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Chapter 6

Continuous Biotechnological Treatment of Cyanide Contaminated Waters by Using a Cyanide Resistant Species of *Aspergillus awamori*

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Additional information is available at the end of the chapter

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1. Introduction

Various industries release a combination of free cyanide 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 in chemical industries [23]. Cyanide is often found in organic, hydrocarbon chains or as inorganic, transition, alkali and alkali earth metal complexes [20]. Many cyanide complexes are highly unstable, thus temperature, pH and light can degrade the components to form free cyanide which is the most toxic form of cyanide [20, 26].

There is an overwhelming popularity in industry for the use of chemical treatment methods for the treatment of free cyanide and cyanide complexes compared to biochemical treatment methods. Chemical remediation methods like alkaline chlorine oxidation are commonly used to treat cyanide contaminated wastewater [23, 24]. Chemical oxidation is particularly ineffective in the treatment of cyanide-metal complexes containing heavy metals, such as copper, nickel and silver, due to the slow reaction rate [23]. The excess quantity of chlorine used in the treatment process increases the chemical oxygen demand (COD) of the wastewater thereby rendering the water undesirable for reuse, toxic to aquatic life and may produce organic substances. In order to reduce operational costs, some manufacturers partially treat the wastewater, resulting in untreated and/or partially decomposed cyanide being discharged. Other methods of treatment include copper catalysed hydrogen peroxide oxida-

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tion, ozonation and electrolytic decomposition [23]. However, these methods are unpopular due to the high capital costs, specialist equipment and maintenance requirements.

Several microorganisms, bacteria such as *Nocardia sp*. and *Rhodococcus sp*., fungi such as *Aspergillus sp.* and *Fusarium sp.* and algae such as *Arthrospira maxima* and *Scenedesmus obliquus*, possess enzymatic mechanisms able to bioremediate free cyanide and cyanide complexes [1, 26]. However, limited studies have been conducted using organic waste and fungal strains in cyanide bioremediation. Several studies have been conducted using varying concentrations of free cyanide, with moderate success being achieved in some cases [1].

Since the early 1970's, progress has been made to develop economically viable continuous remediation processes such as membrane bioreactors (MBRs) [8]. A membrane is generally defined as being a selective barrier. The membrane utilised in a bioreactor can provide either a barrier to limit the transport of certain components, while being permeable to others, thus prevent certain components from contacting a biocatalyst, or contain reactive sites thus being a catalyst itself [5]. The application of MBRs for the production of enzymes has received considerable attention for their diverse industrial use. A number of microorganisms have been studied in MBR applications for wastewater using fungi, such as white-rot fungus, *Phanerochaete chrysosporium* (*P. chrysosporium*) [8].

Solid waste generation in South Africa is a problem growing at an exponential rate with the majority of landfill sites reaching maximum capacity. Approximately $427x10^6$ tonnes of solid waste is generated in South Africa every year, of which 40% by mass is organic waste [10]. The average amount of waste generated per person in South Africa is 0.7 kg/annum, which is close to that of developed countries such as the United Kingdom (0.723 kg/annum) and Singapore (0.87 kg/annum), than for developing countries, such as Nepal (0.3 kg/annum) [10]. It is sensible to bioaugment biotechnological processes to utilise organic waste materials, particularly for industries which produce large quantities of it.

2. Overview: Free cyanide

Free cyanide is the simplest form of cyanide and has two forms, namely as a hydrocyanic molecule or hydrogen cyanide (HCN) which dissociates into an anionic cyanide molecule (CN^-) in solution [20]. By definition, free cyanides are forms of molecular and ionic cyanides that are released in aqueous solution by the dissolution and dissociation of cyanide complexes. Simple and weak acid dissociable cyanides are the most unstable and most likely to form free cyanide in aqueous solution. Simple cyanide compounds are ionically bonded cyanide anions and alkali earth or alkali metals that are neutral, that exist in solid form and dissociate into alkali earth or alkali metals and free cyanide when in aqueous solutions [20].

Accordingly the environmental risk of cyanide wastewater is not limited to the effluent but also the possibility of emitting hydrocyanic gas. Hydrocyanic gas is toxic, colourless, distinctive almond smell at low concentrations, slightly soluble in water and readily dissociates into hydrogen and anionic cyanide at low pH in aqueous solution [20]. For safety reasons, it is

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advised to keep cyanide solutions at a high pH to prevent the evolvement of hydrocyanic gas since there is a direct relationship between the dissociation of hydrocyanic molecule and pH (Figure 1) including temperature [20].

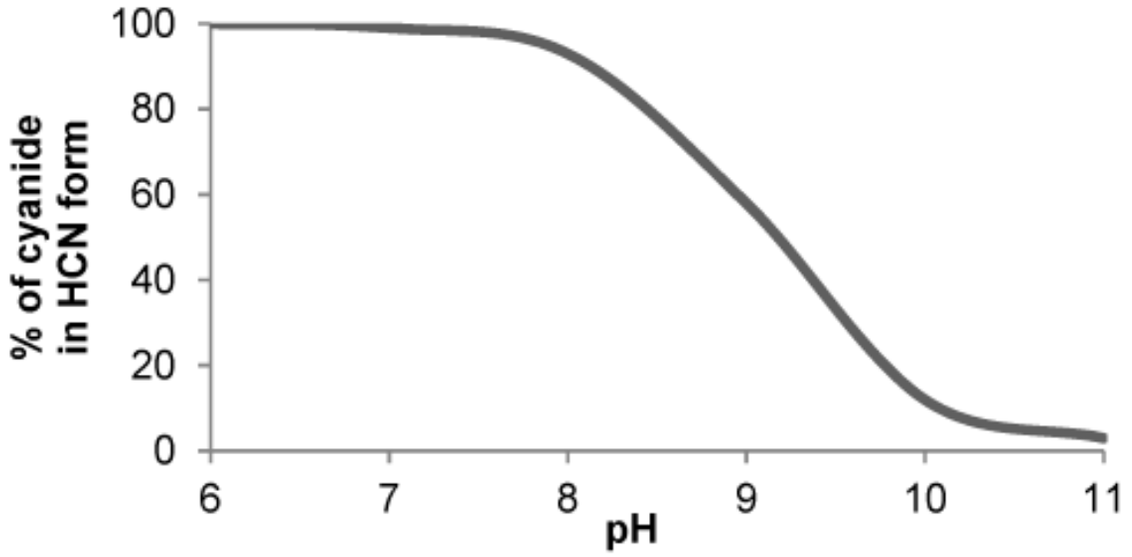

Figure 1. Relationship between HCN in solution and pH [20]

3. Differentiating filamentous fungi: *Aspergillus niger* and *Aspergillus awamori*

Black *Aspergilli* (*Aspergillus* section *Nigri*) species, are aerobic filamentous fungi often derived from soil. They have shown potential in biotechnology, food and medical applications. The trait of these species causing agricultural products to spoil had recently shown to be of benefit. Agricultural waste can be fermented to produce a variety of industrially important extracellular enzymes, such as cellulase, amylase, xylanase, pectinase, elastase, and organic acids, such as citric, galacturonic including gluconic acid. *Aspergillus niger* (*A. niger*) and *Aspergillus awamori* (*A. awamori*) are closely related species and *A. awamori* is often misidentified as *A. niger*. They share similar morphology and growth rates at various temperatures and produce several common enzymes [34].

3.1. Isolation and identification: *Aspergillus awamori*

A black filamentous mould was isolated from cyanide contaminated municipal wastewater discharge drain located in the Western Cape, South Africa. Swab samples were taken at various points along the drain and grown on 1 % (w/v) Citrus Pectin Agar (CPA) plates incubated at 37 °C for 5 days. After incubation, black mycelia of filamentous mould grew on the plate (Figure 2).

This was transferred to Potato Dextrose Agar (PDA) plates with 0.2% (v/v) Penicillin-Streptomycin (PEN-STREP; (10000 units/L of Penicillin and 10 mg of Streptomycin/ml) anti-biotic solution. The plates were again incubated at 37 ˚C for 5 days. One millilitre (1 ml) of 0.1% (w/v) Tween 80 solution was added to each plate and a spatula was used to harvest the spores and mycelium from the plate to form a spore-mycelium suspension. The suspension was then filtered through a glass wool using 20 ml syringes to entrap the mycelium onto the glass wool and produce a spore suspension which was stored at 4 ˚C. Afterwards, serial dilutions were made from the spore suspension and the number of spores in each 1 ml, were determined in duplicate using a Marienfeld Neubauer cell-counter and a Nikon Eclipse E2000 at a phase contrast of one and magnification of 100x. The absorbance of the diluted spore suspension was determined at 750 nm using a Jenway 6715 UV/Visible spectrophotometer with distilled water as a blank [31]. A calibration graph for the spore concentration was determined by plotting the absorbance against the spore concentration (spores/ml), to quantify spore concentration in the inoculum.

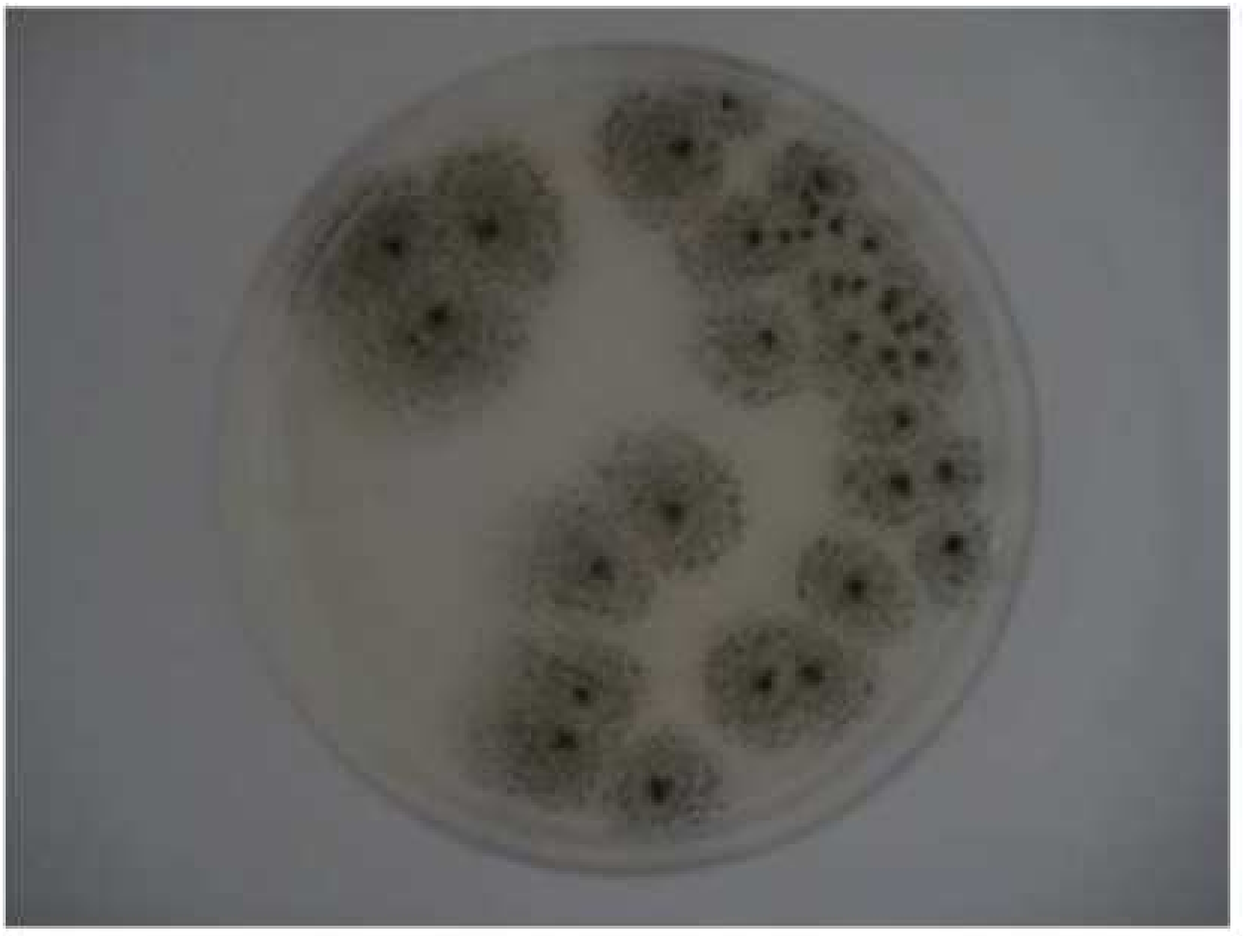

Figure 2. Growth of *A. awamori* on CPA after incubation at 37 ˚C for 5 days

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To observe the morphological characteristics of the fungus, the isolates were inoculated on Malt Extract Agar (MEA) and Czapek Yeast Agar (CYA) and incubated at 26 °C for 7 days. Based on their growth rate, the fungus was presumptively identified as *A. awamori. A. awamori* was reported to show rapid growth on the CYA compared to *A. niger* which exhibited restricted growth. However, the growth and sporulation on MEA was better than CYA in the case of both *A. niger* and *A. awamori*. The fruiting bodies were mounted in lactic acid before they were observed under an oil immersion. The conidial heads for *A. awamori* were not well-defined columns in comparison to conidia heads observed for *A. niger*. The strain showed colony characteristics of both *A. niger* and *A. awamori.* On a general note, the conidiophores and conidia of *A. awamori* and *A. niger* are similar and morphologically indistinguishable, as shown in Figure 3 [34].

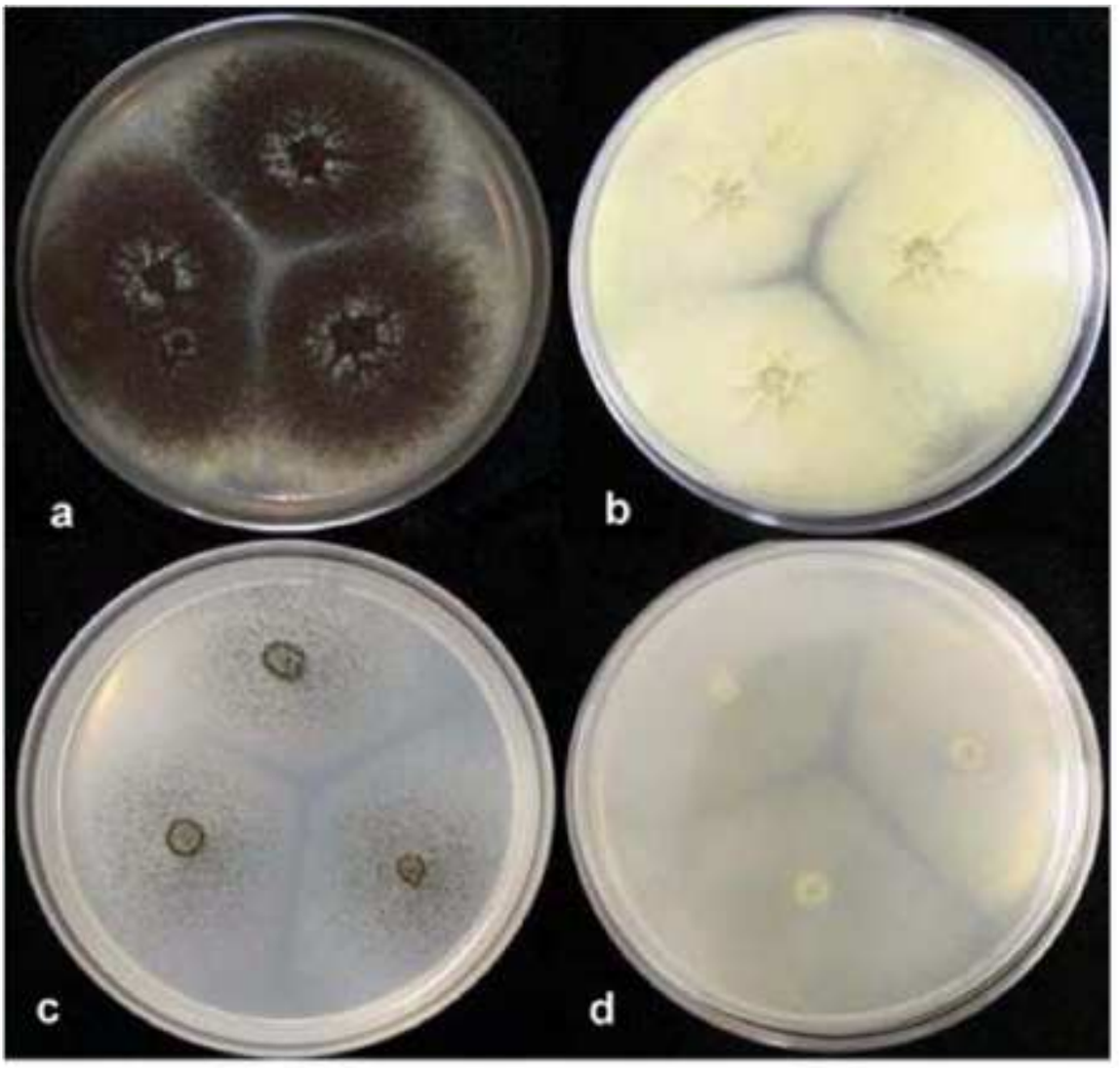

Figure 3. Growth of isolate on (a & b) MEA and (c & d) CYA

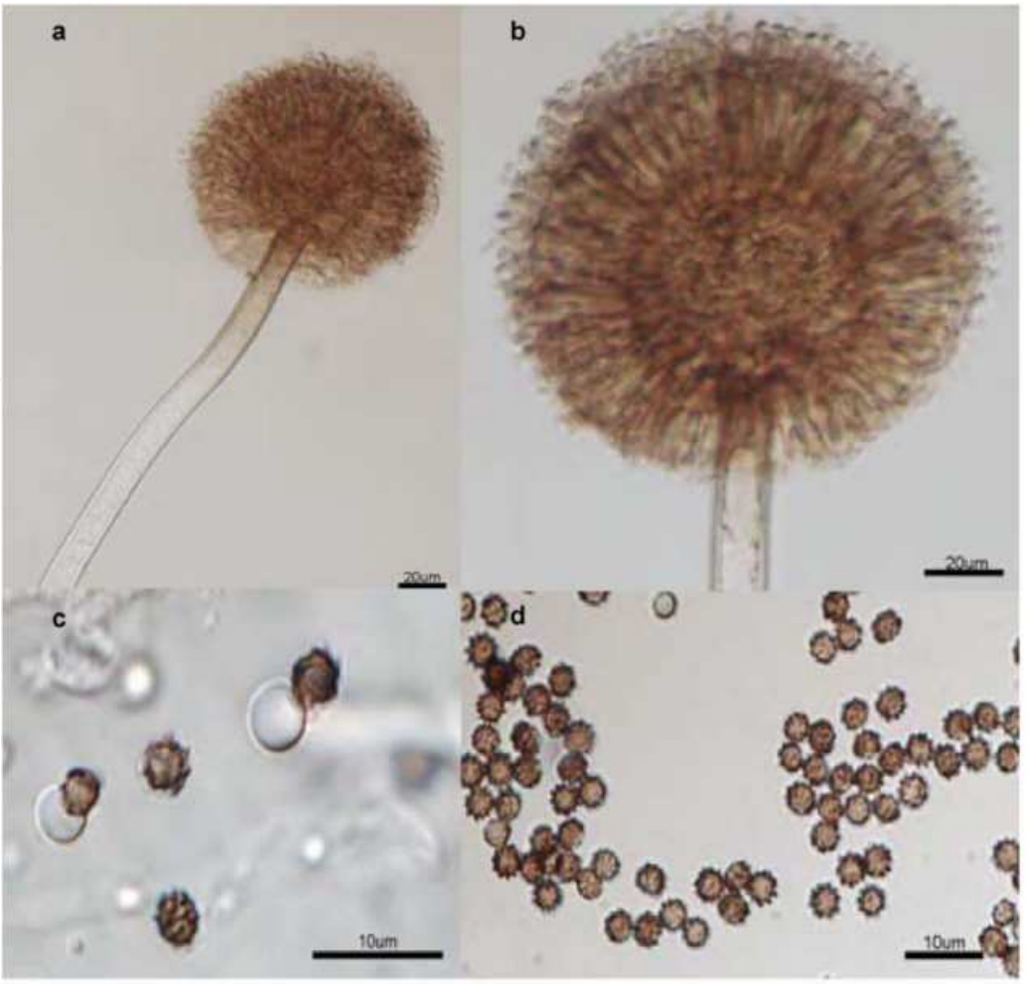

Figure 4. (a & b) Typical *Aspergillus* conidiophores with a radial head and (c & d) roughened, round conidia with regular low ridges and bars

Molecular characterization was carried out in order to confirm the identity of the fungal isolates. DNA was extracted from the pure isolates using the ZR Fungal/Bacterial DNA Kit (Zymo Research, California, USA). The subsequent Polymerase Chain Reaction (PCR) of the ITS1–5.8S–ITS2 rDNA region was prepared with primers ITS1 and ITS4 [36]. The β-tubulin gene was amplified using primers Bt2a and Bt2b [7] and the calmodulin gene with CL1 and CL2A [22], respectively. Sequencing reactions of the PCR products were set-up using a Big Dye terminator cycle sequencing premix kit (Applied Biosystems, CA).

Sequence reactions were analysed with an ABI PRISM 310 genetic analyser. Sequences were compared to those of a recent study by [34]. Datasets were aligned in Se-Al, including a sequence analysis in Se-Al. This was followed by a sequence analysis in PAUP* v4.0b10, using the BioNJ option for calculating a single tree for each dataset. Confidence in nodes was calculated using a bootstrap analysis of 1000 replicates. Only bootstrap values above 90% were indicated on the branches and *Aspergillus flavus* was chosen as the outgroup [34]. The isolated fungus was denoted as *Aspergillus* (CPUT) in Figure 5 and 6.

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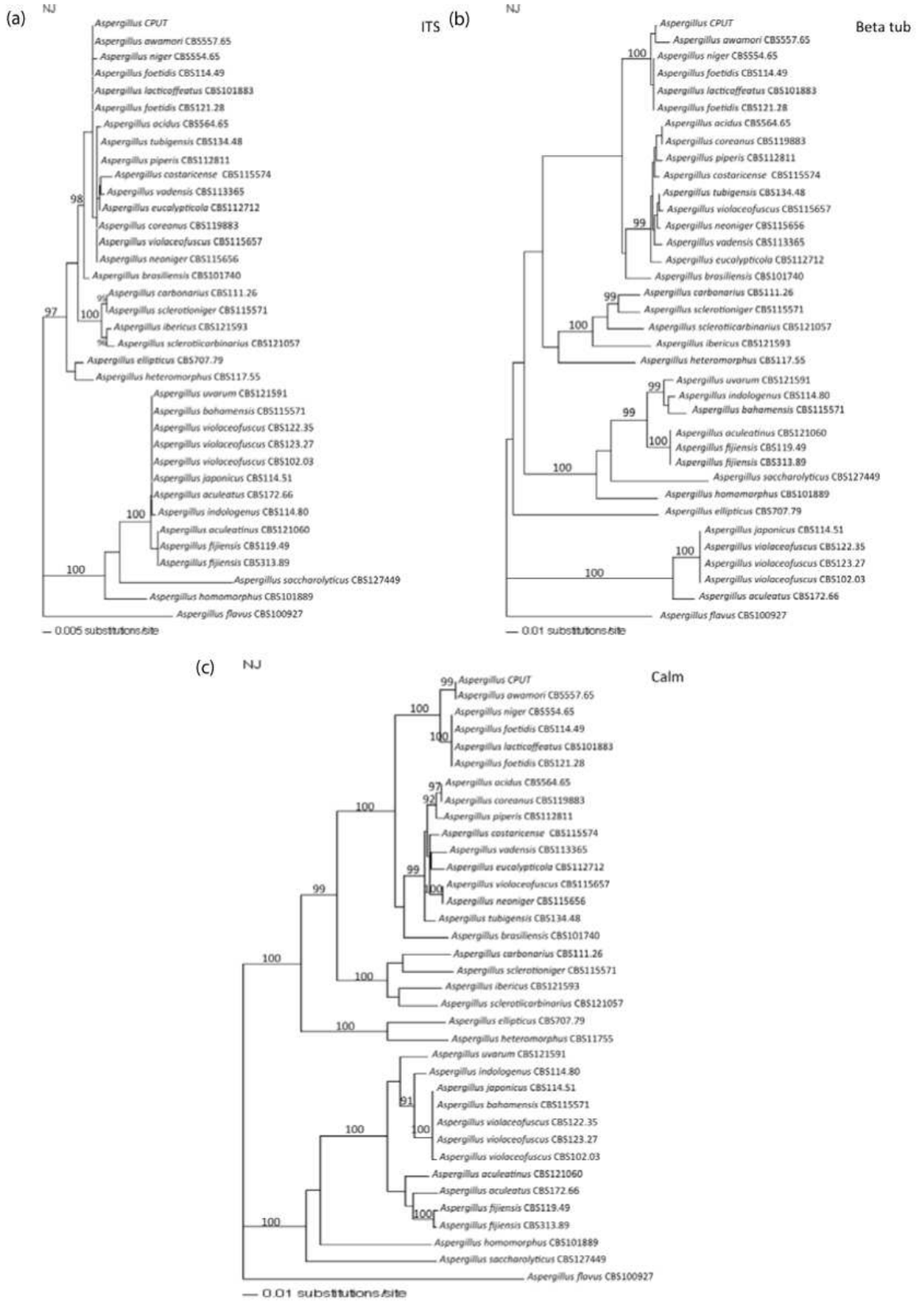

Figure 5. NJ tree based on the analysis of the (a) ITS, (b) β-tublin and (c) calmodulin gene regions

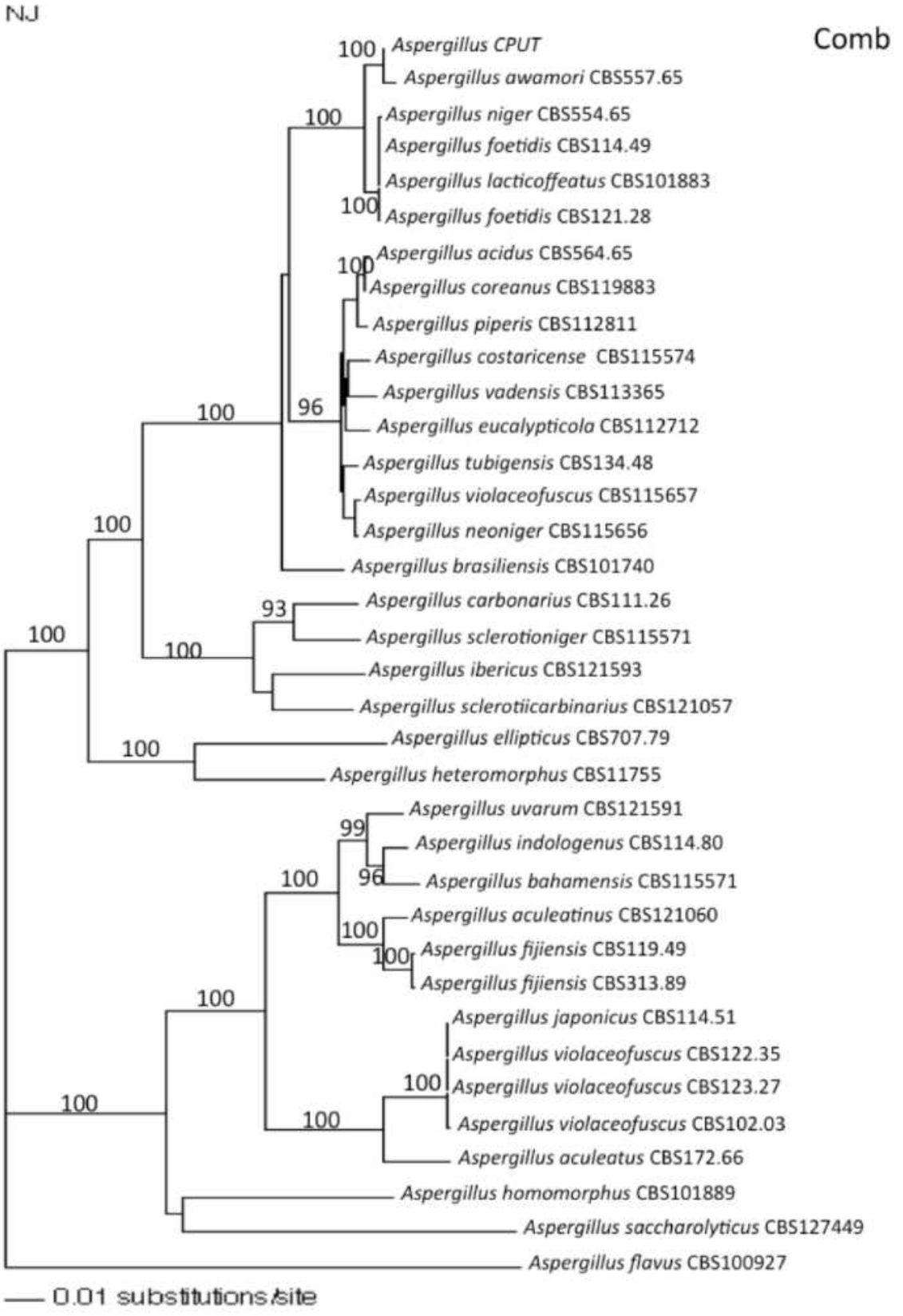

Figure 6. NJ tree based on the analysis of the combined gene regions

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According to [34], the only way to separate *A. niger* and *A. awamori* is through a multi-gene phylogenetic analysis. This was done by using ITS, β-tubulin and calmodulin gene regions, as shown in Figure 5. The combined gene region analysis (Figure 6) indicates that the *Aspergillus* strain was similar and indeed identical to the sequence of the type strain of *A. awamori,* a fungus with diverse properties in biotechnology applications, including the production of nitrilase-a cyanide degrading enzyme.

3.2. Citrus peel supplemented growth medium for cyanide bioremediation using *Aspergillus awamori*

Citrus peels are composed of cellulose, pectin, hemi-cellulose, lignin, chlorophyll pigments, low relative-molecular-mass hydrocarbons including lipids, proteins, simple sugars, starches, water and ash [14, 29]. The major components in citrus peel are cellulose, hemi-cellulose, pectin and lignin which are inter-wound with each other to provide a rigid cell wall structure. *Aspergillus awamori* is able to produce enzymes for the breakdown of cellulose, hemi-cellulose and pectin and leaving lignin as the remaining structural component.

Cellulase, xylanase, pectinase are important enzymes in the hydrolysis of cellulose, hemi-cellulose and pectin into simpler sugars which can be utilised as a carbon source by the fungus. Hydrolysis of the orange peel has shown to yield significant quantities of neutral sugars: glucose, fructose and sucrose with low yields of xylose, arabinose, galactose and mannose. Hydrolysis of citrus peel also yield uronic acids, with galacturonic acid being the major of uronic acid liberated with trace quantities of other uronic acids. The optimum temperature and pH for these enzymes are in the range of 45 to 50 °C and 4.0 to 5.5, respectively [18, 19, 32]. Furthermore, *A. awamori* can produce nitrilase which hydrolyses the nitrile (cyanide) group (R-CΞN) into the corresponding carboxylic acid and ammonia, as shown in Figure 7 [26]. In aqueous solution, ammonia/ammonium equilibrium is observed.

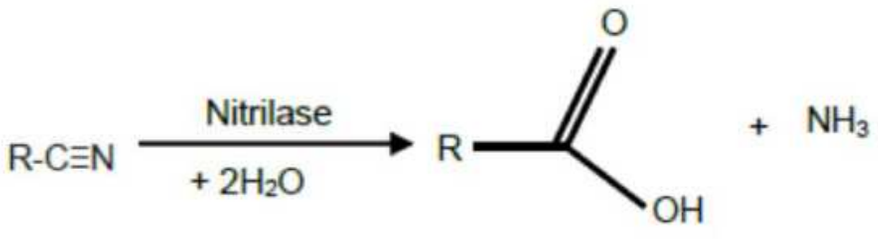

Figure 7. Nitrilase hydrolysis on cyanide group [26]

The majority of the citrus peel components are high carbon source materials and the ammonia produced from the cyanide degradation can be utilised as a nitrogen source by the microorganism. Studies have shown that the optimal pH and temperature for nitrilase production is 8 and 45 °C, respectively [12, 35].

The successful treatment of 400 ppm free cyanide supplemented with refined/readily metabolisable carbon sources has been reported [1]. However, current technology used can

be capital intensive for large scale operations. Most studies on free cyanide bioremediation efficiency measured the free cyanide reduction periodically as opposed to product formation. This may be misleading since free cyanide is very volatile, even at room temperature, and the decline in free cyanide concentration observed may be a result of volatilisation into the atmosphere rather than actual biological remediation. The cyanide tolerance of the *A. awamori* (CPUT) isolate was initially assessed up to a 500 ppm CN^- (1.2515 g KCN/L) in PDA (Figure 8).

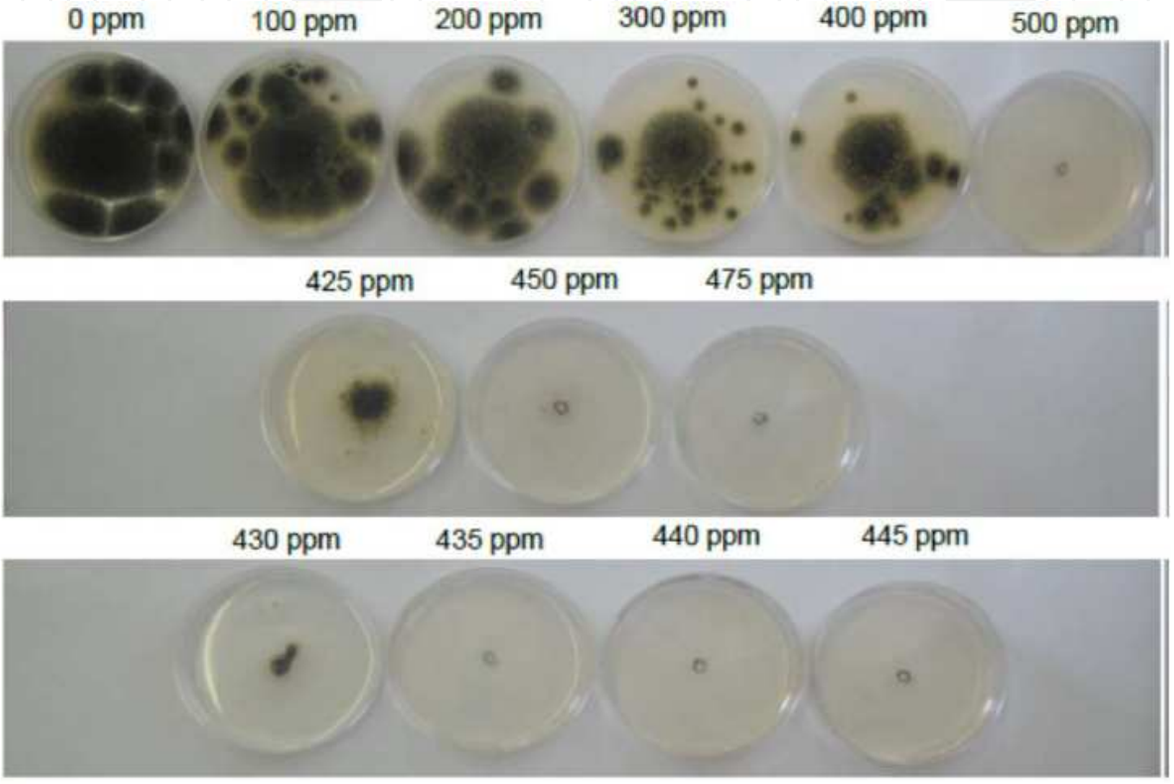

Figure 8. Cyanide tolerance analysis for *A. awamori* (CPUT) isolate

There was a clear decline in the growth of the fungus as the free cyanide concentration was increased. Appreciable growth occurred for the strain for free cyanide concentrations up to 200 ppm. A rapid decline in the growth was observed as the free cyanide concentration exceeded 300 ppm. The toxicity of cyanide reduces the functionality of the fungus metabolic processes, thus its growth. There was limited growth observed at cyanide concentrations above 430 ppm. Preliminary analysis on the effect of growth media on *A. awamori* (CPUT) for free cyanide bioremediation was performed in batch cultures, shaken at 180 rpm and 30 °C in a ZhiCheng (ZHWY-1102) shaking incubator. Media solutions of 42.5 ml of 1% (w/v) refined citrus pectin, 1 % (w/v) powered orange peel, Czapek yeast medium and sterile distilled water (standard) were added into 250 ml flasks. To each of the flasks, 1 ml of spore suspension ($2x10^6$ spores) was added followed by 7.5 ml of a 1 g CN^-/L cyanide solution.. The experiments were run in duplicate in which sampling was every 48 hours. The samples were centrifuged for 13000 rpm for 5 minutes before any analysis was conducted. Merck cy-

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anide (CN^-) (09701) and Merck ammonium (NH^{4+}) (00683) test kits were used to measure the free cyanide and ammonia/ammonium (NH_3/NH^{4+}) concentrations in solution.

The orange peel medium showed considerably higher cyanide reduction compared to the other nutrient media evaluated (Figure 9). The change in the cyanide concentration in the water medium (control) was due to volatilisation. At day 2, 7.5 ml of 1 g CN^-/L free cyanide was again added to each flask to evaluate the robustness of the culture, in each growth media. The orange peel culture had the fastest recovery, even with a sudden increase in free cyanide concentration. The numerous enzymes released by the fungus, sufficiently hydrolysed the orange peel which resulted in better supplementation and maintenance of the fungus compared to the other media for cyanide bioremediation.

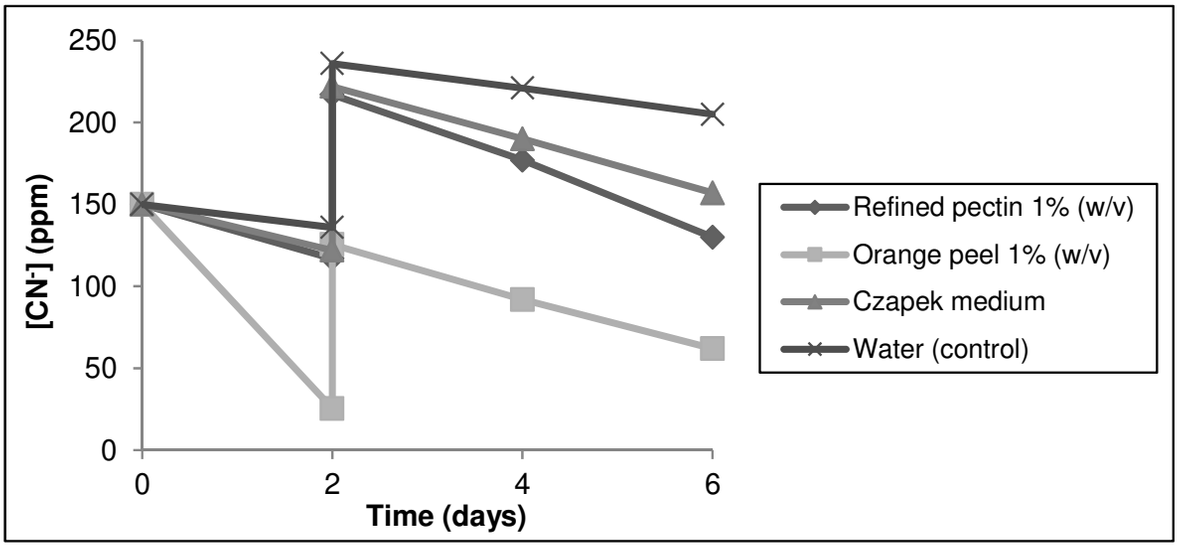

Figure 9. Cyanide bioremediation by *A. awamori* (CPUT) in batch cultures.

NH_3/NH^{4+} can be used as a nitrogen source for most fungi. The presence of quantifiable NH_3/NH^{4+} in solution during biodegradation is indicative of cyanide reduction. However, not all the NH_3/NH^{4+} was consumed by the fungus (Figure 10). Theoretical NH_3/NH^{4+} was calculated based on the quantity of cyanide degraded and the stoichiometry of the cyanide hydrolysis reaction (Figure 7). The experimental [NH_3/NH^{4+}] were lower than that of the theoretical since the fungus metabolised some of the NH_3/NH^{4+} as a nitrogen source. However, the hydrolysis of the cyanide does not result in complete metabolism of NH_3/NH^{4+}. The orange peel medium showed the highest concentration of the ammonia/ammonium in solution compared to the other media which is indicative of cyanide reduction. The rich carbon sources present in the orange peel and the further supplementation by NH_3/NH^{4+}, had shown to be an added advantage of using waste orange peel as a potential nutrient source.

However, for an efficient bioremediation process, a continuous process must be developed to assess the applicability of the *A. awamori* (CPUT) isolate for continuous cyanide bioremediation processes. One of the effective technologies which have been determined to be effective on a large scale is the use of immobilised MBRs, for continuous remediation of

contaminants [8]. The advantage of using MBR technology is that the biomass can be retained for elongated periods while the continuous remediation of the contaminated water is in progress.

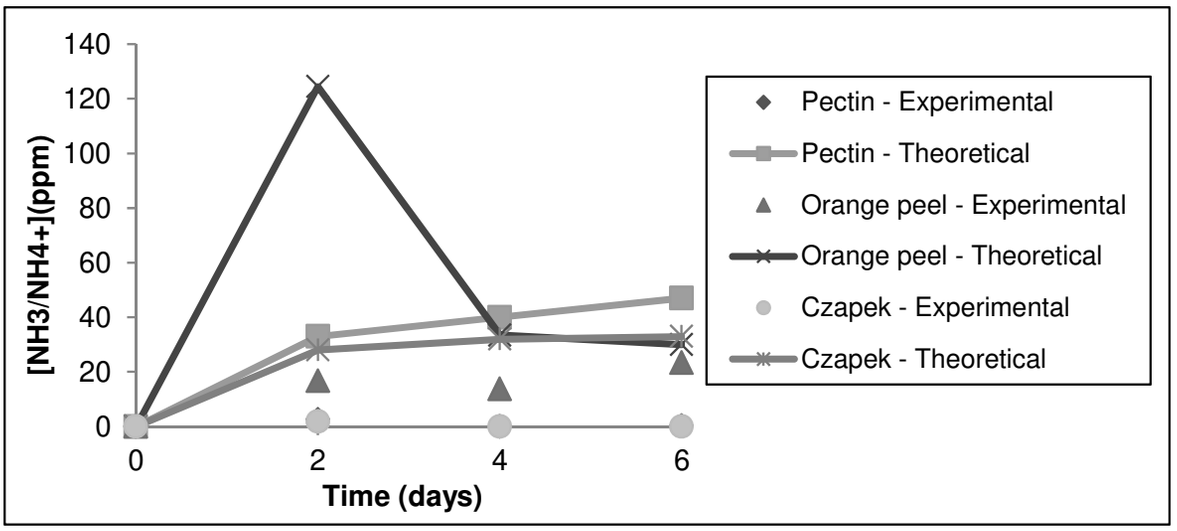

Figure 10. Experimental and theoretical ammonia/ammonium concentration in batch cultures

4. Membrane technology for fixed-film immobilisation in continuous remediation processes

Materials used in the construction of membranes include organic or non-organic (e.g. metal, ceramic), homogeneous (e.g. polymer, metal) and heterogeneous (e.g. polymer mixes, mixed glass) solids and solutions (mostly polymers) [25]. Polymeric membranes are commonly used because they are well developed, competitive in separation performances and economical [25]. Membrane processes are categorised according to the pore size, molecular cut-off and pressure at which they operate. These categories are inter-related, because as the pore size or the molecular cut-off size is decreased the pressure applied to the membrane increases [33]. Membrane separation processes can be broadly categorised into four groups, based on the pore size of the membranes (Figure 11).

Asymmetric membranes have shown to be effective in immobilising biofilms in MBRs since the membranes allow the transport of nutrients to the biomass immobilised on their external surface [27]. Asymmetric refers to the graded porosity of the membrane substructure and indicates that the membranes have an inside coating, a skin layer, and combines the high selectivity of a dense membrane with a high permeation rate of a thin membrane. The exposed microvoids on the externally unskinned surface enable a resilient attachment of the microorganism on the membrane [21]. The production of asymmetric membranes is by manipulating manufacturing parameters during the membrane formation process which results in a unique membrane morphology, characteristics and properties of the membrane [16].

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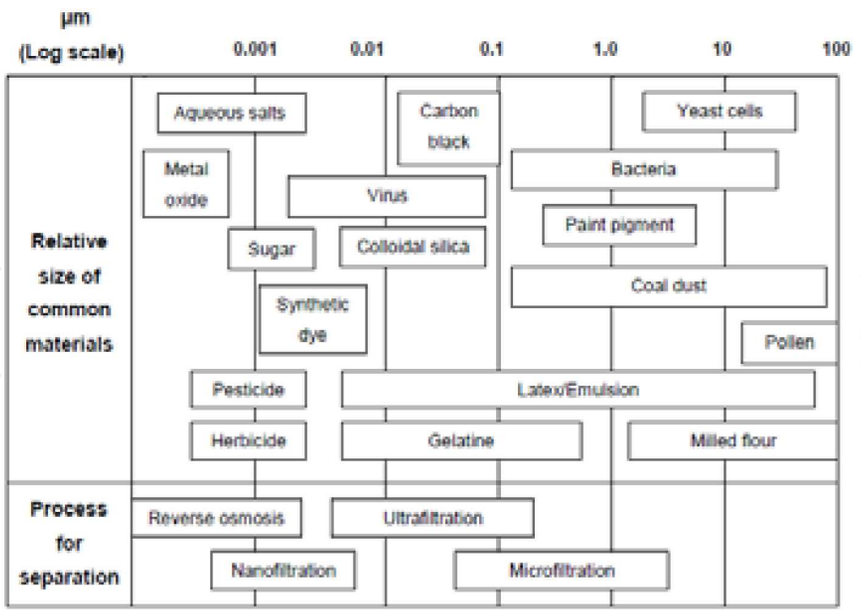

Figure 11. Filtration spectrum [25]

Asymmetric membranes have an excellent mass transfer property which has led to their utilization in numerous industrial applications, especially for MBRs [8, 25]. The most important ultrafiltration (UF) membrane module types are hollow fibre and capillary tube membranes [21, 27]. They are ideal for biofilm growth because of the nutrients permeation gradient along the membrane and provide a shear free environment [8, 15]. The large surface area of these membranes to their small volume allows for high operational capacity which is an advantageous property when used in MBRs [28].

Filamentous microorganisms are commonly used in immobilised MBR systems [3, 21, 27]. These particular microorganisms are able to penetrate the membrane due to their apical growth resulting in effective immobilisation on the surface compared to non-motile bacteria and yeast. Although, comparative studies of ceramic and internally skinned polysulphone (PSu) capillary membranes have shown to provide the best attachment and immobilisation of fungal biofilms than other tubular membrane types [21, 27]. Sheldon and Small [27] showed that the ceramic and internally skinned PSu capillary membrane developed thicker biofilms than tubular membranes. Furthermore, the ceramic capillary membrane can resists mechanical stress caused by the increasing immobilised biofilm build-up on the membrane, as ceramic membranes are mechanically stable and can be chemically and steam sterilized [27].

4.1. Modes of operation and orientation for membrane bioreactors

The membrane modules' mode of operation and orientation are important in determining the overall performance of the process. However, one of the biggest challenges in membrane operations is the effect of fouling and feeding mode, orientation and other mechanisms that would limit their application on a large scale.

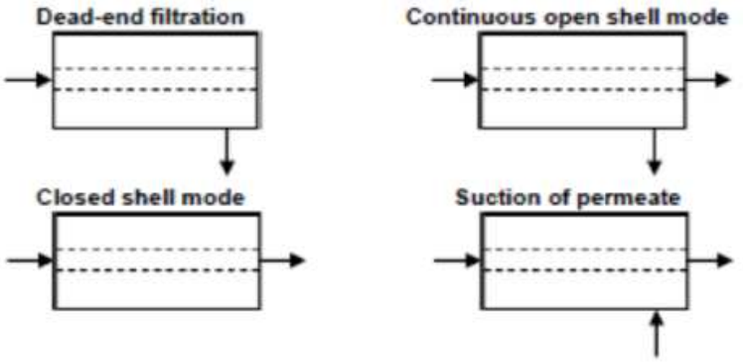

Figure 12. Modes of operation of membrane modules [2]

The effects of membrane modes of operations, as shown in Figure 12, have been studied using both experimental [6, 21, 30] and theoretical approaches [2, 8, 9, 13]. The mode of operation is concerned with the distribution and flow of the fluid in the system, as shown in Figure 12. In a dead-end filtration module the feed stream enters the lumen, permeates through the membrane and exit the shell in a continuous stream, with no retentate stream. In a continuous open shell mode, the feed stream enters the lumen and a portion of the feed permeates through the membrane and leaves the shell in a continuous stream and the other portion exit the lumen in a continuous stream. The amount of feed that will permeate through the membrane is dependent on several factors such as trans-membrane pressure, membrane permeability and feed velocity [25]. The pressure in the lumen is always greater than the shell side pressure in both dead-end filtration and continuous open shell modes [30]. This ensures that the trans-membrane flux, fluid's flow direction between the lumen and shell regions, is directed toward the shell [2].

In a closed shell mode, the feed stream enters the lumen and a portion of the feed permeates through the membrane with no outlet shell stream, while the other portion exits the lumen as a continuous stream. The trans-membrane flux for the initial portion of the membrane is directed towards the shell, but for the end portion it is directed toward the lumen [2]. This is referred to as convective recirculation which results in undesired non-uniform distribution of the biomass in the membrane system [2, 30].

In a suction of permeate, the feed stream enters the shell for an equal distribution along the membrane and permeates through the membrane and exits in a continuous stream [8]. The pressure in the shell is kept at greater than that in the lumen for a trans-membrane flux towards the lumen [30]. There is a negligible pressure gradient in the lumen which results in a relatively uniform trans-membrane flux [30]. The orientation of the MBR is based on its application and is generally operated horizontally or vertically [2, 6, 8, 27]. Studies by Garcin [6] and Ntwampe [21] on Lignin peroxidase and Manganese peroxidase production from *P. chrysosporium* BKMF-1767 (ATCC 24725) in a vertically orientated PSu capillary MBR, had shown the production of these extracellular enzymes were higher in the vertically orientated MBR than in the horizontal orientation. Similarly, the biofilm was denser on the vertically orientated capillary membrane than that in horizontal MBRs [6, 21].

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4.2. Membrane bioreactors: Design and application for cyanide remediation

MBRs are integrated biological and separation units for the production of value added products or the bioremediation of toxic components [8]. Immobilised MBRs refers to the fixed microbial biofilm formation on a membrane matrix with the film being fed a nutrient medium passing through the lumen. These MBR's retain the biomass on the membranes in a low shear environment, separate the nutrient supply from the biomass, as well as continuously remove extracellular metabolic products. These immobilised systems ensure that the biomass can be maintained in a state of low or non-proliferation for extended periods, while still producing the products desired [8]. Filamentous microorganisms have had some degree of success on immobilised MBR systems [3, 21]. However, this has not been evaluated for *A. awamori* biomass for cyanide remediation. Four vertically orientated single fibre membrane bioreactors (SFMBRs) (Figure 13) were constructed as described by Edwards *et a*l. [4].

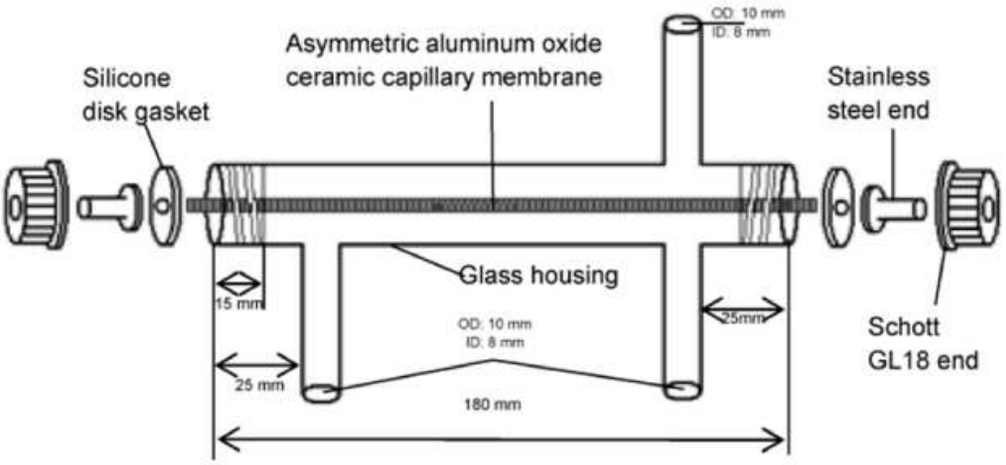

Figure 13. Schematic of SFMBR for filamentous microorganism immobilisation

The glass housing, was produced by Glasschem (South Africa) and the asymmetric aluminium oxide ceramic capillary membranes were produced and supplied by Hyflux CEPAration BV (Netherlands).

Outer diameter (m)	0.0028
Inner diameter (m)	0.0018
Wall thickness (m)	0.0005
Burst pressure (Pa)	5.0×10^{6}
Maximum temperature (˚C)	1000+
Permeability (m/Pa.s)	6.95×10^{-10}

Table 1. Capillary membrane specifications [27]

The two SFMBRs were inoculated with 100 ml of *A. awamori* inoculum ($10x10^6$ spores) and two other SFMBRs were used as controls. High pressure reverse filtration of the inoculation spore solution on the external surface of the membranes was used to immobilise the spores onto the external surface of the ceramic membranes.

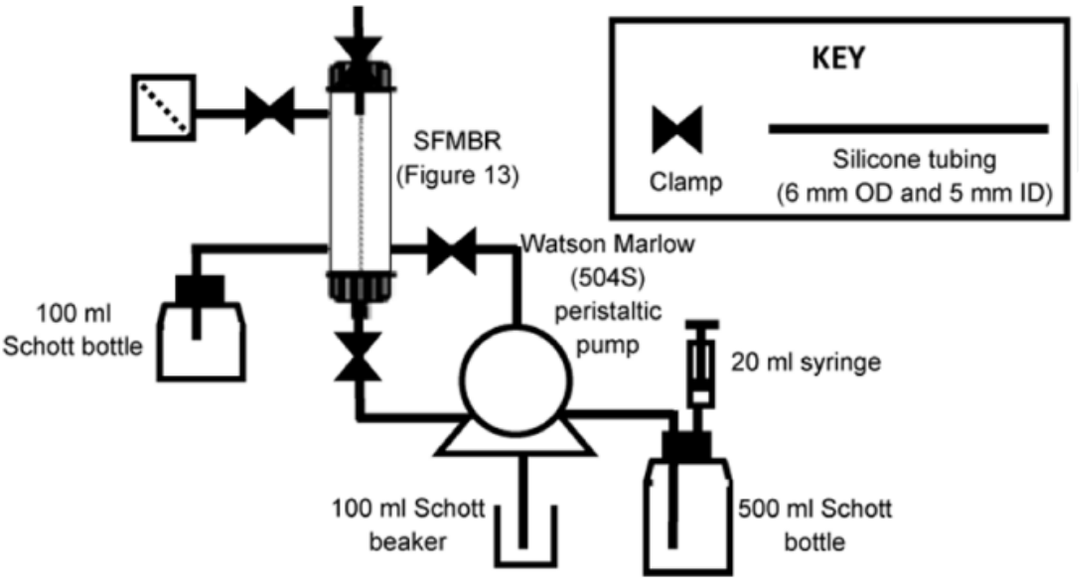

Figure 14. Schematic diagram of the experimental setup

The constructed system was setup in a Scientific Manufacturing (SMC) (160 L unit) low temperature incubator set at 30 °C. A Watson Marlow (504S) peristaltic pump was used to supply the feed solution to the SFMBRs at a flow rate of 3 ml/hr. The SFMBRs were fitted with two way flow 0.2 μm Millipore air filters, to ensure a monoseptic culture in the system and for aeration. The feed solution initially consisted of 1 % (w/v) orange peel solution which was prepared by adding 10 g milled orange peel to a 1 L Schott bottle. The 1 L solution was filtered through a Whatmann No 1 filter paper and the filtrate produced (orange peel extract) was fed to the system for 2 days, in a feed batch mode, to initiate spore germination and biofilm development on the membrane. Thereafter, a feed solution consisting of the orange peel extract and free cyanide solution (280ppm) was fed to the SFMBR.

The total reduced sugars (TRS), such as glucose, fructose, sucrose, were measured using the dinitrosalicylic (DNS) acid colorimetric method [17]. The Merck cyanide (CN^-) (09701) and Merck ammonium (NH^{4+}) (00683) test kits were used to measure the free cyanide and NH_3/NH^{4+} concentrations, since this is a by-product of the cyanide metabolism. The utilisation of the TRS increased for the pure orange peel extract feed in comparison to the control MBRs (Figure 15). The initial feed was rich with TRS and therefore, the metabolism of the TRS was observed. The change in the TRS utilisation for the orange peel extract containing cyanide concentration of 1.7%, 15.2%, 23.7% and 28.8% at day 2, 4, 6 and 8, respectively and therefore the rate of metabolism was drastically hindered which resulted in reduced metabolism of the TRS.

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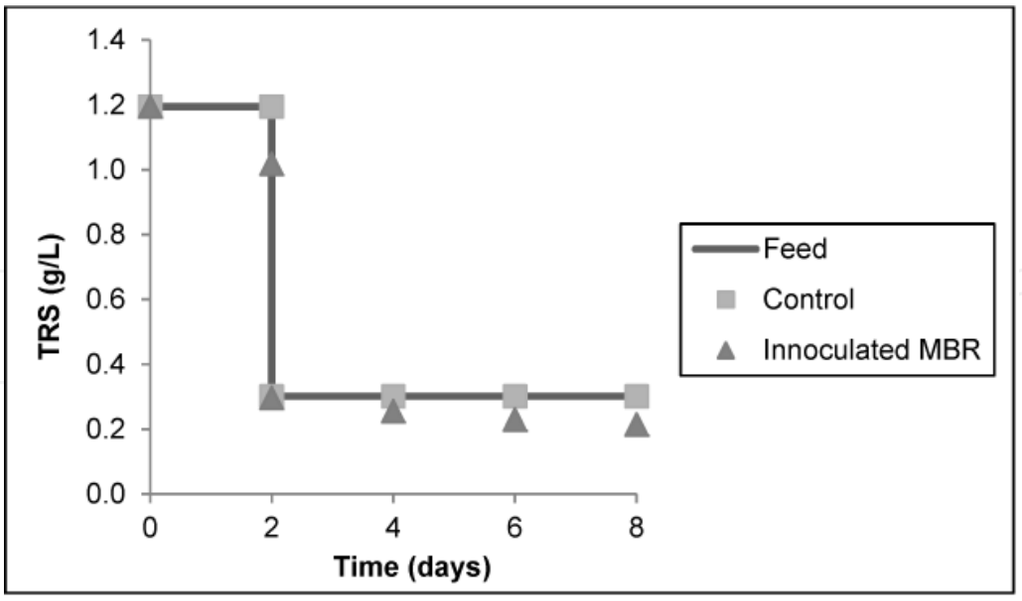

Figure 15. TRS versus time for feed, control and MBR

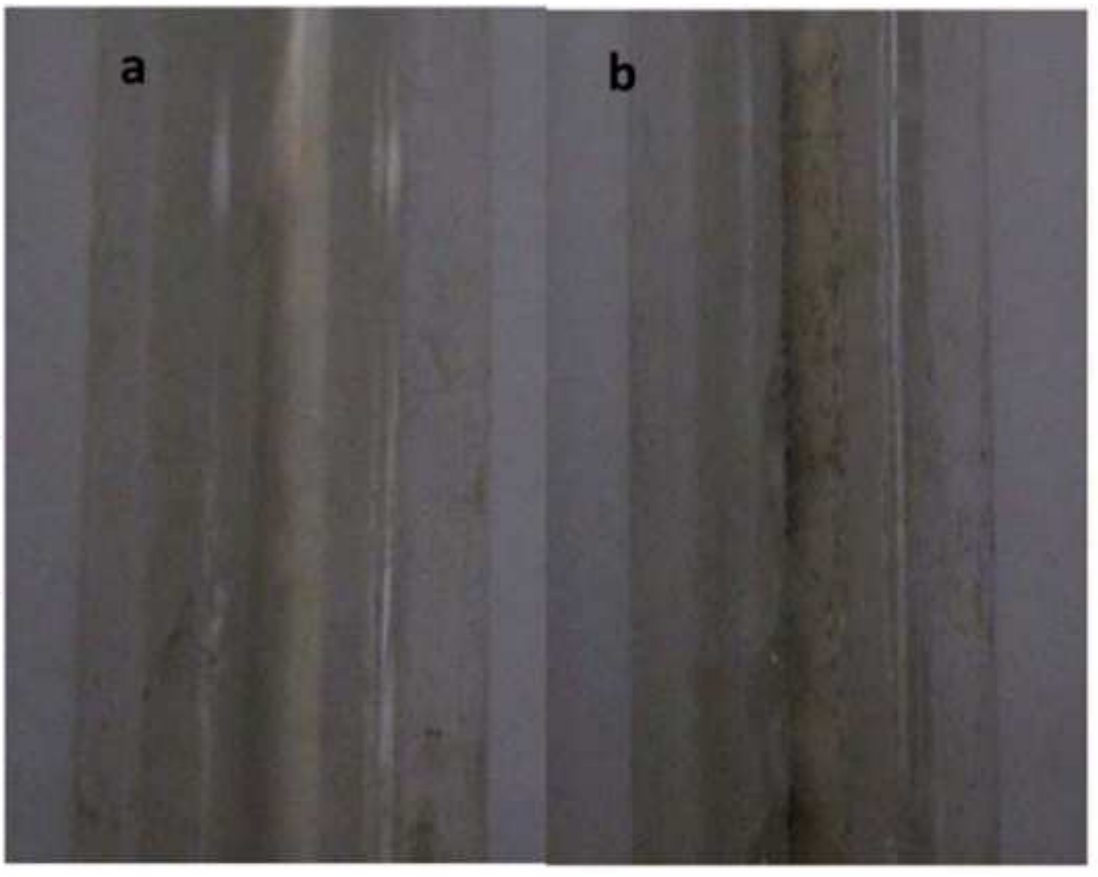

Figure 16. Biofilm development on day (a) 0 and (b) 8

The biofilm development (Figure 16) on the membrane was slow and homogenous along the membrane, as opposed to thick biomass at a particular area of the membrane. The formation of the thin biofilm has its benefits; it allows for better oxygen mass transfer into the biomass, which is vital since *A. awamori*, is an aerobic microorganism. Aerobic microorganisms utilise oxygen for cell maintenance, respiratory oxidation for further growth and for the oxidation of substrates into metabolic products.

The air filter prevented contamination and maintained the oxygen concentration in the MBR due to the gaseous venting, releasing by-products, such as carbon dioxide out of the MBR system and reintroducing oxygen back into the system. The residual cyanide concentration in the permeate decreased with time and a drastic decrease was observed from day 4 as the fungus adapted to the feed (Figure 17). There was also a considerable quantity of cyanide volatilisation compared to actual bioremediation. As shown in the shaken cultures, a majority of the ammonia, produced from cyanide hydrolysis, was consumed by the fungus as a nitrogen source. This was an indication that there was further utilisation of ammonia/ammonium as the fungus adjusts to the cyanide containing feed.

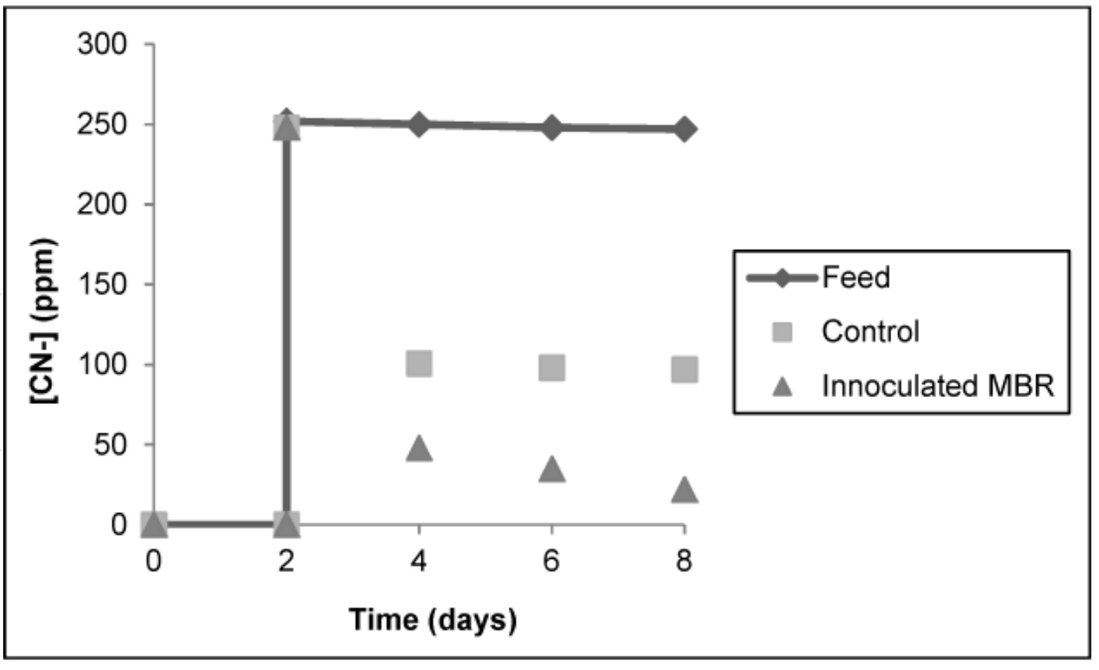

Figure 17. Discharge cyanide concentration versus time for feed, control and MBR

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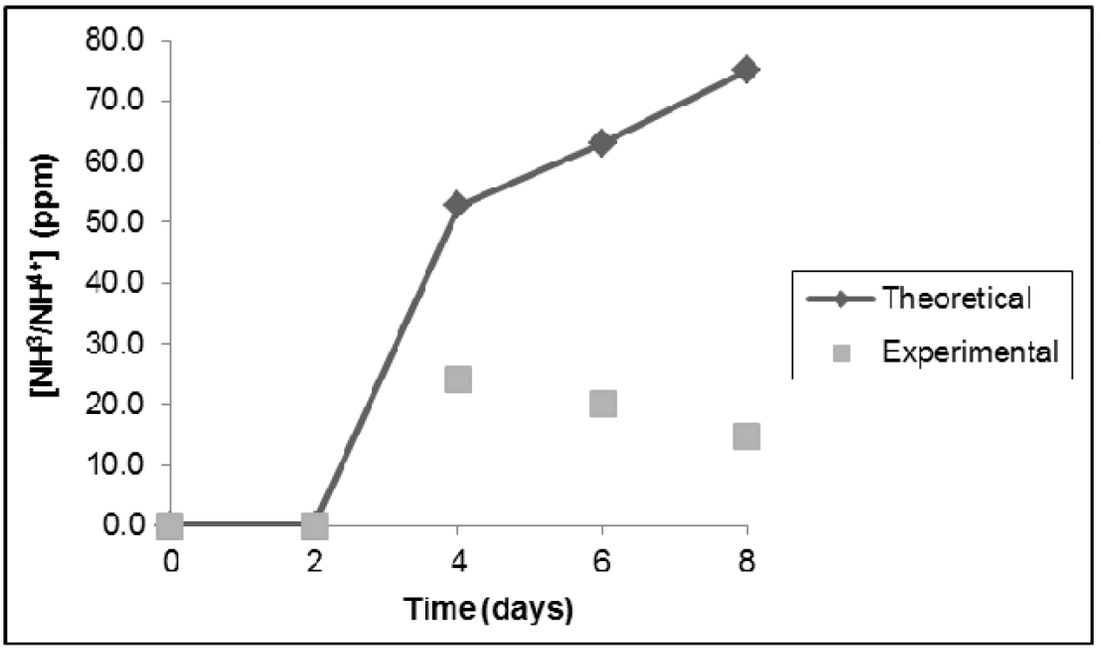

Figure 18. Experimental and theoretical ammonia/ammonium concentration

For the development of a sustainable MBR system for cyanide remediation an alternative MBR system should be used which limits cyanide volatilisation. Immersed MBRs are bioreactors in which the enzyme(s) and/or microorganism(s) or antibiotic(s) are immobilised on membrane(s) and biomass is suspended in the solution and compartmentalised in a reaction vessel [3]. Sidestream MBRs are when the membrane module and bioreactor are separate from each other. Immersed and sidestream MBRs are used for conventional biomass rejection thus allowing for continued biomass utilization [3]. Immersed MBRs require less energy than sidestream MBRs. Membrane modules in a pumped sidestream system utilises more energy due to the high pressures to sustain high volumetric flow rates [11]. However, immersed MBRs may be difficult to operate and remove fouling during operation. The separated system of bioreactor and membrane unit in a sidestream MBRs makes it easier to specifically optimise certain parameters for each unit which cannot be done in immersed MBRs.

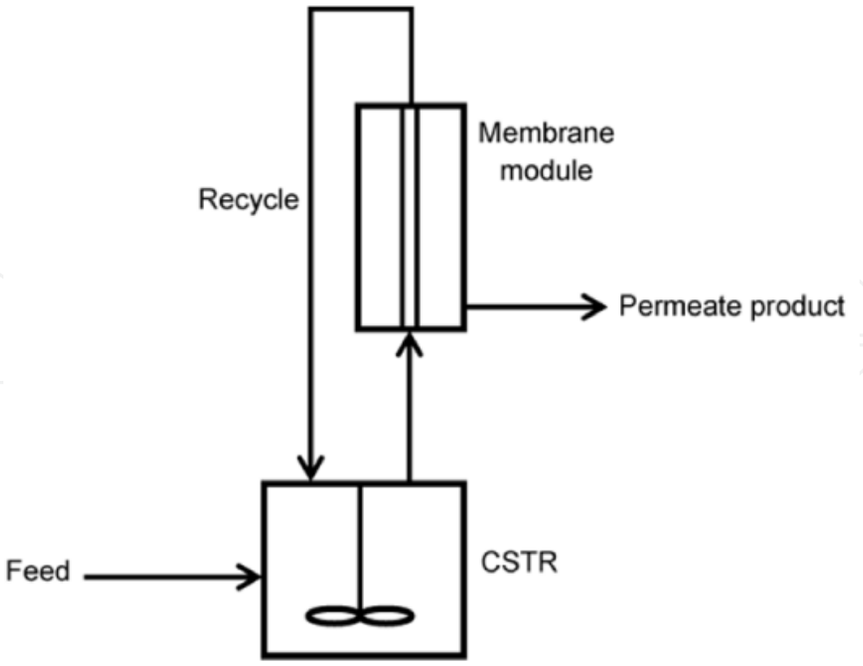

Figure 19. Side stream MBR system

For the continuous bioremediation of cyanide, a sidestream MBR (Figure 19) would be ideal since the solid material, yet to be hydrolysed, can be recycled into the continuous stirred tank reactor (CSTR) and the bioremediated wastewater can be collected as the permeate product. The CSTR can be initially loaded with milled orange peel in a water solution and inoculated with *A. awamori* thus the cyanide containing wastewater can be continuously fed into the system.

5. Conclusion

Large quantities of organic wastes are generated every year, but it has been shown that many of the waste, particularly from the agricultural sector, can be utilised as nutrient source for microbial systems. The use of orange peel has shown to be a rich source for cultivation and supplementation for *A. awamori* (CPUT) isolate for cyanide remediation. The ammonia produced from the cyanide hydrolysis can be used as a nitrogen source by the fungus, although incomplete metabolism of the ammonia was observed. This could be improved by changing operating conditions so that the degradation of cyanide, thus the release and subsequent consumption of the ammonia is improved.

Challenges that are also evident in bioremediation are due to its volatility, especially in open/agitated cultures. The hydrolysis of sugar components from the orange peel by merely boiling it in water is not an effective true solution since this process results in incomplete hydrolysis/liberation of the sugar components and residual solids. This can be improved by hydrolysis using microorganism such as *A. awamori*, as used in this study. The use of an en-

closed sidestream MBR would provide a suitable system for complete hydrolysis of the sugars from the orange peel. The unhydrolysed sugar components in the peel can be recycled and further hydrolysed depending on the molecular-weight cut-off size of the membranes used in the reactor. The sidestream MBR can reduce CN^- volatilization thus, offer a safe to operate reactor unit which is easier to clean with an added advantage of optimising each unit individually.

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