoculated with *F. oxysporum*.

E. Analytical Methods

MERCK® cyanide (CN^-) (09701), ammonium-nitrogen ($NH_4^+ - N$) and nitrate-nitrogen ($NO_3^- - N$) kits were used to analysed the samples for residual free cyanide, ammonium-nitrogen and nitrate-nitrogen using a NOVA 60 spectroquant after incubation for 72 hours. The cyanide test kit measures the free cyanide using the reaction of cyanide with chlorinating agent. The ammonium test kit works using the Berthelot reactions among phenolic compounds, chlorine and ammonia while the nitrate test kits uses the reaction of concentrated sulphuric acid with benzoic acids derivatives to measure nitrate as nitrate-nitrogen. A Crison Basic20 pH meter, calibrated daily was used to measure and/or adjust the pH as stipulated by the CCD.

III. RESULTS AND DISCUSSION

A. Effect of Limiting Substrate on the Growth Rate

The isolate's growth rate increased as the substrate concentration was increased up to a maximum growth rate at which the substrate concentration was 0.3 g/L in both refined and agro-waste carbon source. However, the maximum growth rate on beetroot was higher compared with the growth on glucose -Fig. 1. This could be attributed to the treatment method used for the beetroot which makes more soluble sugars available for the microbial growth as reported previously [18, 19]. Thus, the agro-waste beetroot was selected to be used for this research.

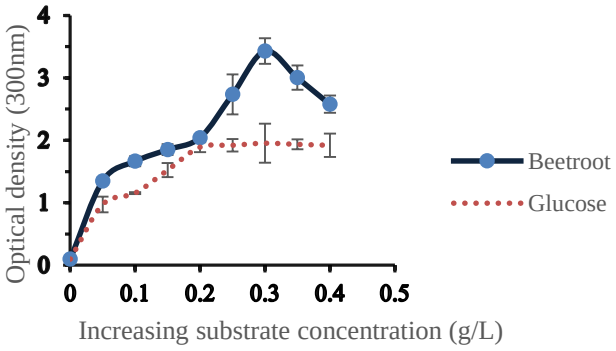

Fig. 1. Effect of limiting substrate concentration on the growth rate of *F. oxysporum*

B. Central Composite Design Response

The response of the individual and interactive effects of the three variables on free cyanide biodegradation is shown in Table II. The results showed random variations in the responses measured, which suggested that the effect of operating conditions and culture components had a direct effect on the isolate's metabolic activity. The highest free cyanide degradation occurred at an axial point (Run 7); at temperature of 25°C, pH of 12.7 and a substrate concentration of 300 mg/L, where 180.90 mg CN^-/L was degraded from an initial 300 mg CN^-/L after 72 h of incubation. The center point (25°C, pH = 8.5, & substrate concentration = 300 mg/L) also showed an appreciable quantity of cyanide degradation of 166.5 mg CN^-/L corresponding to runs 3, 4, 6, 10, 16, and 17. This scenario agreed with previous *F. oxysporum* studies in which it was reported that cyanide degradation was observed at temperature of 25°C and pH of 8.0, with the differentiation in pH observed being attributed to the difference between refined carbon source and agro-waste used in this study. Nonetheless, these studies all indicated that the isolate is alkaline tolerant thus is suitable for cyanide biodegradation [17, 20]. The substrate concentration corresponded to the maximum substrate concentration observed in the substrate-limiting studies shown in Fig. 1. Researchers had shown that *Fusarium sp*. thrives well in cyanide biodegradation at temperature range of 25°C to 30°C and at an alkaline pH [10, 21]. Furthermore, *F. oxysporum* is usually incubated at 25°C under minimal light and with 20°C being used in darkness for optimum growth [22]. The *F. oxysporum* growing on Beetroot produced various enzymes under suitable operating conditions, particularly because these enzymes play major role in cyanide biodegradation [14, 23].

However, some runs (5, 13, 15, and 20) showed extremely low cyanide degradation, an indication of slow and/or minimal microbial growth resulting from low temperature and/or pH which is known to facilitate cyanide efficacy as a metabolic inhibitor [10, 24]. The residual ammonium-nitrogen and nitrate-nitrogen observed were considerably low especially at low pH and/or low temperatures. It was hypothesized that the isolate was utilizing these by-products during the unfavorable conditions. Based on the results in Table II, it would be prudent to optimize around the center point for optimum free cyanide biodegradation, particularly when the influent quality flunctuates.

TABLE II
CODED EXPERIMENTAL DESIGN VARIABLES AND RESPONSES

Run	A	B	C	CN biodeg.(mg/L)		Res.	Res.
				Exp. value	Pred. value	NH_4-N (mg/L)	NO_3-N (mg/L)
1	0	0	α	94.86	100.88	36.40	2.00
2	1	-1	1	102.00	109.36	46.40	15.67
3	0	0	0	166.50	166.62	30.10	2.67
4	0	0	0	166.50	166.62	30.10	2.67
5	-1	-1	-1	76.20	86.61	54.70	2.00
6	0	0	0	166.50	166.62	30.10	2.67
7	0	α	0	180.90	166.90	32.30	36.33
8	0	0	-α	112.29	102.05	37.10	1.00
9	1	1	-1	104.94	129.14	26.10	5.33
10	0	0	0	166.50	166.62	30.10	2.67
11	0	-α	0	120.60	130.39	39.30	2.50
12	α	0	0	117.60	110.99	36.90	4.00
13	-1	1	1	90.00	103.57	65.00	3.67
14	1	-1	-1	128.10	117.51	40.10	2.67
15	-1	-1	1	93.00	71.78	74.70	2.00
16	0	0	0	166.50	166.62	30.10	2.67
17	0	0	0	166.50	166.62	30.10	2.67
18	1	1	1	150.00	142.57	38.90	12.67
19	-1	1	-1	101.19	96.81	54.30	1.00
20	-α	0	0	49.80	52.20	41.40	7.33

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A, B, and C represent the coded level of variables; α represents the axial point with a coded level of 1.682

C. Statistical Model Analysis

The statistical model clarifies the fitness of mean and quadratic models using the Sequential Model Sum of Squares and a Lack-of-Fit Test for the responses measured after 72 h. The free cyanide response was further optimized around the center point since it gives very minimal residual ammonium-nitrogen and nitrate-nitrogen. The responses were analyzed using ANOVA to assess the significance of each variables in the model. A quadratic model was obtained from (1) that relates the free cyanide biodegraded with the independent variables.

TABLE III
ANOVA for free cyanide response quadratic model

Factor	Coeff. Estim	DF	Std. error	F value	Prob>F	Signif
Intercept	166.62	1	5.84	13.51	0.0002	S
A	17.48	1	3.88	20.33	0.0011	S
B	10.85	1	3.88	7.84	0.0188	S
C	-0.35	1	3.88	0.008	0.9304	NS
A^2	-30.06	1	3.77	63.48	<0.0001	S
B^2	-6.36	1	3.77	2.84	0.1230	NS
C^2	-23.03	1	3.77	37.27	0.0001	S
AB	0.36	1	5.06	0.0049	0.9453	NS
AC	1.67	1	5.06	0.11	0.7485	NS
BC	5.40	1	5.06	1.14	0.3116	NS

S = Significant; NS = Not significant; DF = Degree of freedom; "Prob>F" less than 0.05 indicates the model is significant while values greater than 0.1 indicates the model term is insignificant.

The predicted response (Y) for the biodegradation system was:

$$Y = 166.62 + 17.48A + 10.85B - 0.35C - 30.06A^2 - 6.36B^2 - 23.03C^2 + 0.36AB + 1.67AC + 5.40BC \quad (4)$$

Where *A*, *B*, and *C* were the coded values for temperature, pH, and substrate concentration respectively. The coefficient of interaction was estimated from the average of the two confidence levels. ANOVA also showed that five of the ten model terms were significant, and from Table IV, the predicted coefficient of determination (Pred. R^2) is not as close to the adjusted coefficient of determination (Adj. R^2) which is an indicator of a large block effect, thus a model reduction was considered in order to improve the model which gives (5).

$$Y = 166.62 + 17.48A + 10.85B - 30.06A^2 - 23.03C^2 \quad (5)$$

TABLE IV
ANOVA for free cyanide biodegradation in CCD

Source of variation	Sum of squares	DF	Mean square	F-value	Significance
Regression	24948.39	9	2772.04	13.51	S
Residual	2051.44	10	205.14		
Lack of Fit	2051.44	5	410.29	0.000	
Pure error	0.000	5	0.000		
Cor. Total	26999.83	19			

Std.dev. = 14.32; R^2 = 0.9240; Adj. R^2 = 0.8556; Pred. R^2 = 0.3513; Adeq. Precision = 11.325

The quadratic regression model for the free cyanide biodegradation showed that the model is significant at 99.98%, an indication that the total variance in the response could be explained with this model. The Model F-value of 13.51 also support the significance of the model, there is only 0.02% chance of a Model F-value this large could occur due to noise. The adequate precision ratio of 11.325 observed was within a desirable range for signal-to-noise ratio, an indication of an adequate signal which could be used to navigate the design space.

The calculated value of the coefficient of determination (R^2 = 0.9240) showed that 92% of the variations in the actual and predicted values can be explained by the model, with a high degree of correlation between the experimental and predicted values. This showed the accuracy and applicability of the model for predicting free cyanide biodegradation. The suitability of the model was also confirmed by the non-significance of F-value of the Lack-of-Fit Test –see Table IV and normality in the error term as shown in Fig. 2.

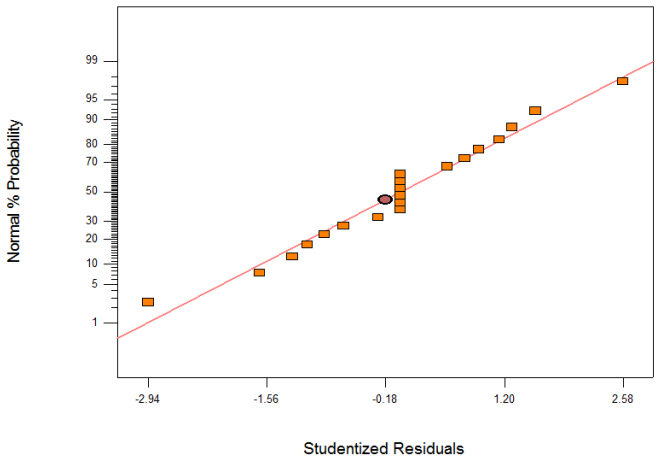

Fig. 2. Normal probability plot of the residuals

D. Graphical Representation of the Model

The level of interaction between independent variables can be determined using 3-D and contour plots. A perfect interaction between two independent variable plots shows an elliptical contour shape while a circular contour represents a non-interactive effect on the system's response [25][25]. For easy of interpretation of experimental results and the prediction of optimum conditions, three-dimensional curves of the system's response was plotted against any two of the variables while keeping the third constant. This allows for the investigation of interactive effects of three independent variables on the system's response. The 3-D and contour plots for all the variable pairs are shown in Fig. 3. The center point was chosen as a constant value. Generally, the quantity of free cyanide biodegraded increases towards the center point. The pair, temperature and pH at constant substrate concentration gave the highest free cyanide biodegradation.

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E. Optimization of Free Cyanide Biodegradation

The optimization of the system's response was done using the numerical option of the Design-Expert® software. The input factors were combined by selecting the desired goal for each variable and the response to achieve peak process performance. In this analysis, all the independent variables, i.e. temperature, pH and substrate concentration were set within range, and the response was set at maximum. Design-Expert® software gave a list of solutions to match the criteria from the least to the most desirable. Fig. 4 shows the desirability ramp generated from 10 solutions through numerical optimization. The optimum point with the highest desirability was selected. Hence, the optimum point with maximum free cyanide biodegradation of 174.148 mg CN^-/L after 72 hours was found at temperature of 26.50°C, pH of 10.77 and substrate concentration of 310.89 mg/L.

IV. CONCLUSION

The *F. oxysporum* growth observed on Beetroot agro-waste showed the importance of mild treatment of the waste for maximum nutrient uptake for microbial growth

The response surface plots identified temperature and substrate concentration as the significant factors affecting free cyanide biodegradation.

The optimum conditions were found at temperature of 26.50°C, pH of 10.77, and a substrate concentration of 310.89 mg/L from the numerical optimization.

The residual ammonium-nitrogen and nitrate-nitrogen formed could serve as a nitrogen source for the isolate when operating conditions are optimized for a single stage nitrification and denitrification.

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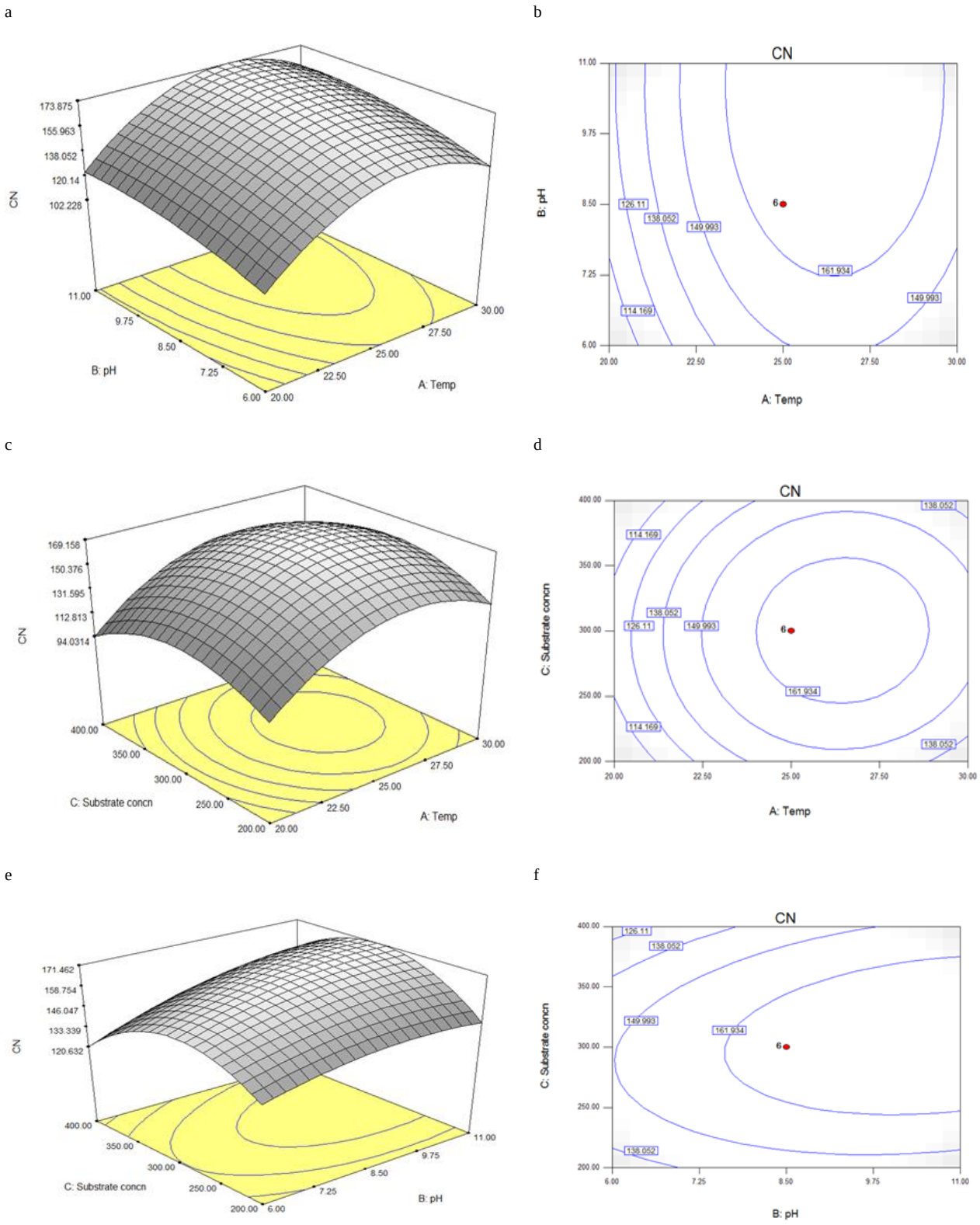

Fig. 3. 3-D plots a, c, and e and contour plots b, d, f showing the effect of independent variables on free cyanide biodegradation

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Fig. 4. Desirability ramp for the numerical optimization of free cyanide biodegradation

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