

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

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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 cyanide F-CN in wastewater using an *Aspergillus awamori* isolate in a process supported solely using *C. sinensis* waste extract. The optimal degradation conditions were at 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 residual 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 effluent recovered, i.e. 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 F-CN and cyanide complexes into the environment via a variety of disposal methods, particularly as wastewater. These industries utilise cyanide-based compounds in various operations, including: the beneficiation of metals, electroplating, case hardening, automotive manufacturing, circuitry board manufacturing, and chemical manufacture (Patil & Paknikar 2000). Cyanide is often found in organic hydrocarbon chains or as inorganic, transition, alkali and alkali 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 treatment methods for the treatment of F-CN and cyanide complexes compared to 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 and have 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 tonnes of solid waste is generated in South Africa every year, of which 40% by mass is organic waste (Greiben & Oelofse 2009). The average amount of 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 Singapore, than for developing countries, such as 0.30 kg/annum in Nepal (Greben & Oelofse 2009). It is sensible to bioaugment biotechnological processes to utilise agro-waste materials, particularly for industries which 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

A. 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 was transferred to potato dextrose agar (PDA) plates supplemented with 0.2% (v/v) Penicillin-Streptomycin 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 *Aspergillus 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 recommend PDA powder, followed by autoclaving the suspension at a temperature of 121 °C for 15min and cooling it to close to 50 °C using a hot plate and magnetic stirrer. A series of 80 mL F-CN PDA liquid media were prepared in a sterile 100 mL Schott bottle using a 1,000 mg F-CN/L solution (2.5 g KCN/L) and sterile distilled water, with gradual addition of cyanide solution and gentle mixing, to create F-CN-PDA plates with varying F-CN concentration. Each of the F-CN-PDA plates was inoculated with the *A. awamori* isolate by transferring it from the PDA using a flame-sterilised stainless loop and pressing this into the in the middle of the plate. To reduce volatilisation of F-CN, the plates were sealed with a parafilm and incubated for 5 days at 37 °C. The F-CN concentration in 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.

Hydrolysed *Citrus sinensis* extract

60 g of powdered *C. sinensis* waste (100 μ m), 800 mL distilled water and 5 mL H₂SO₄ (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 solutions were stirred for 5 minutes and then filtered through a No. 1 Whatman filter paper using in a Büchner funnel under vacuum. The hydrolysed *C. sinensis* extract solution 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 determine 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 responses of interest in this study were the metal biosorption and F-CN degradation, with secondary responses being the sugar, NH₄^P 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 its

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 |

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

The quadratic polynomial function, Equation (1), was the best suited to model the responses and this function in terms of two independent variables can be expressed as follows:

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

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.

Experimental procedure

A 100 mL inoculum solution was made up adding 10 mL hydrolysed *C. sinensis* extract media and 1 mL spore solution (10×10^6 spores) to a 100 mL Schott bottle and a 100 mL solution were 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 24h.

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. The F-CN media was prepared by adding 0.25 g of KCN, 10 mL hydrolysed *C. sinensis* extract and

Table 2 | Regression parameters (b_i), residual sum of squares (R^2) and probability (p) values for coded variables (X_i) responses for F-CN degradation, NH_4^+ and free sugar metabolism Q4

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

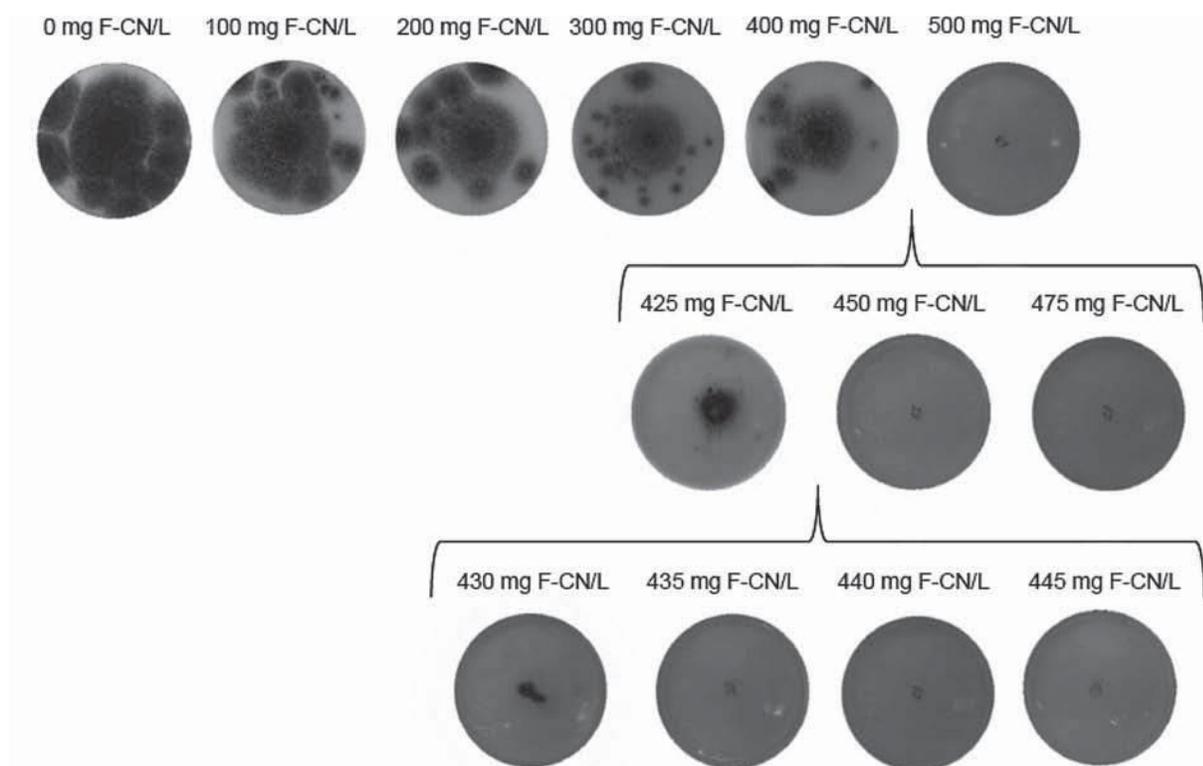


Figure 1 | F-CN tolerance of *A. awamori* isolate.

800 mL phosphate buffer, 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 temperature, 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 analyzed using Merck cyanide (CN 09701) and ammonium (N-NH_4^+ 00683) test kits to quantify the F-CN and NH_4^+ concentrations in solution. The residual total 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 plates in which the 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 was that for wastewater containing F-CN concentrations exceeding range of 400–500 mg F-CN/L, can result in the reduction of the functionality of the fungus metabolic processes, thus its growth and its 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 a 100 mg F-CN/L was degraded within 48 h. Therefore, the design of a bioremediation system will require the F-CN containing wastewater be gradually introduced to aid the fungus's metabolic processes to facilitate the degradation. Based on these

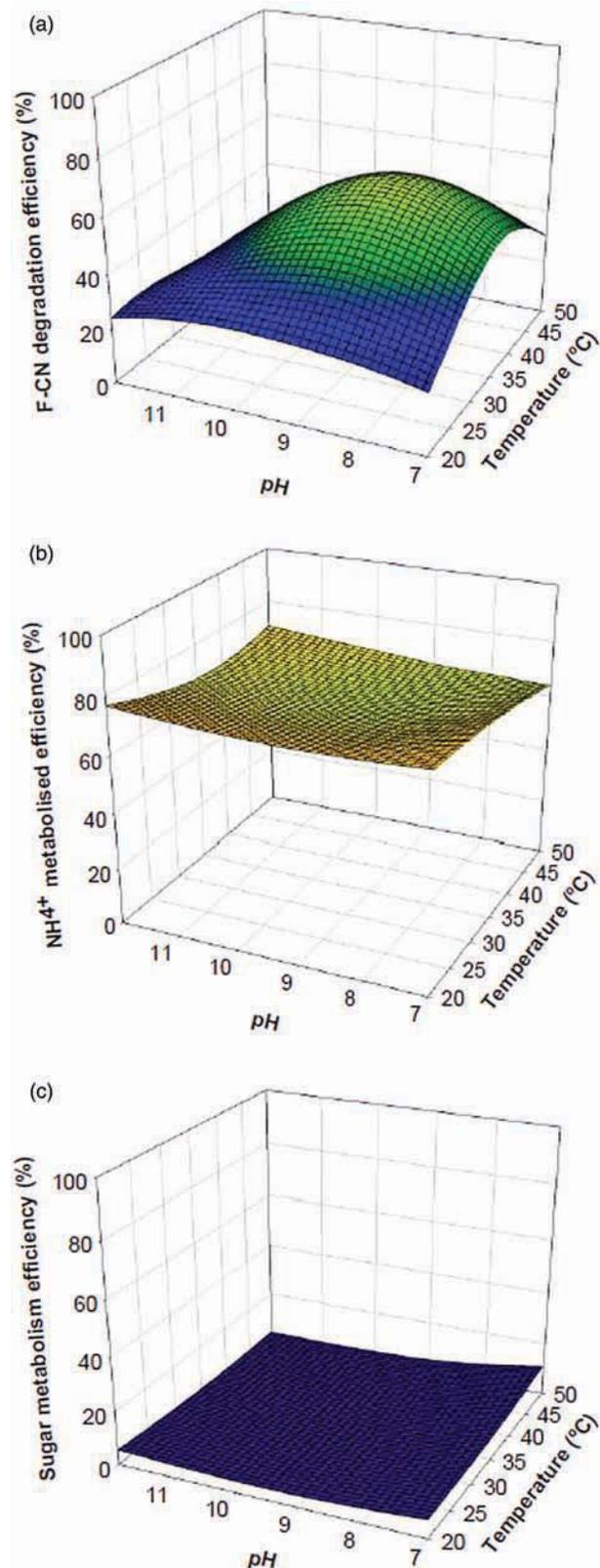


Figure 2 | (a) F-CN degradation, (b) NH_4^+ and (c) sugar metabolism by the *A. awamori* isolate. Q3

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

The metabolism of free sugars and NH_4^{p} was favoured under conditions inversely proportional to that suitable for F-CN degradation, especially at higher temperatures ($>37^{\circ}\text{C}$) and pH approaching the neutral range. This resulted in large quantities of the residual NH_4^{p} being in solution, thus needing to be metabolised, as the fungus was observed to be preferential towards F-CN degradation than to NH_4^{p} metabolism. There was negligible quantifiable Citric acid in the fermented broth detected 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^{p} metabolism and there was negligible formate detection. The readily metabolisable nature of the formate may also have contributed to the minimal sugar metabolism in the broth. Since, the degradation of the 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 solution, produced from acid hydrolysis of waste sweet orange waste, has shown to be a rich and compatible source for the cultivation for the *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^{p} metabolism. The optimal F-CN degradation conditions were at pH 8.75 and 37.02 °C. The minimal usage of the sugars from the hydrolysed *C. sinensis* extract solution, indicating minimal supplementation requirements for the F-CN degradation and NH_4^{p} metabolism would be necessary in a process.

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