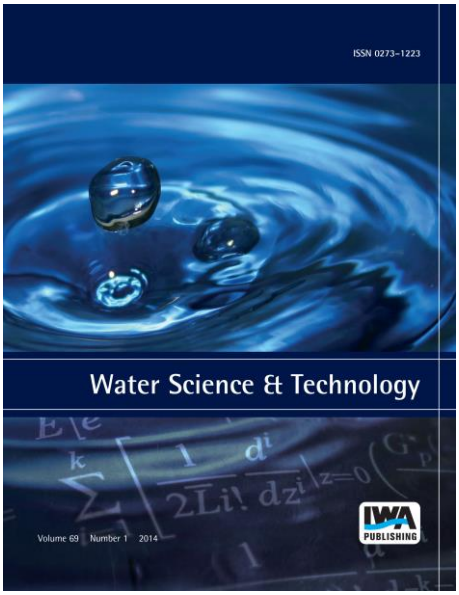

Isolation of high-salinity-tolerant bacterial strains, *Enterobacter* sp., *Serratia* sp., *Yersinia* sp., for nitrification and aerobic denitrification under cyanogenic conditions

N. Mpongwana, S. K. O. Ntwampe, L. Mekuto, E. A. Akinpelu, S. Dyantyi and Y. Mpentshu

ABSTRACT

Cyanides (CN^-) and soluble salts could potentially inhibit biological processes in wastewater treatment plants (WWTPs), such as nitrification and denitrification. Cyanide in wastewater can alter metabolic functions of microbial populations in WWTPs, thus significantly inhibiting nitrifier and denitrifier metabolic processes, rendering the water treatment processes ineffective. In this study, bacterial isolates that are tolerant to high salinity conditions, which are capable of nitrification and aerobic denitrification under cyanogenic conditions, were isolated from a poultry slaughterhouse effluent. Three of the bacterial isolates were found to be able to oxidise NH_4-N in the presence of 65.91 mg/L of free cyanide (CN^-) under saline conditions, i.e. 4.5% (w/v) NaCl. The isolates I, H and G, were identified as *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively. Results showed that 81% (I), 71% (G) and 75% (H) of 400 mg/L NH_4-N was biodegraded (nitrification) within 72 h, with the rates of biodegradation being suitably described by first order reactions, with rate constants being: 4.19 h^{-1} (I), 4.21 h^{-1} (H) and 3.79 h^{-1} (G), respectively, with correlation coefficients ranging between 0.82 and 0.89. Chemical oxygen demand (COD) removal rates were 38% (I), 42% (H) and 48% (G), over a period of 168 h with COD reduction being highest at near neutral pH.

Key words | cyanide, denitrification, *Enterobacter* sp., nitrification, salinity, *Serratia* sp., *Yersinia* sp.

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INTRODUCTION

Nitrification and denitrification are two significant processes that play a crucial role in the reduction of total nitrogen (TN) in industrial and domestic wastewater treatment plants (WWTPs) (Chen *et al.* 2014; Fajardo *et al.* 2014; Ge *et al.* 2015). These processes are performed by microorganisms that have the ability to oxidise NH_4-N and NO_2-N. These microorganisms are known as ammonium and nitrite oxidising bacteria (AOB and NOB) and can reduce NH_4-N to nitrogen gas (Shoda & Ishikawa 2014). Nitrification is carried out by AOB while denitrification is carried out by NOB (Mousavi *et al.* 2014). However, a single stage nitrification and denitrification process is difficult to achieve, particularly under saline and cyanogenic conditions. Recently, Mekuto *et al.* (2015) achieved nitrification and subsequent aerobic denitrification in a single stage process, using a consortium of *Bacillus* sp. without assessing the salinity tolerance of the process designed. Furthermore, high salinity can reduce the effectiveness of nitrification and denitrification processes (Campos 2002) although other toxicants such as free cyanide (CN^-), which have been reported as contaminants that possess the greatest inhibition effect to nitrification and denitrification microorganisms, can further exacerbate inhibitory effects, resulting in redundant TN removal (Han *et al.* 2013). CN^- does this by inactivating the respiration metabolic system of microorganisms (Chen *et al.* 2008). The aim of this study was to screen bacteria that were isolated from poultry slaughterhouse effluent, and assess their ability to nitrify and denitrify under high salinity and cyanogenic conditions.

MATERIALS AND METHODS

Microbial isolation and identification

Microorganisms used in this study were isolated from poultry slaughterhouse wastewater (Western Cape, South

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Africa). Initially, the wastewater samples were diluted, which was followed by spread plating small volumes of the water onto nutrient agar containing: 100 mg/L CN^-, 500 mg/L NH_4-N and initially 2% (w/v) NaCl, which was the salinity concentration of the slaughterhouse wastewater. The plates were incubated at both 34 °C and then at 37 °C for a period of 72 h, with 34 °C being identified as a suitable growth temperature. Minimal microbial growth was observed at temperatures below 30 °C. Single colonies were transferred into fresh agar plates for colony purification. Gram staining was performed on the pure isolates. An extraction kit (Promega) was used to isolate genomic DNA of the isolates according to the method developed by Miller *et al.* (1988), which was used with minor modifications. The 16S rDNA of the isolates was amplified using a forward primer 27f (5 0 -AGAGTTTGATCATGGCTCAG-3 0) and a reverse primer 1492r (5 0 -TACGGTTACCTTGTTACGACTT-3 0). The polymerase chain reaction (PCR) programme used was: 94 °C for 2 min, 30 cycles at 95 °C for 40 s, 55 °C for 30 s, 72 °C for 1.5 min, and the final extension being at 72 °C for 10 min. The PCR products were sequenced at the University of Stellenbosch (South Africa). Sequences obtained were thereafter compared with available 16S rDNA gene sequences in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Toxicant tolerance experiments

Nutrient broth (100 mL in 250 mL Erlenmeyer flasks) was inoculated with a loopful of 24 h old agar cultures followed by incubation in a Labwit ZWY240 shaking incubator at a speed of 160 rpm at 34 °C for a further 24 h. Subsequently, serial dilutions were performed using samples (1 mL) obtained from the culture and grown on nutrient agar containing toxicants: 500 mg/L NH_4-N and 100 mg/L CN^- at different salinity (2–6% w/v) using NaCl. Colony counts were used to assess the toxicant tolerance of the isolates. Thereafter, response surface methodology was used to determine the maximum tolerance limit, i.e. 400 mg/L NH_4-N, 65.91 mg/L CN^- and 4.5% (w/v) NaCl, based on cell concentration under different saline conditions.

Effect of pH experiments

A loopful of the isolates was inoculated into 250 mL multiport Erlenmeyer flasks with 150 mL basal medium containing: 1.5 g KH_2PO_4, 7.9 g Na_2HPO_4, 0.5 g $MgSO_4.7H_2O$ and 1 mL trace elemental per litre. The trace element solution had: 50 g EDTA, 2.2 g $ZnSO_4.7H_2O$, 5.5 g $CaCl_2$, 5.06 g $MnCl_2.4H_2O$, 5.0 g $FeSO_4.7H_2O$, 1.1 g $(NH_4)_6Mo_7O_2.4H_2O$, 1.57 g $CuSO_4.5H_2O$ and 1.61 g $CoCl_2.6H_2O$, made up to 1 L with sterile distilled water. Disodium succinate (7.8 g/L) was used as the sole carbon source. The medium was filter-sterilized using a 0.22 μm filter and the pH was adjusted to pH 2, 4.5, 7, 10 and 12. After inoculation, the flasks were incubated in a shaking incubator (160 rpm) at 34 °C for 48 h to allow microbial growth. Toxicants, i.e. 400 mg/L NH_4-N, 65.91 mg/L CN^- and 4.5% (w/v) NaCl, were added to the 48 h old cultures subsequent to incubation under the same conditions for a further 72 h. Samples (10 mL) were collected in duplicate using 20 mL syringes after 72 h to analyse for NH_4-N, NO_2-N, NO_3-N and chemical oxygen demand (COD). The multiport Erlenmeyer flasks had sampling ports which were sealed to avoid cyanide volatilisation and the flasks were not opened during the course of experiment.

Total nitrogen removal experiments

The same medium that was used in the experiments to evaluate the effect of pH was also used in TN removal experiments. The experiments were done in a similar manner, although pH 7 was chosen as all isolates performed significantly better at this pH. All experiments were carried out for 168 h. Samples (10 mL) were collected in duplicate using 20 mL syringes daily (24 h) to analyse for NH_4-N, NO_2-N, NO_3-N and CN^-. These experiments were repeated to ascertain the reliability of the results initially obtained.

Analytical methods

Residual NH_4-N, CN^-, NO_2-N and NO_3-N concentration was measured as per manufacturers' instruction using Merck ammonium (NH_4^+) (00683), cyanide (CN^-) (09701), nitrite (NO_2^-) (110057) and nitrate (14773) testkits. A Merck Spectroquant Nova 60 instrument was used to quantify the concentration of the analytes. The cyanide testkit functions on the basis that cyanide reacts with chloramines-T and pyridine-barbituric acid (Lambert *et al.* 1975). The ammonium testkit is based on the fact that the Berthelot reagent reacts with ammonium, chlorine and phenolic compounds, to form indophenol dyes (Patton & Crouch 1977). The nitrate testkit uses concentrated sulfuric acid in the presence of a benzoic acid derivative to form a colorimetrically quantifiable by-product, while the nitrite testkit is based on the concept that nitrite ions react with sulfanilic acid in order to form diazonium salt (Hassan *et al.* 2003). The COD, which functions on the basis that the inorganic substances in the sample are oxidised by potassium

dichromate in 50% sulfuric acid solution at a suitable temperature, was also quantified using Merck testkits (14555). The microbial growth rate in the cultures was determined using a UV–visible spectrophotometer at 660 nm.

Modelling of NH_4-N degradation and accumulation

Third polynomial equations were used to model degradation and accumulation of NH_4-N for each of the isolates using an ordinary differential equation solver, Polymath®. The equations are as follows:

Isolate H:

$$\frac{dy}{dx} = -12.4x^2 + 123x - 245 \quad (1)$$

Isolate G:

$$\frac{dy}{dx} = -12.7x^2 + 125x - 250 \quad (2)$$

Isolate I:

$$\frac{dy}{dx} = -13.7x^2 + 133x - 256 \quad (3)$$

RESULTS AND DISCUSSION

Isolation and characterisation of strains

A Gram stain was performed on each of the isolates and all three strains were found to be Gram-negative rods. The 16 s rRNA of the isolates was sequenced, with the results indicated that strain I, H and G had 95, 93 and 97% similarities to *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively. Figure 1 illustrates both genomic DNA and PCR product bands in agarose gel used during the isolate identification process.

Salinity tolerance

Nitrification and denitrification are inhibited by high salinity; however, inhibition of nitrification and denitrification by salt concentration is not well documented in literature (Dinçer & Kargi 2001). Some authors have proposed the use of halophilic organisms to achieve highest treatment efficiency of

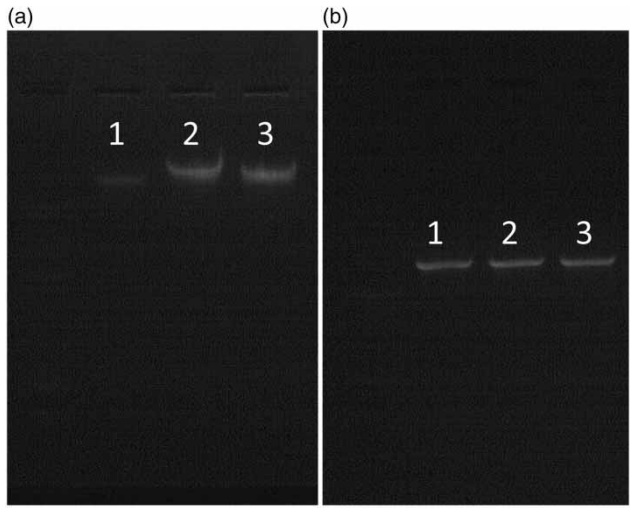

Figure 1 | (a) Genomic DNA – Lane 1: *Enterobacter* sp., Lane 2: *Yersinia* sp. and Lane 3: *Serratia* sp. (b) PCR products – Lane 1: *Enterobacter* sp., Lane 2: *Yersinia* sp. and Lane 3: *Serratia* sp.

wastewater containing high NaCl concentrations (Campos 2002). In this study, bacteria were isolated from poultry slaughterhouse wastewater, where large quantities of quaternary ammonium cleaning reagents (Gantzhorn *et al.* 2014) and aluminium salts (Ikeda *et al.* 2002) were used for cleaning and sterilisation. These strains were confirmed for their ability to facilitate nitrification and aerobic denitrification in the presence of NaCl and cyanide. Initially, numerous bacteria were isolated, with the determination of a suitable growth temperature for the isolates; however, only three strains were capable of oxidising ammonium nitrogen under these conditions at 34 °C and 37 °C (Figure 2(a) and (b)). High colony counts were observed at 34 °C for all three isolate and in salinity conditions <4% (w/v). Although these strains were able to tolerate up to 6% (w/v) NaCl, growth at a salinity concentration of 6% (w/v) NaCl was minimal. Isolate I had the lowest colony counts at 37 °C and no growth was observed for isolate H at 37 °C and 6% NaCl, with 4.5% NaCl being chosen as the limit for subsequent experiments.

Effect of pH on nitrification, denitrification and COD reduction

In WWTPs, pH plays an important role during the reduction of TN in wastewater. Ammonium nitrogen can exist as either ammonium ion or ammonia and hydrogen ions depending on pH. When the pH of wastewater increases above 7.0 it occurs in the form of NH_4^+-N and if the pH increases to values above 10.0, it will occur as ammonia

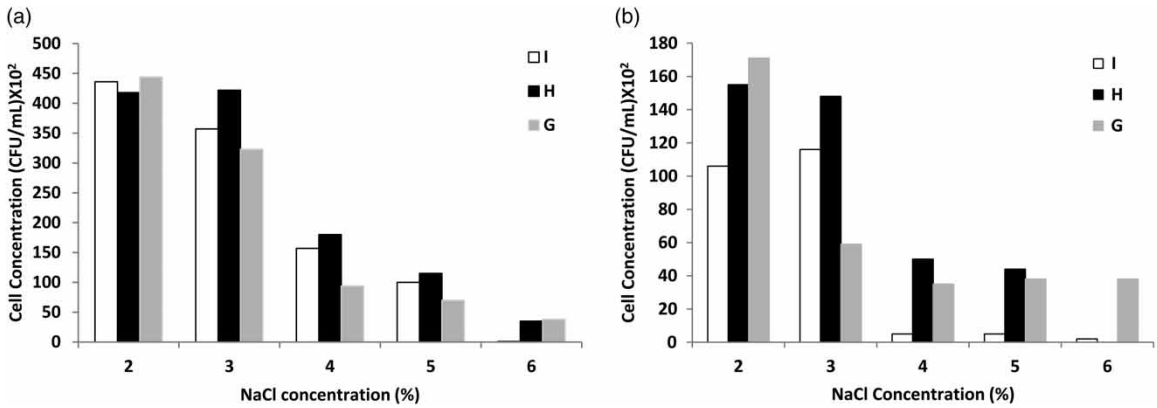

Figure 2 | Cell concentration at different salinity concentration at (a) 34 °C and (b) 37 °C, in plates containing 500 mg/L NH_4-N and 100 mg/L CN^-.

and hydrogen ions. A large percentage, i.e. 85%, of ammonium nitrogen present in wastewater can be liberated into the air through agitation when the pH is above 10 (Bonmatı & Fotats 2003; Guštin & Marinšek-Logar 2011). Hence, evaluation of the effect of pH in nitrification and denitrification in conditions containing high salt and CN^- concentrations is paramount. Many studies have shown that nitrification occurs at neutral pH. This observation was also confirmed, with nitrification observed for the isolates being used at pH 7 (Figure 3(a)). The highest COD

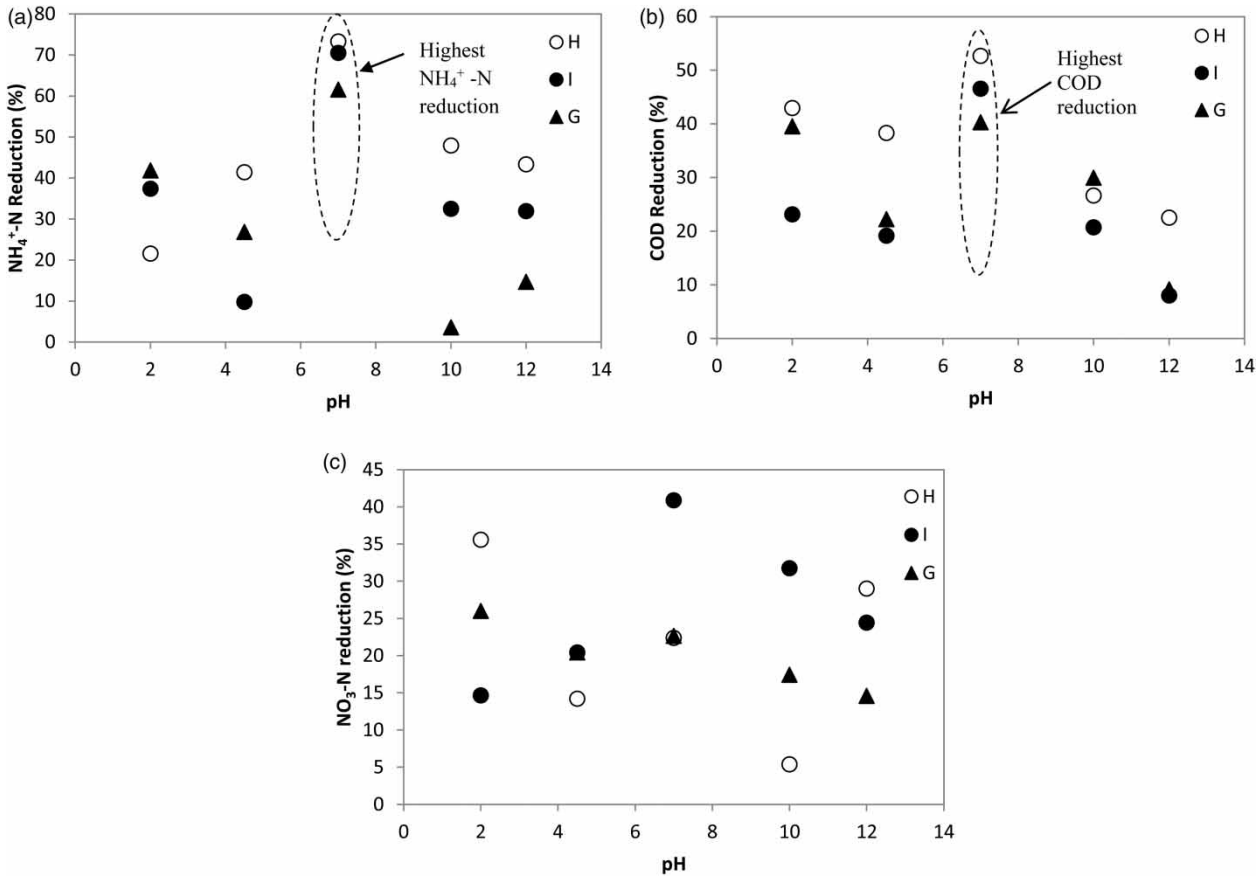

Figure 3 | Effect of pH on nitrification and denitrification including the reduction of COD. (a) NH_4^+-N reduction (nitrification), (b) COD reduction, and (c) NO_3-N reduction (denitrification). Isolates I, H and G are *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively.

reduction was also observed at pH 7 for all the isolates (Figure 3(b)). However, the reduction of NO_3-N was low at pH 7 for *Yersinia* sp. and *Serratia* sp. when compared to the isolate *Enterobacter* sp. – see Figure 3(c).

Nitrogenous compound removal efficiency and modelling

Ammonium nitrogen degradation by the three isolates from the poultry wastewater effluent was studied in batch cultures in the presence of 65.91 mg CN^-/L and 4.5% NaCl. The maximum efficiency for the combination of the studied contaminants was 400 mg/L NH_4-N, 65.91 mg/L CN^- and 4.5% (w/v) NaCl. The maximum ammonium nitrogen removal was observed to be until 72 h (Figure 6) with first order reaction rate constants (k) being 0.57, 0.53, and 0.52 h^{-1}, respectively. Ammonium utilisation was approximately 81% (I), 75% (H) and 71% (G) by 72 h and drastically increased after 96 h by 75, 62.9, and 56.6%, as the isolates started to degrade the CN^- to NH_4-N which accumulated in the bioreactors when the residual NH_4-N reached a concentration below 100 mg/L from an initial concentration of 400 mg/L. Furthermore, bacterial decay was observed at OD_{660} from day 5 (Figure 4(d)). Dinçer & Kargi (2001) reported a decrease in nitrification from 2.9 mg N/L.h to 2.6 mg N/L.h and further to 2.2 mg N/L.h when the salinity concentration was increased from 0% to 3% and further to 5%, respectively. Additionally, Uygur & Kargı (2004) observed a similar decrease in nitrification efficiency from 96% to 39% when salinity increased from 0 to 6%. Therefore, it appears that the isolates from this study had a higher nitrification rate than previously studied. The characteristics of the isolates are similar to those of *Rhodococcus* sp. CPZ24 (Chen *et al.* 2012), with low accumulation of NO_2-N and NO_3-N being observed between 48 and 96 h (Figure 4(a) and (b)) subsequent to their disappearance after 120 h. Moreover, there was no correlation between ammonium oxidation and NO_2-N accumulation within the cultures. Chen *et al.* (2012) observed a similar phenomenon with *Rhodococcus* sp. CPZ24, with <2% and 7% of NO_2-N and NO_3-N accumulating during the study, respectively.

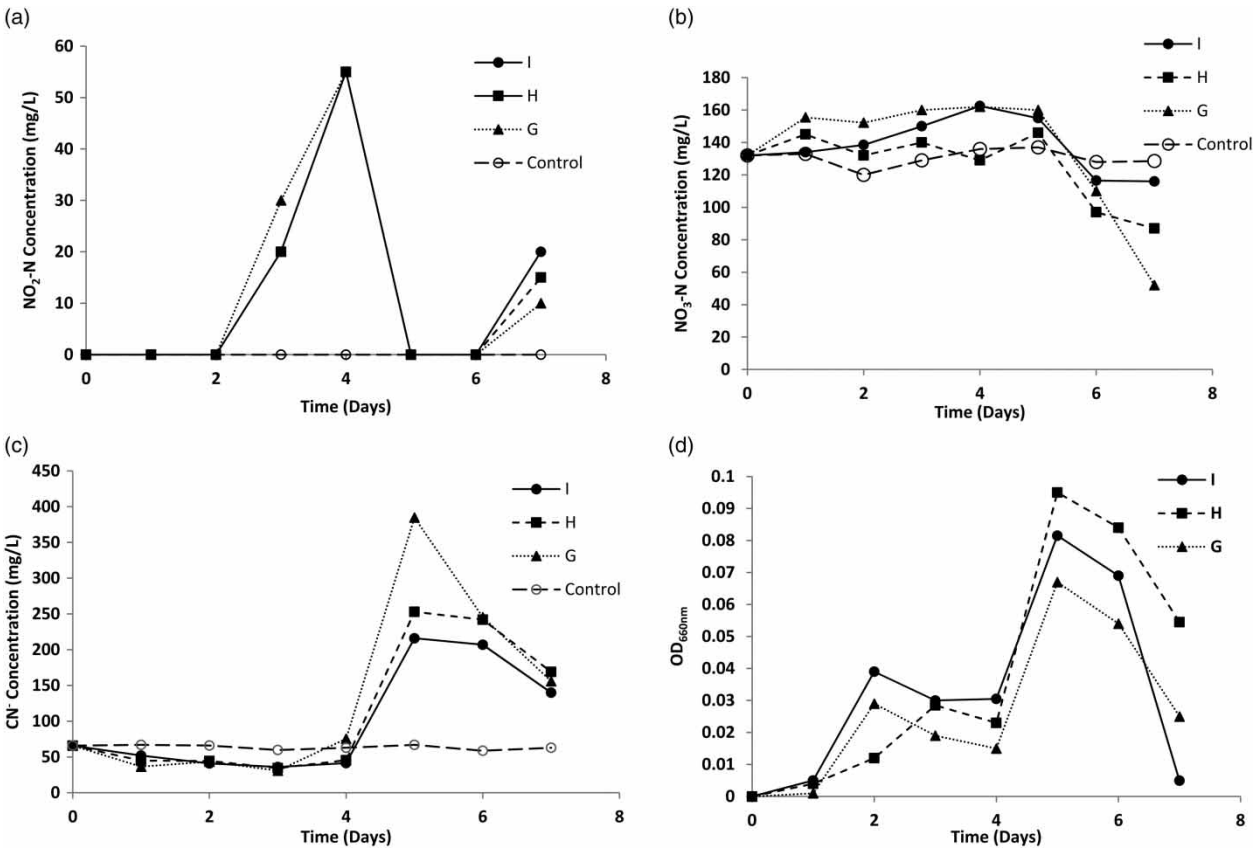

Figure 4 | (a) NO_2-N accumulation and subsequent degradation, (b) NO_3-N removal, (c) CN^- production and degradation, and (d) cell growth, for isolates I, H and G and control.

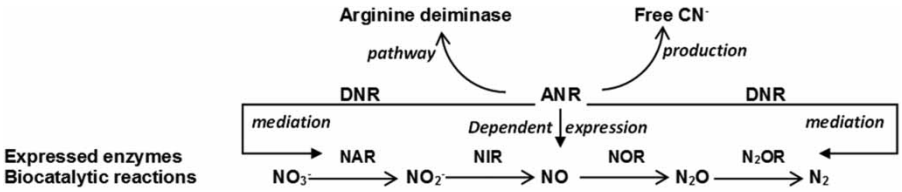

Figure 5 | Diagram representing regulation cascade of ANR and DNR in *Pseudomonas aeruginosa*, the production of cyanide, the arginine deiminase (ADI) pathway and the expression of denitrification enzymes (based on Arai *et al.* 1997). NAR: nitrate reductase; NIR: nitrite reductase; NOR: nitric oxide reductase: N_2OR: nitrous oxide reductase.

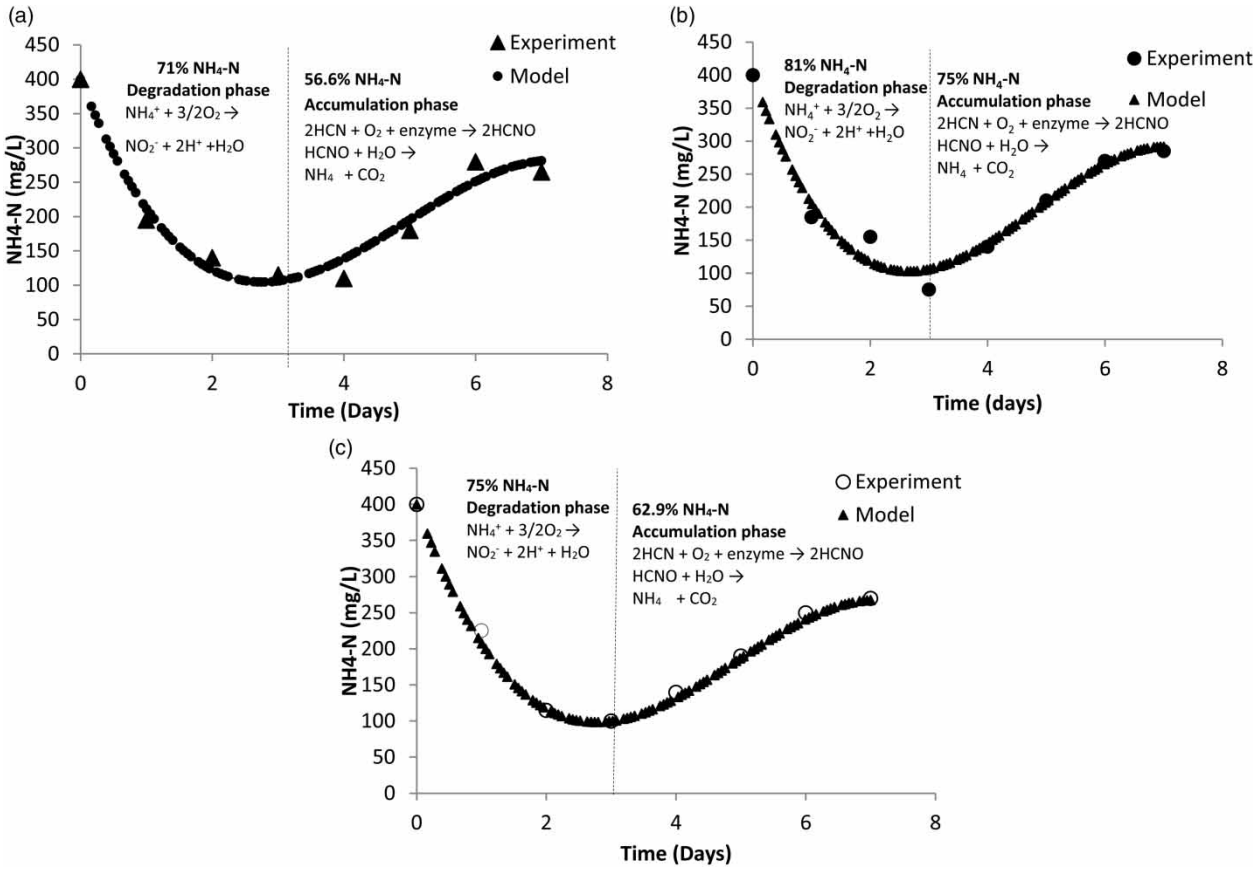

Figure 6 | Comparison between the model and NH_4^+-N degradation and accumulation for isolate G (a), isolate I (b), and isolate H (c). This includes mechanisms for each part of the reaction.

Therefore, NH_4-N removal could be due to rapid bacterial assimilation or simultaneous conversion of TN to nitrogen gas – an observation reported for *Acinetobacter* sp. Y16 (Huang *et al.* 2013) and *P. stutzeri* YZN-001 (Zhang *et al.* 2011) cultures, whereby ammonium was oxidised with minimal nitrite or nitrate accumulation. Wild *et al.* (1994) showed that despite CN^- toxicity, some microorganisms can adapt to high concentration of CN^- after being exposed for prolonged periods to high CN^- concentrations. This was also confirmed by Do *et al.* (2008) who observed ammonium oxidation after 28 days of non-reactivity during a prolonged lag phase period in cultures containing 10 mg/L of CN^-. The ability of isolates I, H and G to facilitate nitrification in the presence of salts and CN^- could be linked to the environment, which contains significant quantities of nitrogen, phosphorus and other heavy metals from which they were isolated. Furthermore, Magaji & Chup (2012) observed that salt, CN^- and other heavy metals were higher than the US Environmental Protection Agency acceptable standards in river banks

close to the slaughterhouse located in Gwagwalada-Abuja (Nigeria). Overall, the presence of CN^- did not affect nitrification as it was unutilised from day 0 to day 4, with a drastic increase from 96 h (Figure 4(c)), an observation which was deemed incomprehensible.

However, the increase in CN^- concentration from 96 h may be ascribed to the expression of anaerobic regulation of arginine (ANR). Arai *et al.* (1997) reported production of cyanide by *Pseudomonas aeruginosa* when ANR is expressed. This protein is responsible for the dissimilative nitrate respiration regulator (DNR) proteins which express enzymes necessary for denitrification. This can confirm the expression of the denitrification pathway (Figure 5); moreover, with a decrease in NO_3-N at 120–168 h, it was plausible that the denitrification pathway was activated in the isolates used. The increase in cyanide resulted in the deactivation of all three isolates as observed in the rapid decrease in the OD_{660} for the cultures (Figure 4(d)). The isolates were also determined to utilise CN^-, with all three isolates modifying their metabolism, starting to degrade CN^- after 120 h, subsequent to the accumulation of NH_4-N (Figure 4(c) and Figure (6)). This observation was modelled using Polymath®. The degradation and accumulation of NH_4-N was described by a third order polynomial with R^2 values of 0.96 (I), 0.99 (H) and 0.95 (G), respectively (see Figure 6).

CONCLUSION

The 16S rRNA sequence revealed that I, H, and G are 95, 93 and 97% similar to *Enterobacter* sp., *Yersinia* sp. and *Serratia* sp., respectively, with overall results showing that isolates I, H, and G can degrade up to 81, 75 and 71% of NH_4-N within 72 h of bioreactor operation, with minimal NO_2-N and NO_3-N accumulation, in the presence of CN^- and 4.5% (w/v) NaCl. Additionally, COD and ammonium nitrogen reduction was determined to be optimal at pH 7. Therefore these isolates demonstrated their potential for full scale applications.

ACKNOWLEDGEMENTS

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