Biodegradation Kinetics of Free Cyanide in *Fusarium oxysporum-Beta vulgaris* Waste-metal (As, Cu, Fe, Pb, Zn) Cultures under Alkaline Conditions

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The kinetics of free cyanide biodegradation were investigated under simulated winter (5 °C) and optimum conditions (22 °C and pH of 11) using a *Fusarium oxysporum* isolate grown on *Beta vulgaris* waste as a sole carbon source in the presence of heavy metals, *i.e.* As, Fe, Cu, Pb, and Zn. The highest free cyanide degradation efficiency was 77% and 51% at 22 °C and 5 °C respectively, in cultures containing free cyanide concentration of 100 mg F-CN/L. When compared with the simulated winter conditions (5 °C), the specific population growth rate increased 4-fold, 5-fold, and 6-fold in 100, 200 and 300 mg F-CN/L, respectively, for cultures incubated at 22 °C in comparison to cultures at 5 °C; an indication that the *Fusarium oxysporum* cyanide degrading isolate prefers a higher temperature for growth and cyanide biodegradation purposes. The estimated energy of activation for cellular respiration during cyanide degradation was 44.9, 54, and 63.5 kJ/mol for 100, 200, and 300 mg F-CN/L cultures, respectively, for the change in temperature from 5 to 22 °C.

Keywords: Beta vulgaris; Biodegradation; Free cyanide; Fusarium oxysporum

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INTRODUCTION

The ability of cyanide to lixiviate metals has made it a suitable reagent in mineral processing industries; thus its presence in wastewater from these industries is unavoidable (Patil and Paknikar 1999; Dash *et al.* 2009). Currently, free cyanide release into the environment in various forms is estimated to be more than 14 million kg per annum worldwide (Gupta *et al.* 2010). Several treatment methods for cyanide-containing wastewater have focused largely on chemical oxidation methods (Adams *et al.* 2001). However, due to capital and/or operating costs and the production of by-products that require further treatment, these methods are considered ineffective (Gupta *et al.* 2010; Santos *et al.* 2014). Due to environmental concerns associated with chemical oxidation methods for cyanide treatment, biological methods are an appropriate alternative.

Some of the biological treatment methods of treating cyanide have focused on the application of cyanide degrading fungi such as *Aspergillus* sp. and *Fusarium* sp. (Pereira *et al.* 1996; Barclay *et al.* 2002; Santos *et al.* 2013), with several studies establishing sustainable biological processes using bacterial strains including *Klebsiella* sp., *Pseudomonas* sp., and *Bacillus* sp. (Kao *et al.* 2003; Luque-Almagro *et al.* 2005; Chen *et*

al. 2008; Mekuto *et al.* 2013). Although these biological processes have been found to be inexpensive and environmentally friendly (Potivichayanon and Kitleartpornpairoat 2014), their use on a large scale is hampered by nutrient requirements needed to sustain the performance of such processes. In most cyanide degradation studies, the use of refined carbon sources such as glucose and sucrose for microbial growth is reported, which will result in the escalation of operating costs if the designed processes are used on a large scale (Ntwampe and Santos 2013). A suitable alternative to mitigate such costs is to use agricultural waste, which is often discarded. Globally, approximately 1.6 billion tonnes of agricultural waste (agrowaste) are generated annually (Gustavsson *et al.* 2011), some of which contains trace elements, reducible sugars, proteins, and minerals that can sustain bioprocesses designed for cyanide degradation (Mussatto *et al.* 2012). The compatibility of agrowaste and the biocatalyst needed to degrade the cyanide in wastewater will be an important design parameter for such a process to be used on a large scale. One such reported symbiotic relationship is that of *Fusarium oxysporum* and *Beta vulgaris*.

Fusarium oxysporum has been shown to produce numerous enzymes when grown on *Beta vulgaris* (Anuradha *et al.* 2010), and this can be investigated under varying cyanide concentrations. The degradation by-products may also be utilised by the microbial species, depending on process conditions. Since cyanide is a known metabolic inhibitor, the microorganism must be able to overcome the inhibiting effect of cyanide prior to growth; thus a higher activation energy for cellular respiration is required for cyanide degradation to be active. During cellular respiration the microorganism generates energy that is used to breakdown nutrients into usable components. This energy must be greater than the activation energy for microbial growth to occur especially in the presence of an inhibitor, such as cyanide (Erecińska and Wilson 1982; Parolini and Carcano 2010). For free cyanide biodegradation and thus the remediation of the wastewater, other influential operational parameters include temperature and the presence of metals commonly found in cyanide-containing wastewater.

Therefore, the objective of this study was to assess the biodegradation kinetics of free cyanide in wastewater in the presence of metals, *i.e.* iron, zinc, copper, lead, and arsenic, using *Fusarium oxysporum-Beta vulgaris* waste under optimum temperature conditions in comparison to simulated winter conditions existing on the gold reef of South Africa - an area associated with large quantities of effluent containing free cyanide. South Africa's known gold reserve is second only to Australia, representing 11% of the world's total gold reserve (Edelstein 2014). Her gold reef extends across Gauteng province and the Free State province. The minimum temperature during winter in 2014 was ±3 °C in Johannesburg – capital city of Gauteng province and ±8 °C for Bloemfontein – capital city of Free State province, according to the South African Weather Service. Since, the averaged mean minimum winter temperature for the gold reef was estimated at 5 ±0.5 °C in 2014; therefore, an average temperature of 5 °C was used to represent winter temperature conditions in this study.

MATERIALS AND METHODS

Source of the Fungal Isolate

Isolated *Fusarium oxysporum* from an environment saturated with cyanide containing pesticides was cultured on potato dextrose agar (PDA) plates and incubated at 37 °C for 5 days. The spores and mycelia from the plates were harvested using sterile

distilled water, subsequent to filtration through a glass wool to separate the spores from the mycelia. A dilution series was performed to quantify the spore concentration. The absorbance and spore concentration for each of the dilutions was quantified in duplicate using a Jenway 6715 UV/Visible spectrophotometer set at a wavelength of 300 nm using sterile distilled water as a blank, while the latter was performed using a direct count system in a Marienfeld Neubauer cell-counter and a Nikon Eclipse E2000, phase contrast 1 and 100 X magnification, to develop an absorbance-spore concentration calibration graph.

Preparation of Metal-Containing Synthetic Wastewater Samples

The wastewater sample used in this study was adapted from Acheampong's research, which indicated that cyanide containing gold mining wastewater contains heavy metals (upto concentration listed); arsenic (7.1 mg/L), iron (4.5 mg/L), copper (8.0 mg/L), lead (0.2 mg/L), and zinc (0.2 mg/L) (Acheampong *et al.* 2013).

Beta vulgaris Preparation

Beta vulgaris waste was obtained from a fruit and vegetable company, Cape Town, South Africa. The agrowaste was used as a sole carbon source in the experiments. Subsequent to the collection of the waste, it was dried at 80 °C for seven days and milled to less than 100 µm using a grinder (Bosch MKM 7000). The agrowaste was mixed with distilled water and autoclaved at 116 °C for 15 min and then cooled to room temperature. The solution was filtered through a No. 1 Whatman filter paper in a Bruchner funnel under vacuum and the filtrate was used for the experiments.

Nutrient Media and Culture Conditions

Optimisation of bioreactor conditions for maximum cyanide biodegradation

To optimize cyanide biodegradation efficiency, a central composite design (CCD) of response surface methodology (RSM) was used to determine the optimum operating conditions for maximum cyanide biodegradation at elevated cyanide concentrations of 500 mg F-CN/L. The Design-Expert® software version 6.0.8 (Stat-Ease Inc., USA) was used to generate the experimental design. Each independent variable (temperature-*A* and pH-*B*) was analysed at five different coded levels; -β, -1, 0, +1, and +β, representing a factorial, centre, and axial points (Table 1). Each sample was inoculated with 2% (v/v) of the inoculum in multiple-port airtight shake flasks to minimize volatilization of cyanide as HCN gas. The uninoculated broth served as a control at varying specified condition. The pH of the samples was adjusted using 1 M NaOH or 1 M HCI, accordingly. All experiments were in duplicate, and the mean of measured values was used to generate the response (*Y*), which is the cyanide biodegradation. The response (*Y*) of the biodegradation process can be represented by the quadratic model,

$$Y = \propto_o + \sum_{i=1}^{n} \propto_i X_i + \sum_{i=1}^{n} \propto_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \propto_{ij} X_i X_j + \varepsilon \quad (1)$$

where $X_i, X_j \ldots\ldots, Xn$ are independent variables, ε is the random error, $\propto_o$ is the offset term, and the variables $\propto_i, \propto_{ii}$ and $\propto_{ij}$ are the linear, squared, and interaction effects, respectively.

Table 1. Coded Experimental Design Variables

Run	A	B
1	0	0
2	-1	1
3	0	0
4	1	1
5	1	-1
6	0	0
7	-1	-1
8	0	0
9	0	β
10	β	0
11	-β	0
12	0	-β
13	0	0
14	0	0

A and B represent the coded level of variables while β represents the axial point with coded level of 1.4142

Synthetic wastewater of 20 mL with 1 mL spore solution (2.25×10^6) of *Fusarium oxysporum* was added to 10 mL of the *Beta vulgaris* waste medium (0.0098 g/mL) as the sole carbon and energy source and incubated for 48 h in a rotary shaker at 70 rpm under operating conditions as specified in Table 1. Afterwards, 20 mL of dissolved KCN in distilled water was added to the flask to make a culture with a final cyanide concentration of 500 mg CN^-/L and culture volume of 51 mL. Subsequently, the culture broth was incubated for 72 h in the rotary shaker ZHCHENG model (ZHYWY-1102) incubator at 70 rpm, to determine the optimum temperature conditions with the highest cyanide biodegradation of from an initial concentration of 500 mg F-CN/L over a period of 72 hours incubation.

Comparison of optimum and simulated winter conditions for cyanide biodegradation

For optimisation studies, maximum cyanide biodegradation was achieved at a temperature of 22.32 °C and pH of 11. A temperature of 5 °C was used to represent average winter temperature conditions observed in 2014 on the gold reef of South Africa. In this part of the study, 20 mL of the *Beta vulgaris* waste extract (13% v/v) was used, to which 1 mL of an overnight grown inoculum - 0.7 % (v/v), was added. A volume of 49 mL of the metal containing synthetic wastewater, and 80 mL of dissolved potassium cyanide (KCN) in phosphate buffer (pH = 11) was added to the flask to make a final volume of 150 mL. The free cyanide concentrations used for this part of study were 100, 200 and 300 mg F-CN/L. The cultures were incubated in a rotary shaker ZHCHENG model (ZYYWY-1102) for optimum conditions while a SR13 SHELL LAB BOD refrigerated incubator model LI5-2 was used to simulate conditions. All cultures were at 70 rpm for a week. Free cyanide volatilisation was quantified using Eq.(s) 2 and 3. Two sets of control cultures were prepared; one without cyanide inhibition to monitor the growth of *Fusarium oxysporum*, and the other without *Fusarium oxysporum* under similar conditions as those used for cyanide containing cultures.

$$CN^-_B = CN^-_I - CN^-_R - CN^-_V \quad (2)$$

$$CN^-_V = CN^-_{IC} - CN^-_{FC} \quad (3)$$

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

Where CN_B^- was the free cyanide bioremediated; CN_I^- was the initial free cyanide concentration in the culture broth; CN_V^- was the free cyanide that volatilised during culture incubation; CN_R^- was the residual free cyanide concentration measured after incubation; CN_{IC}^- was the initial free cyanide concentration in control culture (100, 200, and 300 mg F-CN/L); and CN_{FC}^- was the final free cyanide concentration in the control culture. The control was prepared under the same conditions as other cultures except for the absence of *Fusarium oxysporum*. The biodegradation efficiency (classified as the removal efficiency) was calculated using Eq. 4.

$$Biodegradation\ Efficiency\ (Removal\ rate) = \frac{CN_B^-}{CN_{FC}^-} * 100 \quad (4)$$

Analytical Methods

Samples were collected every 24 h, then centrifuged at 13000 rpm for 5 min using a Haraeus Megafuge 1.0 before analysis using MERCK® cyanide (CN^-) (09701), ammonium-nitrogen (NH_4^+-N) (00683) and nitrate-nitrogen (NO_3^--N) (14773) kits to quantify the residual concentration of free cyanide, ammonium-nitrogen and nitrate-nitrogen using a NOVA 60 spectroquant. The cyanide test kits measure cyanide as free cyanide via the reaction of a chlorinating agent with cyanide. The ammonium test kits works on the reaction of ammonia with chlorinating agents, and ammonium is measured as ammonium-nitrogen. The nitrate test kits measures nitrate as nitrate-nitrogen in the reaction of concentrated sulphuric acid with benzoic acid derivatives.

RESULTS AND DISCUSSION

Free Cyanide Degradation Efficiency

Under optimum temperature (22 °C) conditions, the *Fusarium oxysporum* cultures showed a removal efficiency of 77%, 58%, and 62% for cultures containing 100, 200, and 300 mg F-CN/L respectively (Fig. 1), within 144 h. Free cyanide loss due to volitilisation was about 2.7%, 2.2%, and 3.2% for 100, 200, and 300 mg F-CN/L, respectively (Fig. 2). This is an improvement from a previous study when white rot fungus, *Trametes versicolor* removal efficiency of F-CN was less than 30% (Cabuk *et al.* 2006). Some researchers reported higher cyanide removal efficiency of 90% (Ezzi and Lynch 2005; Campos *et al.* 2006). The residual ammonium-nitrogen concentration fluctuated between 70 to 210 mg NH_4^+-N/L throughout the experiments. A high residual ammonium-nitrogen concentration in cultures inhibited cyanide degradation due to the microorganism's preference for it as a nutritional source in the presence of cyanide. The residual nitrate-nitrogen formed was between 61 mg NO_3^--N/L and 102 mg NO_3^--N/L over the period under observation. Under simulated winter conditions (5 °C), the free cyanide removal efficiency was between 40 and to below 60% with residual cyanide concentrations being 39 mg F-CN/L for 100 mg F-CN/L, 104 mg F-CN/L for 200 mg F-CN/L, and 155 mg F-CN/L for 300 mg F-CN/L, respectively, for an observation period lasting 144 h (Fig. 3 and 4). Volatilisation of free cyanide was about 3.3%, 2.8%, and 4.2% for 100, 200, and 300 mg F-CN/L, respectively. The simulated winter conditions affected the activity of the microorganism. Previous research has recognised that a temperature drop in winter is a key inhibitor to microbial

activity, thus resulting in low removal of contaminants (Zilouei *et al*. 2006; Zou *et al*. 2014). The high amount of residual ammonium-nitrogen (up to 50 mg NH_4^+-N/L) and nitrate-nitrogen (up to 140 mg NO_3^--N/L) showed the impact of the temperature on cyanide biodegradation efficiency.

Free Cyanide Degradation Kinetics

Assuming first order kinetics, *i.e. d[CN])/dt= -k[CN]*, was used in an Ordinary Differential Eq. (ODE) solver (Polymath version 5.0) to simulate the cyanide biodegradation kinetics. The rate of cyanide degradation was higher at F-CN concentration of 100 mg F-CN/L, which was observed to be reduced as the cyanide concentration was increased to 200 mg F-CN/L and subsequently to 300 mg F-CN/L. Similarly, as the temperature was reduced, the rate of cyanide degradation also reduced (Table 2).

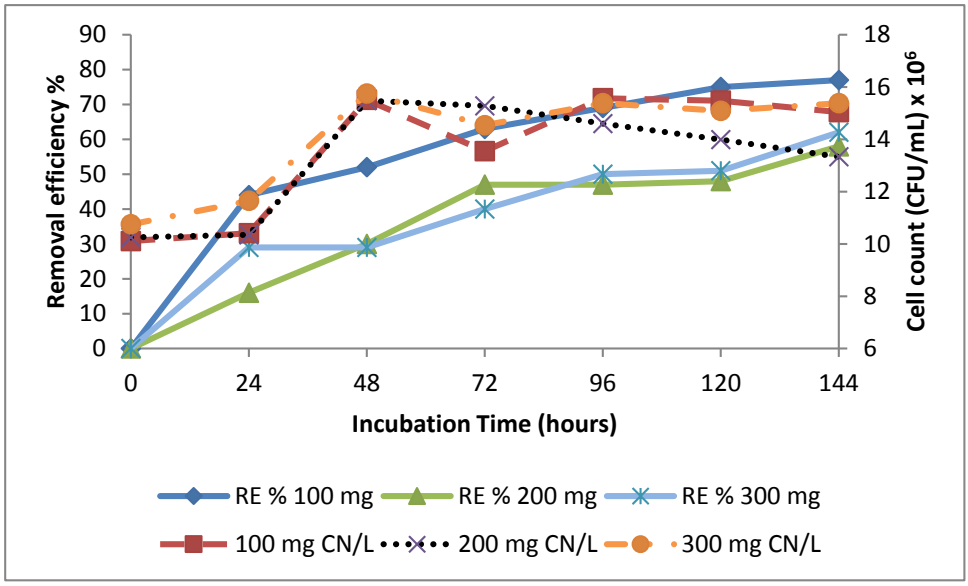

Fig. 1. Relationship between free cyanide degradation efficiency and *Fusarium oxysporum* growth at operating temperature of 22 °C

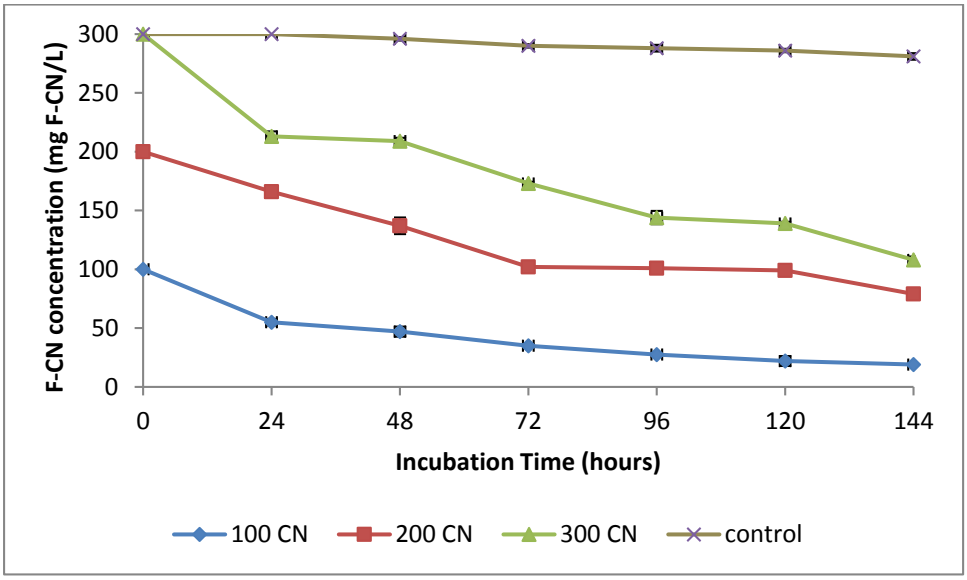

Fig. 2. Free cyanide degradation in the culture at temperature 22 °C

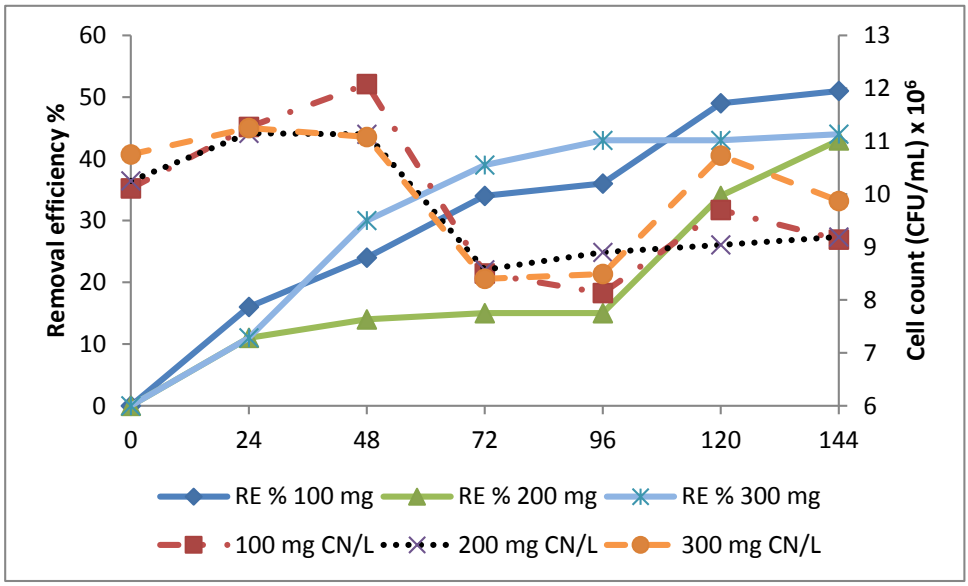

Fig. 3. Relationship between free cyanide degradation removal efficiency and *Fusarium oxysporum* growth at operating temperature of 5 °C

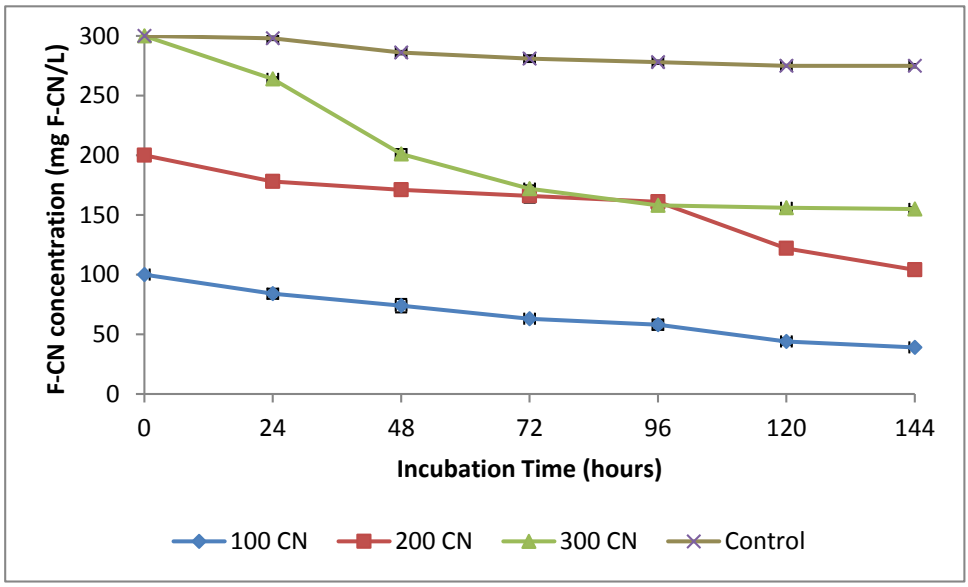

Fig. 4. Free cyanide degradation in the culture at temperature 5 °C

Table 2. Ordinary Differential Equation Solver Input Parameters

Rate Eq.: $\frac{d[CN]}{dt} = -k[CN]$		
Time: 0 h < t < 144 h		
C(0) = $[CN^-]_{t=0}$	Temperature (22 °C)	Temperature (5 °C)
100	k = 0.0115 h^{-1}	k = 0.0065 h^{-1}
200	k = 0.0065 h^{-1}	k = 0.0045 h^{-1}
300	k = 0.0071 h^{-1}	k = 0.0046 h^{-1}

The high degradation rate observed may be attributed to the sufficient enzyme activity aided by suitable bioreactor conditions and the utilisation of *Beta vulgaris* waste extract which contains soluble sugars, minerals and proteins to support enzyme production.

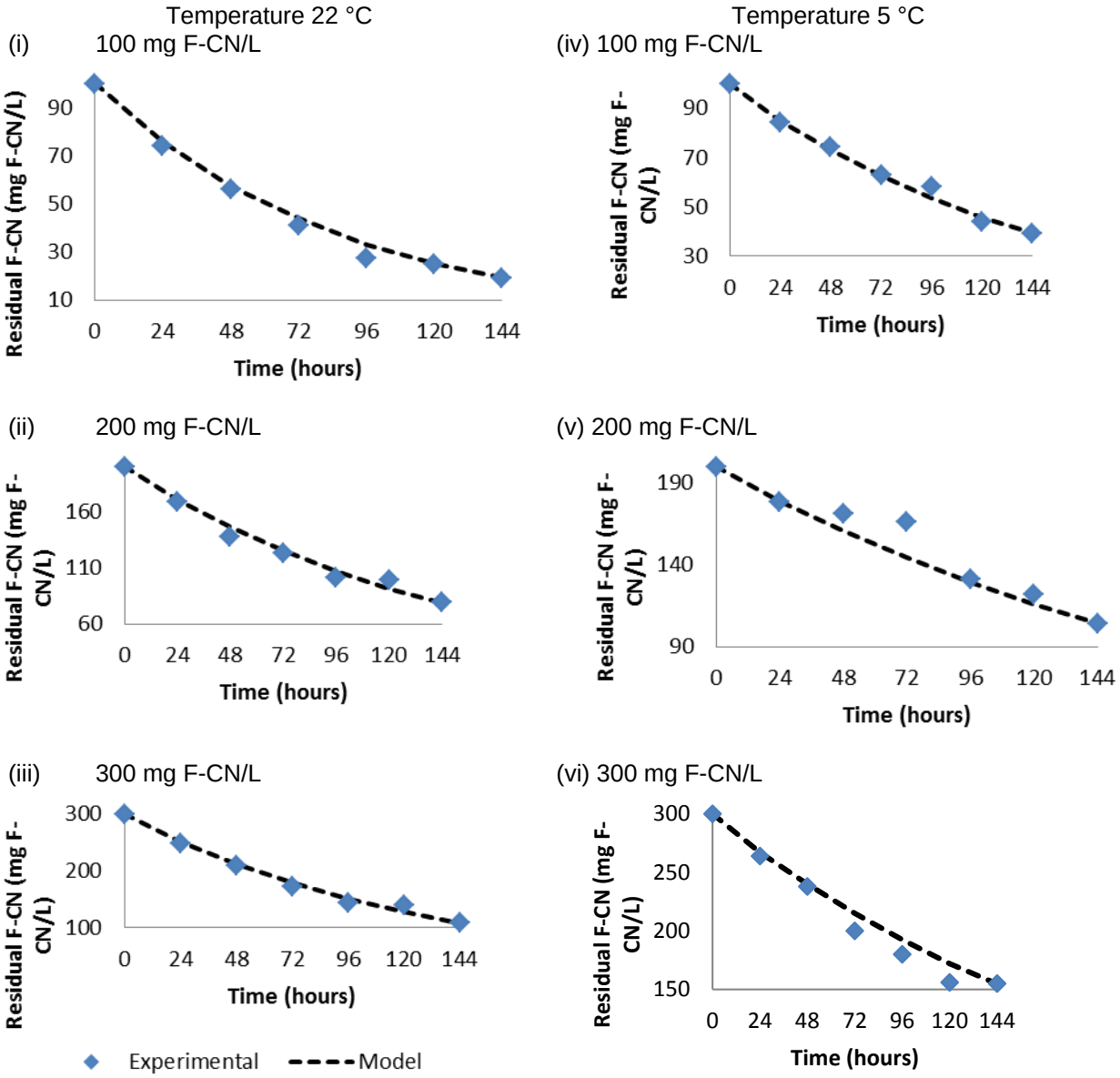

Fig. 5. Comparison between experimental and modelled bioremediation kinetics at temperatures 22 °C (i, ii and iii) and 5 °C (iv, v and vi)

Earlier reports have shown that substrates such as *Beta vulgaris* have a large content in pectin, reducing sugars, cellulose, and protein (Mulligan 2005; Anuradha *et al*. 2010; Amodu *et al*. 2014). Anuradha *et al*. (2010) showed that *Fusarium oxysporum* is capable of producing numerous enzymes when grown on *Beta vulgaris*, orange peel, carrot peel, and pineapple peel. Figure 5 shows the accuracy between the model and experimental values with correlation of coefficients (R^2) between the model and experimental values being 0.9567 (100 mg F-CN/L), 0.9856 (200 mg F-CN/L), and 0.9828 (300 mg F-CN/L)

at 22 °C while for the simulated winter conditions were determined to be 0.9856 (100 mg F-CN/L), 0.9930 (200 mg F-CN/L) and 0.9927 (300 mg F-CN/L).

Fusarium oxysporum Growth Kinetics in Cyanide

The cell concentration in the medium varied depending on the quantity of cyanide concentration in the culture. From Fig. 6 and 7, it was apparent that the cyanide concentration inhibited the fungus microbial growth. Under a temperature of 22 °C, the maximum cell concentration in 100, 200, and 300 mg F-CN/L cultures was 1.557 x 10^7 CFU/mL, 1.548 x 10^7 CFU/mL, and 1.575 x10^7 CFU/mL, respectively (Fig. 6). The control experiment with no cyanide in the culture has maximum cell concentration of 2.317 x10^7 CFU/mL.

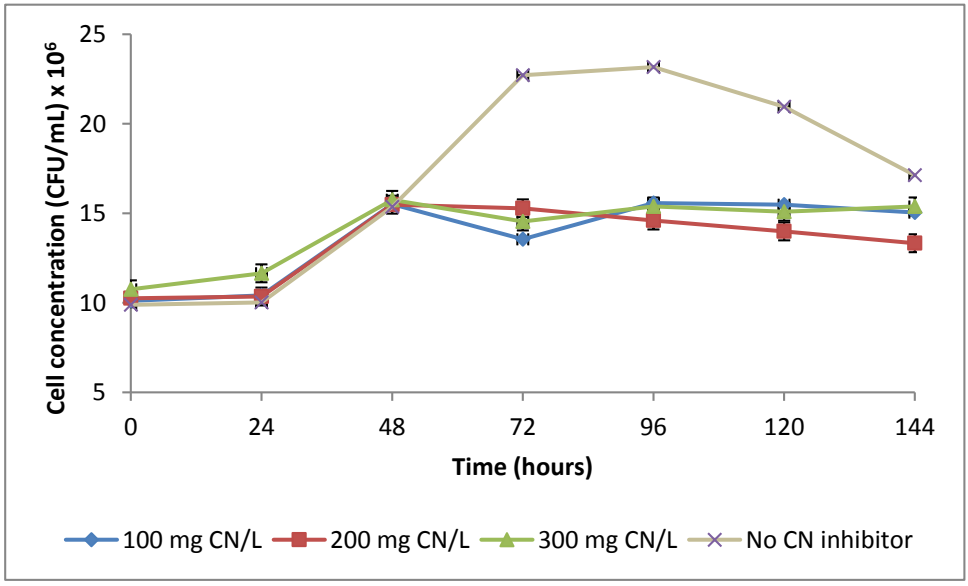

Fig. 6. *Fusarium oxysporum* growth at temperature 22 °C

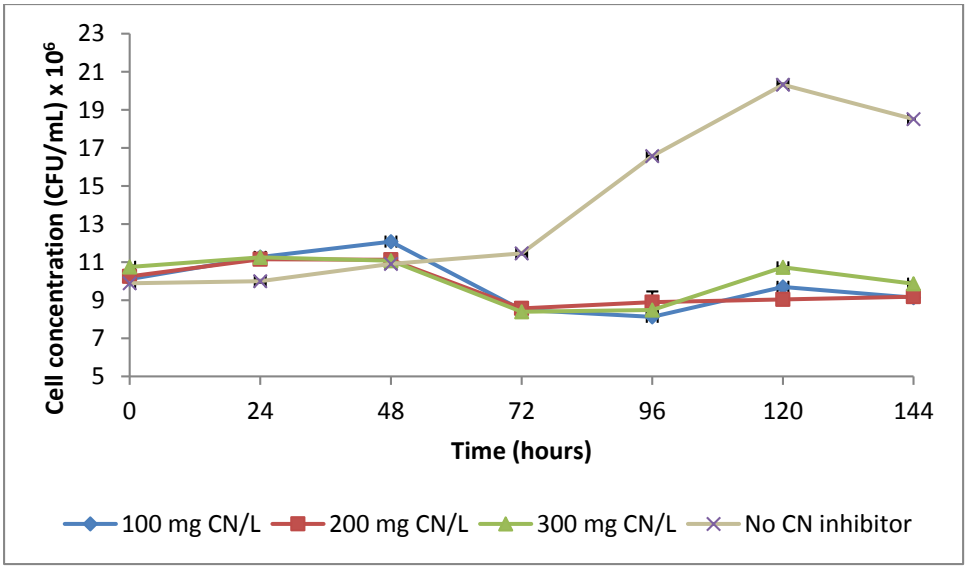

Fig. 7. *Fusarium oxysporum* growth at temperature 5 °C

The maximum growth rate observed was 0.0166 h^{-1}, 0.0168 h^{-1}, and 0.012 h^{-1} for 100, 200, and 300 mg F-CN/L cultures, respectively. Under a temperature of 22 °C, the microorganism had an ability to grow in medium containing cyanide at concentration up to 200 mg F-CN/L, without any observable impediments, which was also reflected in the doubling time (Table 3).

Table 3. *Fusarium oxysporum* Growth Kinetics Parameters

F-CN concentration (mg F-CN/L)	Temperature 5°C		Temperature 22°C	
	μ_m (h^{-1})	t_d (h)	μ_m (h^{-1})	t_d (h)
100	0.0045	154	0.0166	42
200	0.0035	198	0.0168	41
300	0.0019	365	0.0120	58
No F-CN	0.0154	45	0.0178	39

The maximum cell concentration under simulated winter conditions (5 °C) for 100, 200, and 300 mg F-CN/L was 1.208 x10^7 CFU/mL, 1.115 x10^7 CFU/mL, and 1.125 x 10^7 CFU/mL, respectively, while for non-cyanide cultures was 2.032 x10^7 CFU/mL (Fig. 7). The maximum growth rate of 0.0045 h^{-1}, 0.0035 h^{-1}, and 0.0019 h^{-1} for 100 mg F-CN/L, 200 mg F-CN/L, and 300 mg F-CN/L, cultures was observed. As a result of the reduction in growth rates under a cold temperature, the microbial doubling time considerably increased (Table 3).

The effect of temperature on cellular respiration was investigated by estimating the activation energy for metabolic respiration using a modified Arrhenius model for microbial activity, *i.e.* Eq. 5 (Shuler and Fikret 2002),

$$ln\left(\frac{\mu_{m1}}{\mu_{m2}}\right) = \left(\frac{1}{T_2} - \frac{1}{T_1}\right)\frac{E}{R} \quad (5)$$

where μ_{m1} and μ_{m2} are the maximum specific growth rate at temperature 22 °C and 5 °C, respectively.

Table 4. Activation Energy for Metabolic Respiration in *F. oxysporum* Cultures

F-CN concentration (mg F-CN/L)	Activation energy, *E* (kJ/mol)
100	44.9
200	54.0
300	63.5
No F-CN	4.9

The values of activation energy obtained (Table 4) reflect the increase in the specific growth rate with temperature increases from 5 to 22 °C, as observed in Table 4. The value of the activation energy increased with an increase in the concentration of free cyanide. The highest value of activation energy (63.5 kJ/mol) recorded in 300 mg F-CN/L cultures explains the reason for low growth rates observed resulting from impaired microbial activity due to the high cyanide concentration. Generally, temperature affects the configuration of microbial cell constituents, especially membrane components. In most

cases, for every 10 °C rise in temperature, there is a two-fold increase in the specific growth rate (Shuler and Fikret 2002). In this study, for the 17 °C rise in temperature from 5 to 22 °C, the specific growth rate increased approximately 4-fold, 5-fold, and 6-fold in 100 mg F-CN/L, 200 mg F-CN/L and 300 mg F-CN/L, respectively; an indication that the *Fusarium oxysporum* isolate used in this study from a cyanide containing pesticide saturated environment, is most likely suitable for cyanide degradation processes operated at a higher temperature.

CONCLUSIONS

1. The degradation efficiency of free cyanide under optimum conditions was higher at temperature 22 °C and a pH of 11 in comparison to simulated winter conditions (5 °C); which led to a higher concentration of residual ammonium-nitrogen and nitrate-nitrogen formed especially at elevated concentrations of free cyanide. Although the residual by-products can serve as a nitrogen source for the fungus, there was an indication of incomplete metabolism. This can be enhanced by changing the operating conditions such that the microbial system can subsequently nitrify and denitrify residual by-products after cyanide biodegradation.
2. The ordinary differential Eq. (ODE) model used to describe the cyanide removal rate was determined within a 95% confidence level, with means and standard deviations for k values at 5 °C and 22 °C being: 0.0052 (± 0.0011) h^{-1} and 0.0084 (± 0.0027) h^{-1}, respectively.
3. The application of the Arrhenius model described the estimation of the energy of activation for cyanide biodegradation. The activation energy for metabolic respiration was estimated at a minimum of 44.9 kJ/mol was needed for microbial growth in order to achieve maximum cyanide degradation rates in cultures with low cyanide concentrations.

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