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Potential of Agro-Waste Extracts as Supplements for the Continuous Bioremediation of Free Cyanide Contaminated Wastewater

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***Abstract*—**Different agricultural waste peels were assessed for their suitability to be used as primary substrates for the bioremediation of free cyanide (CN^-) by a cyanide-degrading fungus *Aspergillus awamori* isolated from cyanide containing wastewater. The bioremediated CN^- concentration were in the range of 36 to 110 mg CN^-/L, with Orange (*C. sinensis*) > Carrot (*D. carota*) > Onion (*A. cepa*) > Apple (*M. pumila*), being chosen as suitable substrates for large scale CN^- degradation processes due to: 1) the high concentration of bioremediated CN^-, 2) total reduced sugars released into solution to sustain the biocatalyst, and 3) minimal residual NH_4-N concentration after fermentation. The bioremediation rate constants (k) were $0.017h^{-1}$ ($0h < t < 24h$), with improved bioremediation rates ($0.02189h^{-1}$) observed after 24h. The averaged nitrilase activity was ~10 U/L.

***Keywords*—**Agricultural waste, Bioremediation, Cyanide, Wastewater.

I. Introduction

LARGE volumes of free cyanide (CN^-) containing wastewater are produced during various industrial activities such as the production of electroplated products and processing of ore from the mining industry [1]. Cyanide is one of the most toxic and lethal chemicals. Its release into the environment in the form of wastewater, results in the destruction of ecosystems. Currently, the release of cyanide worldwide is estimated to be >14 million kg per annum [2]. Although, there are several ways in which cyanide can be treated, commonly used methods such as chemical oxidation techniques require significant capital investment including large quantities of reagents such as chlorine, and hydrogen peroxide [3], which further exacerbate environmental pollution.

Bioremediation technologies have been developed for the treatment of cyanide, in which some microorganisms are used to degrade cyanide. These microorganisms include *Pseudomonas* sp., *Acinetobacter* sp., and *Aspergillus* sp. [4]. However, the use of these microorganisms requires readily metabolisable substrates such as glucose and sucrose, which further hinders the application of bioremediation technologies on a large scale, thus necessitating the need to develop processes in which low cost materials, including waste materials, are used to bioaugment the biodegradation processes. A viable option is to use organic agricultural waste, which is discarded and/or used in the animal feed industries. Globally, 998 million tonnes of agricultural waste is produced per annum and most of it is disposed into landfills. Some of this waste contains residue which has various biomolecules including metallic trace elements, which can sustain various microorganisms thus serve as a carbon source for microbial growth and for the production of cyanide hydrolysing enzymes [5]. The resultant by-products from cyanide degradation such as ammonia can be further metabolised depending on the nutritional needs of the immobilised microorganism.

The black moulds, *Aspergillus* sp., are an important group of species in food mycology, and biotechnology. Although, many of these species cause food spoilage, they are used in the fermentation industry to produce hydrolytic enzymes and organic acids [6]. As most agricultural waste contains cellulose, hemi-cellulose, pectin, lignin, chlorophyll pigments, low molecular weight hydrocarbons including lipids, proteins, simple sugars, starches, water, and ash, *Aspergillus* sp. can grow and use these compounds to produce enzymes such as nitrilase for the breakdown of contaminants at temperatures and pH in the range of 45 to 50˚C and 4.0 to 5.5, respectively [7]-[9].

For this research, *Aspergillus awamori,* a microorganism which is able to produce various enzymes to hydrolyse complex sugars to simple sugars from agricultural waste [10], was isolated from industrial wastewater containing high cyanide concentrations, primarily to identify suitable substrates from the agricultural sector to bioaugment a process designed for the bioremediation of cyanide without supplementation with refined carbohydrates such as glucose and sucrose.

II. Materials and Methods

A. Microorganism Isolation and Inoculum Preparation

Various fungal isolates from cyanide containing wastewater were cultured on potato dextrose agar (PDA) plates containing a 0.2% (v/v) Penicillin-Streptomycin antibiotic to limit bacterial growth. The isolates were transferred to plates containing 1% (w/v) refined citrus pectin, agar, and varying cyanide (KCN) concentrations of up to 500mg CN^-/L. The

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pectin-agar-CN^- plates were incubated at 37˚C for 120h. To reduce volatilisation of cyanide, the plates were sealed with a parafilm and the cyanide concentration in the reference plates (control) was verified at the end of the incubation cycle using a Merck cyanide (CN^- 09701) test kit to ascertain the integrity of the experiments.

One fungal isolate, which grew on pectin-agar-CN^- plates up to a concentration of 400mg CN^-/L, was selected as being suitable for the cyanide degradation studies. The strain was identified using a combined ITS, β-tubulin, and calmodulin gene regions, a technique that distinguishes between various *Aspergillus* sp. which share similar morphological characteristics. This strain was identified as *Aspergillus awamori-cput* [11].

The inoculum was prepared by re-culturing the isolate on PDA plates with subsequent incubation at 37˚C for 120h. Thereafter, between 1 to 5ml of a 0.1% (w/v) Tween 80 solution was added to each PDA plate containing conidia including mycelia, with ensuing swirling of the solution in rotational movements for one minute, to ensure that spores were suspended in the solution. Thereafter, the spore/mycelia solution collected was filtered using a heat sterilised (autoclaved) syringe containing glass-wool to entrap the mycelia onto the glass-wool with the conidia remaining in solution. The quantity of spores per ml was determined by counting using a Marienfeld Neubauer counting chamber. An inoculum size of $2x10^6$ conidia was added to the cyanide containing broth prepared as 1% (w/v) of milled agricultural waste in sodium acetate buffer (pH 5, prepared with 54.4g of sodium acetate in 50ml of warm sterile distilled water to which 10ml of glacial acetic acid including water was added to make a 100ml buffer solution) as a large percentage of cyanide remains in KCN form at pH values below 6.

B. Nutrient Broth for Cyanide Biodegradation

Potato, Carrot, Pear, Beetroot, Orange, Apple, Pineapple, Sweet potato, and Onion waste peel, including Corn cobs, were collected and autoclaved at 116°C for 15min (to prevent the thermal breakdown of reducing sugars) and dried for 168h at 80°C, in a sterile SMC incubator. Thereafter, broth containing the dry milled waste peel (<100µm), was prepared by weighing 0.5g of each waste peel into 250ml Erlenmeyer flasks containing 1ml spore solution ($2x10^6$ spores) and 24ml of the sodium acetate buffer, which was subsequently fermented for 48h to release carbohydrates and metallic components into solution. Subsequently, 25ml of dissolved KCN in sodium acetate buffer was added to the flasks to make a culture with a final cyanide concentration and culture volume of 175mg CN^-/L and 50ml, respectively. Subsequent to the addition of the cyanide solution, the fermentation was carried out for an additional 72h in a ZHCHENG model (ZHWY-1102) incubator at 120rpm for an additional 72hrs at a temperature of 40°C, with 2ml of samples taken every 24h for analysis. The collected samples were centrifuged at 13000 rpm before analysis can commence. Thereafter (second set of experiments), three substrates, i.e., Orange (*C. sinensis*), Onion (*A. cepa*), Apple (*M. pumila*), were chosen to assess kinetic parameters associated with the biodegradation process based on their performance for both cyanide biodegradation and residual ammonium (NH_4-N) consumption in the first set of experiments.

C. Total Reducing Sugars

The total reducing sugars (TRS) were quantified according to the method developed by [12], in which dinitrosalicylic acid is used. This was done using a Jenway 6715 UV/visible spectrophotometer.

D. Cyanide and Ammonium Concentration

Merck cyanide (CN) (09701) and Merck ammonium (NH_4-N) (00683) test kits were used to quantify the cyanide and ammonium concentration using a NOVA 60 spectroquant. All samples were appropriately diluted before analysis.

E. Nitrilase Activity

Nitrilase activity was measured using a rapid semi-quantitative technique by using crude samples without concentrating and purifying the samples. This was done by monitoring the change in absorbance at 238nm (ε_{238} = 3300 $M^{-1}.cm^{-1}$), as this indicates the conversion of benzonitrile to benzoic acid, in a total reaction mixture of 891µl which contained 16.5µl of a crude enzyme supernatant, 825µl of TRIS/HCL buffer (50mM, pH = 8), and 49.5µl of 10mM benzonitrile. The reaction mixture was quenched with 99µl of 1M HCL after 30min, with absorbance readings being measured at intervals of 5min [13]. The hydrochloric acid was added at the onset of the experiments for blank samples. All crude samples were centrifuged before use.

F. Bioremediation Kinetics: Mass Balance

In order to account for free cyanide volatilisation, an appropriate mass balance equation was developed and used in all calculations (1) and (2):

$$[CN_S^- - (CN_R^- + CN_V^-)] = CN_B^- \quad (1)$$

$$CN_V^- = (CN_{V0}^- - CN_{Vf}^-) \quad (2)$$

where, CN_s^- is the initial free cyanide concentration in the fermentation broth; CN_r^- is the residual free cyanide measured after fermentation; CN_v^- is the free cyanide that volatilised during culture incubation and fermentation; CN_b^- is the free cyanide that is bioremediated; CN_{v0}^- is the initial free cyanide in control cultures (unfermented broth, incubated with a cyanide concentration of 175mg CN^-/L); CN_{vf}^- is the final free cyanide in control cultures (unfermented broth, incubated with a cyanide concentration of 172.7mg CN^-/L after 3 days). The loss of free cyanide due to volatilisation was between 2 to 3%. All experiments were in duplicate.

III. Results and Discussion

The use of agricultural waste in bioremediation processes can advance the acceptability of these low performance processes, as the use of waste materials will generally minimise capital and operational costs. It is therefore, prudent

to design and evaluate the feasibility of using agro-waste for processes designed to decontaminate environmental matrices, in particular polluted industrial wastewater. In this study, several agricultural waste, i.e. Sweet orange (*C. sinensis*), carrot (*D. carota* L. var. *sativus*), Onion (*A. cepa*), Potato (*S. tuberosum*), Pineapple (*A. comosus*), Apple (*Malus pumila*), Beetroot (*B. vulgaris*), Sweet potato (*I. batatas*), Pear (*P. communis*), cobs from Corn (*Z. mays*), were used to assess their compatibility as sole carbon sources for the degradation of cyanide as KCN using *Aspergillus awamori*, an isolate which was determined to degrade free cyanide even in solutions having a high cyanide concentration.

This fungus, commonly known as *Black koji* mould, is well known to spoil agricultural produce. The *A. awamori* isolate obtained from cyanide containing wastewater, had to be able to grow on agro-waste extracts, tolerate and sufficiently degrade cyanide including cyanide degradation by-products produced from the remediation process. Although, several *Aspergillus* sp., in particular *Aspergillus niger*, had been shown to degrade cyanide, the processes were mostly grown on supplemented media containing refined carbohydrates. This is not feasible for environmental biodegradation processes on a large scale. Furthermore, most *Aspergillus* sp. cannot tolerate cyanide concentration exceeding 200mg CN^-/L [14], whereas the *A. awamori* isolate used in this study was shown to tolerate cyanide concentration up to 400mg CN^-/L, with stunted growth being observed when cyanide concentrations exceeded 200mg CN^-/L [11]. Most microbial species which can tolerate high cyanide concentrations are bacterial, i.e. *Bacillus* sp., in cultures also supplemented with refined carbohydrates. Therefore, there is a need to curb the reliance on refined carbohydrates.

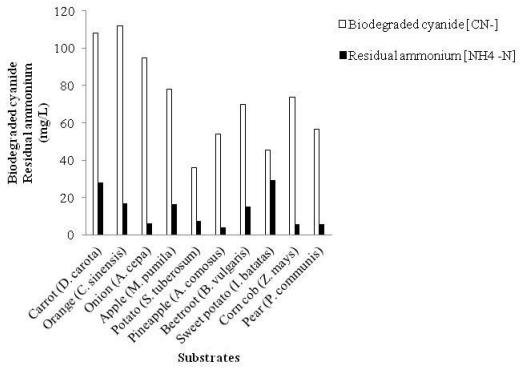


Fig. 1 Biodegraded cyanide and residual ammonium concentrations after 72 h

Fig. 1 illustrates the first batch of experiments in which the bioremediation of cyanide using different agricultural waste as substrates was assessed; with the best performing substrates being Orange (110mg CN^-/L), Carrot (108mg CN^-/L), Onion (95mg CN^-/L) and Apple (78mg CN^-/L) with minimal residual NH_4-N being observed in the cultures. The residual ammonium concentration ranged between 4 (low for Pineapple) to 29mg/L (high for Sweet potato).

These results indicated that there is a need to assess the degradation of the ammonium in the cultures, as residual ammonium can further accumulate in environmental matrices, thus cause pollution. Ammonia, which is in equilibrium with ammonium in the environment, is generally considered as one of the most toxic by-products of wastewater treatment. This means that, although, sufficient biodegradation rates can be achieved using cyanide degrading organisms, the resultant ammonium should be reduced before the treated water is disposed in to surface water sources or through municipal wastewater drainage systems.

As agricultural residues such as that of *C. sinensis* is generated in large quantities every year, it was apparent that this type of waste can be utilised as a sole nutrient supplement for the *A. awamori* isolate for treatment of cyanide contaminated water. The experimental analysis conclusively indicated that citrus based agricultural residues, when compared to high starch based agricultural waste, namely *Z. mays* cob and *I. batatas* waste, can support microbial growth in suitably designed processes thus sustain processes for the biodegradation of highly toxic contaminants. Although it is desirable to have readily available source of reducing sugars to support metabolic activities of microorganisms in bioremediation systems, the presence of residual reducing sugars in the effluent from a bioaugmented bioreactor system can be problematic, particularly for downstream processes. Most wastewater treatment facilities use tertiary treatment methods to further improve the quality of the treated water for reuse. Having a high concentration of residual sugars can render these tertiary treatment systems ineffective, as the sugars can promote biofilm formation and the deposition of exopolysaccharides which can further increase operational costs to clean and replace affected apparatus.

Although, the *A. awamori* cultures without cyanide had the lowest reducing sugar present after fermentation, cultures supplemented with *M. pumila*, *A. comosus*, and *P. cummonis* to support the biodegradation of cyanide, had a high concentration of residual total reducing sugars in the range of 0.48 to 0.93mg/L from the 1% (w/v) media used, when compared to others –Fig. 2.

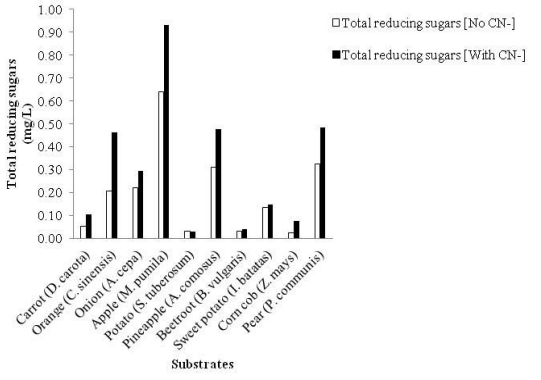


Fig. 2 Total reducing sugars (TRS) in batch cultures after 72 h

This suggested that this type of waste can be used in small quantities when compared to others used in this study. The availability of reducing sugars resulted in low cyanide degradation efficiency for these cultures, with minimal residual ammonium concentrations being observed in the cultures (Fig. 1).

Although, it is desirable to ferment waste in order to release embedded reducing sugars in the waste peel to develop sufficient biomass and the release of suitable enzymes prior to the commencement of a degradation process, there is a need to ascertain optimum conditions which will be suitable for a large scale operation.

For the second set of experiments, four types of waste peels were selected from the nine different wastes used in the initial experiments, to determine the biodegradation reaction kinetics of cyanide using the isolated *A. awamori.* The basis for the selection was that the waste material should support a high cyanide and residual ammonium degradation efficiency with minimal total reducing sugars remaining after fermentation. Additionally, the waste must be readily available and must support quicker conversion rates of free cyanide in solution. *C. sinensis*, *A. cepa*, *D. carota*, and *M. pumila* were selected as they were clearly the most suitable substrates for the high conversion rates of cyanide. However, due to the high ammonium concentration at the end of the biodegradation cycle for *D. carota* cultures, it was therefore prudent to consider *C. sinensis*, *A. cepa*, and *M. pumila* exclusively. The experiments were done in repeated batches which were used to determine the cyanide degradation rate assuming first order decay kinetics. Furthermore, the experiments were used to quantify the nitrilase activity using benzonitrile as a substrate.

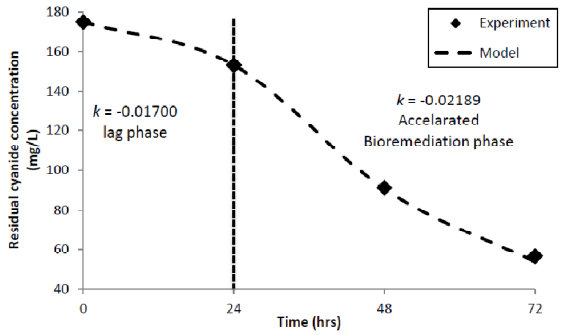
Fig. 3 shows cyanide degradation kinetics for *C. sinensis*, *A. cepa*, and *M. pumila* cultures in which cyanide degradation was assessed for 24 to 72 h. A similar start-up strategy was employed whereby the biomass was allowed to grow and accumulate in the bioreactors for 48 h subsequent to the addition of cyanide (KCN, 175 mg CN^-/L).

Assuming first order kinetics, a model, i.e. $\frac{d[CN]}{dt} = -k[CN]$, was used in a Ordinary Differential Equation (ODE) solver, to simulate the cyanide degradation kinetics. This was done using the following ODE parameters – Table I: 1) boundary conditions: 0 h < t < 24 h; t(0) = 0 h with t(f) = 24 h; with the rate of decay (k) being 0.017h^{-1} using C(0) = $[CN^-]_{t=0\ h}$ = 175mg/L. Furthermore, at 24h < t < 72h; t(0) = 24h; t(f) = 72h, the rate of decay (k) was determined to have improved to 0.02189h^{-1} using C(0) = $[CN^-]_{t=24h}$, i.e. $[CN^-]_{t=24h}$ being the averaged concentration of cyanide in the cultures after 24h of catalysed cyanide degradation. This represented, an averaged degradation rate >50% in 72h. This rapid degradation rate can only be achieved under conditions which facilitate sufficient enzyme activity using substrate extracts which have a chemical composition which includes soluble sugars and trace minerals to support extracellular hydrolytic enzyme production. Previously, it was determined that substrates such as orange peel, have a large content in reducing sugars, pectin and cellulose with minimal starch and protein [15]. In other research studies, an *A. awamori* strain, was determined to have multienzyme producing capabilities to grow on orange peel, jack rind fruit, carrot peel and beetroot peel [16]. Similarly, the *A. awamori* isolate used in this study demonstrated that it can successfully grow on multiple substrates while sufficiently degrading the cyanide, a characteristic which is associated with directed evolution, particularly for microorganism living under harsh conditions, which in this case, include being able to form biofilms on matrices exposed to cyanide containing wastewater.

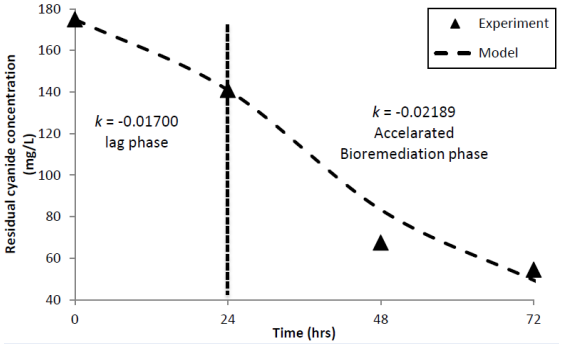
TABLE I
ORDINARY DIFFERENTIAL EQUATION SOLVER INPUT PARAMETERS

Rate equation: $\frac{d[CN]}{dt} = -k[CN]$	
0 h < t < 24 h	24 h< t < 72 h
t(0) = 0 h	t(0) = 24 h
t(f) = 24 h	t(f) = 72 h
k = - 0.01700 h^{-1}	k = - 0.02189 h^{-1}
C(0) = $[CN^-]_{t=0\ h}$ = 175 mg/L	C(0) = $[CN^-]_{t=24h}$

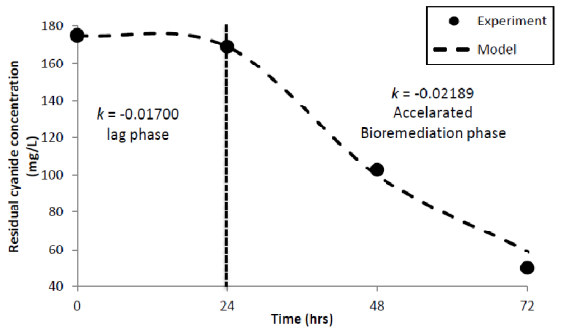
Nitrilase activity was quantified as the rate of benzonitrile conversion to benzoic acid as illustrated in Fig. 4. The accumulation of benzoic acid showed the presence of nitrilase, as benzoic acid is a product formed during the enzymatic conversion of benzonitrile [5]. Nitrilase activity was determined to be ± 10 U/L. Generally, hydrolytic conversions involved during the degradation of cyanide involve hydratases which form an intermediate amide subsequent to the formation of a carboxylic acid, while nitrilases convert nitriles in a single step to form end-products such as ammonium and carboxylic acids,-a process which is highly dependent on the pH.



(a) Apple (*M. pumila*)



(b) Orange (*C. sinensis*)



(c) Onion (*A. cepa*)

Fig. 3 Comparison between experimental and modelled bioremediation kinetics in (a) Apple, (b) Orange, (c) Onion cultures

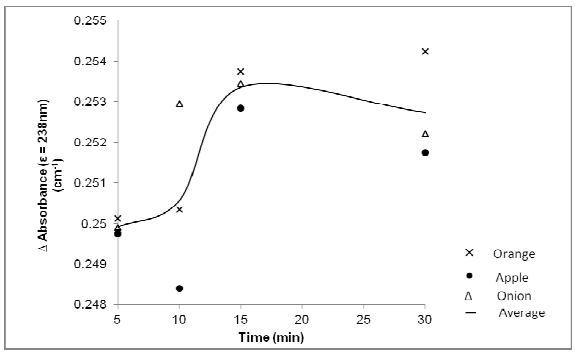


Fig. 4 Averaged rate of benzoic acid formation monitored at 238 nm using crude extracts from *C. sinensis*, *A. cepa* and *M. pumila* cultures

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