Operating conditions for the continuous bioremediation of free cyanide contaminated wastewater using *Aspergillus awamori*

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ABSTRACT

Generation of cyanide-containing wastewater is a growing problem worldwide as numerous cyanide complexes are highly unstable and degrade to form free cyanide (F-CN), the most toxic form of cyanide. Agro-waste materials, such as sweet orange (*Citrus sinensis*) waste from the citrus industry, are rich in readily metabolisable carbohydrates that can supplement microbial activity and thus support biodegradation of toxic compounds in wastewater. This study reports on optimal operating conditions for the continuous biodegradation of F-CN in wastewater using an *Aspergillus awamori* isolate in a process supported solely using *C. sinensis* waste extract. The optimal degradation conditions were pH 8.75 and 37.02 °C with the isolate's F-CN tolerance being observed up to 430 mg F-CN/L. Furthermore, the ammonium produced as a by-product of F-CN degradation was also metabolised by the *A. awamori*, with negligible residual citric acid and formate being observed in the effluent post treatment. This study demonstrates the feasibility of using agricultural waste as a primary and sole carbon source for the cultivation of a cyanide-degrading *A. awamori* species for F-CN degradation under alkaline conditions.

Key words | *Aspergillus awamori*, bioremediation, *Citrus sinensis*, free cyanide

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INTRODUCTION

Various industries release a combination of free cyanide (F-CN) and cyanide complexes into the environment via a variety of disposal methods, particularly wastewater. These industries utilise cyanide-based compounds in various operations, including the beneficiation of metals, electroplating, case hardening, automotive manufacture, circuit board manufacture and chemical manufacture (Patil & Paknikar 2000). Cyanide is often found in organic hydrocarbon chains or as inorganic, transition, alkali and alkaline earth metal complexes (Nesbitt 1996). Many cyanide complexes are highly unstable, thus temperature, pH and light can degrade the components to form F-CN, which is the most toxic form of cyanide (Nesbitt 1996; Rao *et al.* 2010).

There is an overwhelming popularity in industry for the use of chemical methods for the treatment of F-CN and cyanide complexes rather than biochemical treatment methods. However these methods can: (1) be ineffective for certain cyanides; (2) produce by-products that require further treatment to meet discharge standards; (3) involve high reagent/chemical costs; (4) use reagents/chemicals that are toxic to the environment; and (5) require specialised equipment and maintenance with high capital or royalty costs (Nesbitt 1996; Patil & Paknikar 2000, Gupta *et al.* 2010).

Several microorganisms, bacteria such as *Nocardia* sp. and *Rhodococcus* sp., fungi such as *Aspergillus* sp. and *Fusarium* sp. and algae such as *Arthrospira* sp. and *Scenedesmus* sp., possess enzymatic mechanisms able to bioremediate F-CN and cyanide complexes (Akcil *et al.* 2003; Rao *et al.* 2010). However, limited studies have been conducted using organic waste and fungal strains in cyanide bioremediation (Rao *et al.* 2010).

Solid waste generation in South Africa is a problem growing at an exponential rate, with the majority of landfill sites reaching maximum capacity. Approximately 427×10^6 t of solid waste is generated in South Africa every year, of which 40% by mass is organic waste (Greben & Oelofse 2009). The average amount of solid waste generated per person in South Africa is 0.7 kg/annum, which is closer to that of developed countries such as 0.723 kg/annum in the United Kingdom and 0.87 kg/annum in

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Singapore, than for developing countries, such as 0.30 kg/annum in Nepal (Greben & Oelofse 2009). It is therefore sensible to bioaugment biotechnological processes to utilise agro-waste materials, particularly from industries that produce large quantities of organic waste, as a nutrient supplement for a biological process such as the bioremediation of F-CN.

MATERIALS AND METHODS

Microorganism isolation and identification

Aspergillus awamori was isolated from a cyanide-contaminated municipal wastewater discharge facility located in the Western Cape, South Africa. Swab samples were taken at various points along the discharge points and grown on 1% (w/v) citrus pectin agar plates incubated at 37 °C for 5 days. After incubation, the mycelia were transferred to potato dextrose agar (PDA) plates supplemented with 2% (v/v) penicillin (10^4 U/mL)-streptomycin (10 mg/mL) and incubated at 37 °C for 5 days. The strain was identified using a combined ITS, β-tubulin and calmodulin gene regions technique, a technique that distinguishes between *A. niger* and *A. awamori*, which share similar morphological characteristics (Varga *et al.* 2011; Santos *et al.* 2013).

F-CN-PDA plate preparation

Double-strength PDA was prepared by measuring twice the amount of recommended PDA powder, followed by autoclaving the suspension at a temperature of 121 °C for 15 minutes and cooling it to 50 °C using a hot plate and magnetic stirrer. A series of 80 mL F-CN-PDA solutions were prepared in sterile 100 mL Schott bottles using a 1,000 mg F-CN/L solution (2.5 g KCN/L), with gradual addition of the cyanide solution, to create F-CN-PDA petri dishes with varying F-CN concentrations. Each of the F-CN-PDA petri dishes was inoculated with the *A. awamori* isolate by transferring it from F-CN free PDA petri dishes using a flame-sterilised loop. To reduce volatilisation of F-CN, the petri dishes were sealed with a parafilm and incubated for 5 days at 37 °C. The F-CN concentration in the controls was verified at the end of the incubation period using a Merck cyanide (CN^- 09701) test kit to ascertain the integrity of the experiments. The F-CN-PDA petri-dish cultures were used to assess the isolates tolerance to high F-CN concentrations and for subsequent use in liquid cultures.

Hydrolysed *Citrus sinensis* extract

Sixty grams of powdered *C. sinensis* waste ($\leq$100 μm), 800 mL distilled water and 5 mL H_2SO_4 (98%) were mixed together and a 1 L solution was made using distilled water. The solution was autoclaved at 116 °C for 13 minutes and cooled to room temperature (Talebnia *et al.* 2008). Thereafter, the pH was adjusted to 4.5 with 1 M NaOH. The solution was stirred for 5 minutes and then filtered through a No. 1 Whatman filter paper using a Büchner funnel under vacuum. The hydrolysed *C. sinensis* extract was prepared by making a 2 L solution with the extract recovered from the preparation of the hydrolysed *C. sinensis* waste in a 2 L Schott bottle by adding sterile distilled water.

Experimental design

Central composite design is one of the most widely used approaches to determining optimums or analysing responses to changes in variable(s) for various processes. It utilises the responses covered in a developed experimental design, which makes the optimising or analysis very efficient. The primary response of interest in this study was F-CN degradation efficiency, with secondary responses being the free sugar, NH_4^+ and formate metabolism efficiency. The temperature (X_1) and pH (X_2) were selected as the independent variables since both of these variables are likely to affect the response.

Expert design V8.0 (Stat Ease, USA) was used to generate an experimental map, as shown in Table 1, with

Table 1 | Experimental map for pH and temperature variables

Run	Temperature (°C)	pH
1	35.00	9.50
2	35.00	12.00
3	50.00	9.50
4	24.39	11.27
5	45.61	7.73
6	20.00	9.50
7	35.00	9.50
8	24.39	7.73
9	35.00	7.00
10	35.00	9.50
11	35.00	9.50
12	35.00	9.50
13	45.61	11.27

temperature and pH ranges of 20–50 °C and 7–12 respectively.

The quadratic polynomial function, Equation (1), was best suited for modelling the responses. The function in terms of two independent variables can be expressed as follows:

$$Y_i = b_0 + b_{1(1)}X_1 + b_{1(2)}X_2 + b_{2(1)}X_1^2 + b_{2(2)}X_2^2 + b_3X_1X_2 \pm \varepsilon \quad (1)$$

where b_0 is the constant term, $b_{1(i)}$ are the linear coefficients, $b_{2(i)}$ are the quadratic coefficients, b_3 is the interactive coefficient and ε is the error; see Table 2 for values.

Experimental procedure

A 100 mL inoculum solution was made by mixing 10 mL hydrolysed *C. sinensis* extract and 1 mL spore solution (10×10^6 spores) in a 100 mL Schott bottle and a 100 mL solution was made using sterile distilled water. 1 mL of the inoculum solution was added to 1.5 mL Eppendorf tubes and incubated at 35 °C at 120 rpm for 24 hours.

The 1 mL incubated inoculum solution and 50 mL (100 mg/L) F-CN media were added to each of the 100 mL Schott flasks (n = 13; duplicate samples). The F-CN medium was prepared by adding 0.25 g of KCN, 10 mL hydrolysed *C. sinensis* extract and 800 mL phosphate

Table 2 | Regression parameters (b_i), residual sum of squares (R^2) and probability (ρ) values for coded variables (X_i) responses for F-CN degradation, NH_4^+ and free sugar metabolism

Efficiency		F-CN degradation	NH_4^+ metabolism	Free sugar metabolism
Regression parameters	$b_0 \pm \varepsilon$	−359.50999	+202.39062	+40.00715
	$b_{1(1)}$	+8.07503	−2.16771	+0.064540
	$b_{1(2)}$	+62.31717	−19.95216	−7.32572
	$b_{2(2)}$	−0.10668	+0.017014	$+5.644 \times 10^{-3}$
	$b_{2(2)}$	−3.52097	+0.91273	+0.43794
	b_3	−0.020236	+0.072994	−0.048583
	R^2	0.9828	0.8344	0.9835
	ρ	<0.0001	0.0109	<0.0001

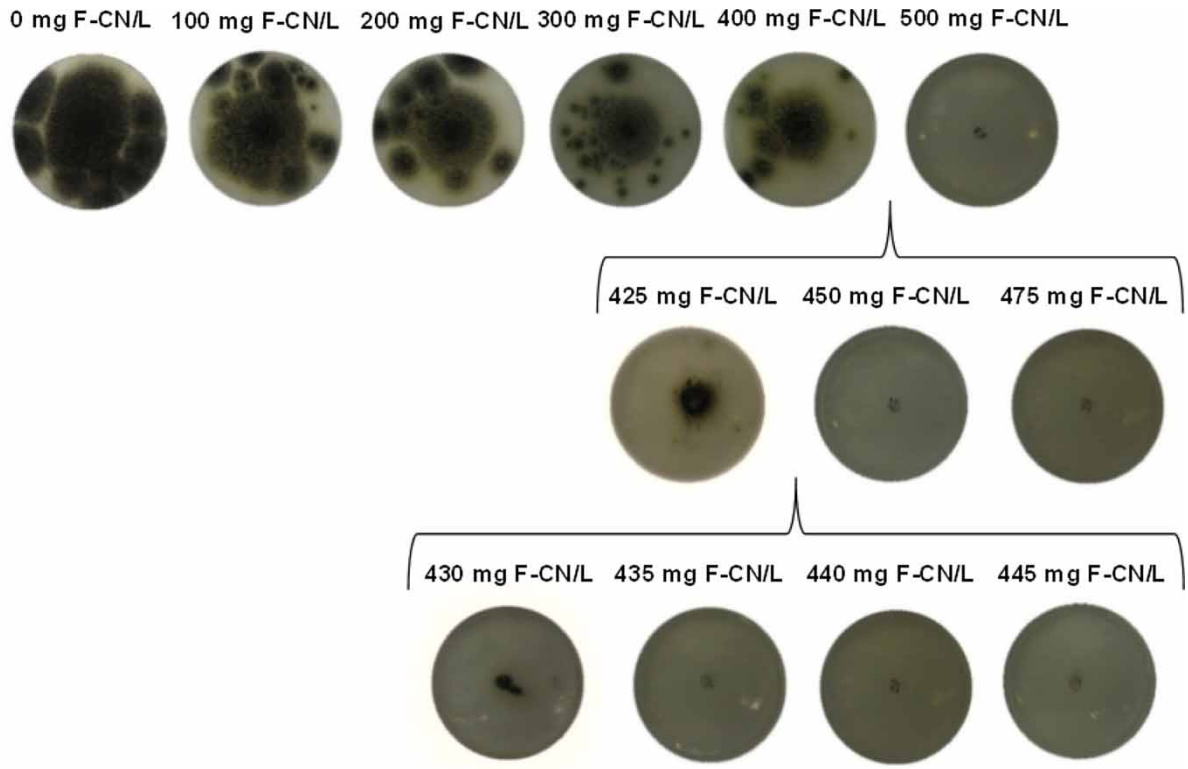

Figure 1 | F-CN tolerance of *A. awamori* isolate (Santos *et al.* 2013).

buffer, at the pH values stipulated in Table 1, to a 1 L Scott bottle and a 1 L solution was made using sterile distilled water. The experiments were performed in duplicate and the flasks were shaken at 120 rpm at the required temperatures, stipulated in Table 1, for 48 hours.

Analytical methods

All samples were centrifuged at 13,000 rpm using a Haraeus Megafuge 1.0 and filtered through a 0.2 μm filter before being analysed using Merck cyanide (CN^- 09701) and ammonium ($N\text{-}NH_4^+$ 00683) test kits to quantify the F-CN and NH_4^+ concentrations in solution. The residual free sugars, formate and citric acid were quantified according to methods developed by Miller (1959), Sleat & Mah (1984) and Marier & Boulet (1958) respectively, using a Jenway 6715 UV/Visible spectrophotometer at appropriate settings.

RESULTS AND DISCUSSION

F-CN tolerance of *A. awamori*

The tolerance of F-CN by the *A. awamori* isolate was initially assessed using F-CN concentration up to 500 mg F-CN/L, as shown in Figure 1. There was a clear decline in the growth of the fungus as the F-CN concentration was increased. Appreciable growth occurred when the strain was grown in F-CN-PDA petri dishes in which concentrations of up to 200 mg F-CN/L were used. Rapidly declining growth was noted when the F-CN concentration exceeded 300 mg F-CN/L and minimal growth was observed up to 430 mg F-CN/L, an indication of the fungus's maximum F-CN tolerance. The implication of this is that wastewater containing F-CN concentrations exceeding 400–500 mg F-CN/L can result in the reduction of the functionality of the fungus's metabolic processes, and thus its growth and potential for F-CN degradation.

Effect of pH and temperature

The degradation of F-CN by the *A. awamori* isolate was highly dependent on pH and temperature, with optimal conditions inferred to be pH 8.75 and 37.02 °C, as shown in Figure 2. Under these conditions, 62.37% of 100 mg F-CN/L was degraded within 48 hours. Therefore the design of a bioremediation system will require the F-CN-containing wastewater to be gradually introduced to aid the fungus's metabolic processes and facilitate the degradation. Based

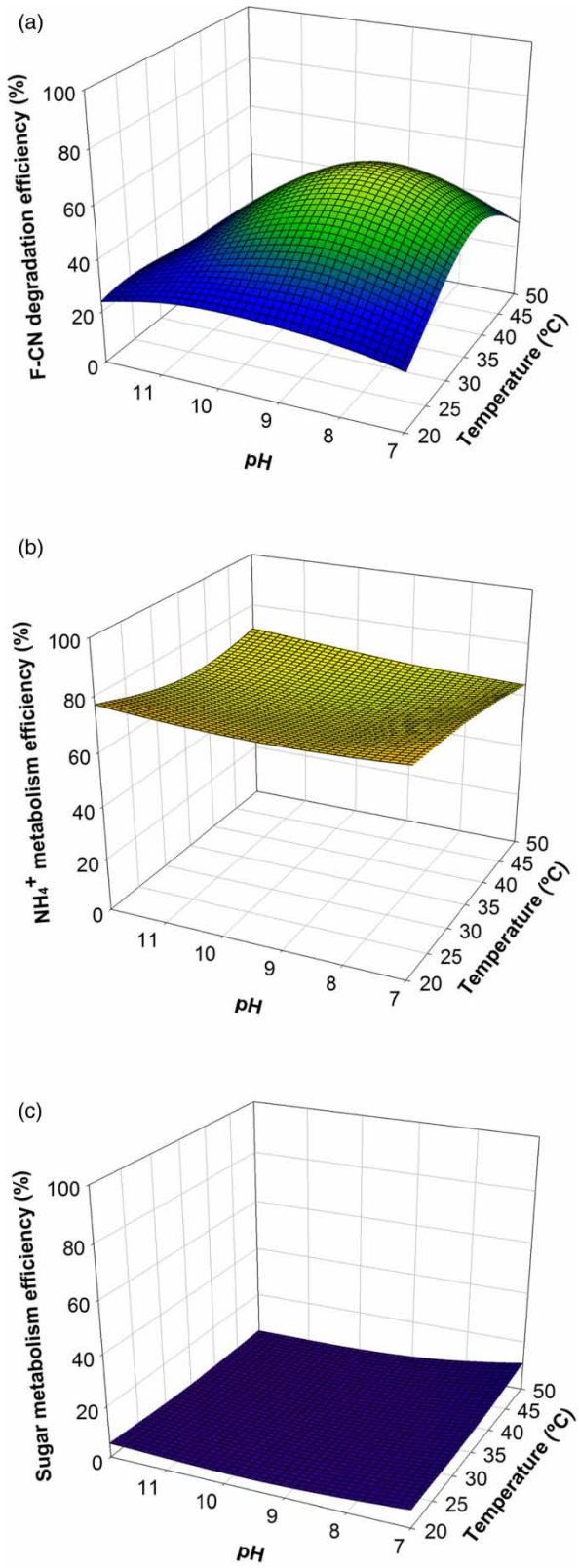

Figure 2 | (a) F-CN degradation, (b) NH_4^+ and (c) sugar metabolism efficiency by the *A. awamori* isolate.

on these results, a multi-step degradation process design would be recommended, to increase the overall F-CN conversion and rapid metabolism of by-products, such as NH_4^+ and formate. In this study, more than 75% (w/w) of the NH_4^+ produced was metabolised by the fungus.

The metabolism of free sugars and NH_4^+ was favoured under conditions inversely proportional to those suitable for F-CN degradation, especially at higher temperatures (>37 °C) and pH approaching the neutral range. This resulted in large quantities of the residual NH_4^+ being in solution, and thus needing to be metabolised, as the fungus was observed to be preferential towards F-CN degradation rather than NH_4^+ metabolism. There was negligible quantifiable citric acid detected in the fermented broth which was attributed to the low carbon source supplementation of the cultures (Papagianni 2007).

However, >85% (w/w) of the sugars quantified in the broth were not consumed during the F-CN degradation and NH_4^+ metabolism and there was negligible formate detected. The readily metabolisable nature of the formate may also have contributed to the minimal sugar metabolism in the broth. Since the degradation of F-CN and metabolism of NH_4^+ has been shown to occur with low primary carbon source metabolism, the *A. awamori* isolate was proposed as a suitable biocatalyst for medium- to large-scale operations. This is advantageous, since a process utilising the fungus would require minimal augmentation by a carbon source supplement, such as the hydrolysed *C. sinensis* extract used in this study.

CONCLUSIONS

The use of a hydrolysed *C. sinensis* extract, produced from acid hydrolysis of *C. sinensis* waste, has shown to be a rich and compatible source for cultivation for *A. awamori* isolate, with the isolate's F-CN tolerance observed up to 430 mg F-CN/L, for concurrent F-CN degradation and NH_4^+ metabolism. The optimal F-CN degradation conditions were at pH 8.75 and 37.02 °C. The minimal usage of the free sugars from the hydrolysed *C. sinensis* extract, indicated minimal supplementation requirements for F-CN degradation and NH_4^+ metabolism would be necessary in a small- to medium-sized process.

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