Biodegradation of free cyanide and subsequent utilisation of biodegradation by-products by *Bacillus* consortia: Optimization using Response Surface Methodology

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ABSTRACT

A mesophilic alkali-tolerant bacterial consortium belonging to the *Bacillus* genus was evaluated for its ability to biodegrade high free cyanide (CN^-) concentration (up to 500 mg CN^-/L), subsequent to the oxidation of the formed ammonium and nitrates in a continuous bioreactor system solely supplemented with whey waste. Furthermore, an optimisation study for successful cyanide biodegradation by this consortium was evaluated in batch bioreactors (BB) using response surface methodology (RSM). The input variables, that is, pH, temperature and whey-waste concentration, were optimised using a numerical optimisation technique where the optimum conditions were found to be: pH 9.88, temperature 33.60 °C and whey-waste concentration of 14.27 g/L, under which 206.53 mg CN^-/L in 96 h can be biodegraded by the microbial species from an initial cyanide concentration of 500 mg CN^-/L. Furthermore, using the optimised data, cyanide biodegradation in a continuous mode was evaluated in a dual-stage packed-bed bioreactor (PBB) connected in series to a pneumatic bioreactor system (PBS) used for simultaneous nitrification, including aerobic denitrification. The whey-supported *Bacillus sp.* culture was not inhibited by the free cyanide concentration of up to 500 mg CN^-/L, with an overall degradation efficiency of ≥99% with subsequent nitrification and aerobic denitrification of the formed ammonium and nitrates over a period of 80 days. This is the first study to report free cyanide biodegradation at concentrations of up to 500 mg CN^-/L in a continuous system using whey waste as a microbial feedstock. The results showed that the process has the potential for the bioremediation of cyanide containing wastewaters.

Keywords: Cyanide, *Bacillus*, biodegradation, nitrification-aerobic denitrification, response surface methodology, dual-stage packed-bed reactor.

1 Introduction

Environmental legislation focusing on wastewater disposal has become increasingly stringent on industries that utilise cyanide and/or cyanide-related compounds. The International Cyanide Management Code (ICMC) of the International Cyanide Management Institute (ICMI) (www.cyanide-code.org) which is associated with the approval of process certification for industries that utilise cyanide has enforced such industries to develop alternative waste management practices (Gibbons, 2005, Akcil, 2002). The mineral processing, photo finishing, metal plating, coal processing, synthetic fibre production and extraction of precious metals, that is, gold and silver, contribute significantly to cyanide contamination in the environment through wastewater (Acheampong et al., 2013, Akcil, 2006, Akcil, 2003). As fresh water reserves throughout the world are low, cyanide contamination in water reserves is a major threat, not only to the economy, but also to the lives of living organisms, including humans, that feed from these sources (Harper and Goldhaber, 1997, Kjeldsen, 1999, Mudder and Botz, 2004). In the mining industry, dilute cyanide solutions are utilised for the recovery of base (e.g. Cu, Zn, Ni, etc.) and precious metals (e.g. Au, Ag, etc.), thus forming cyanide–metal complexes which are

classified as weak-acid dissociable and strong-acid dissociable cyanides (Akcil, 2002, Mudder et al., 2001). The presence of these metallocyanides and thiocyanate adds to the complexity of the solution matrix. However, for technical reasons, the water utilised for these processes cannot be recycled upstream of the mineral bioleaching circuit as the microorganisms employed in mineral bioleaching are sensitive to cyanide and its complexes, and thus the presence of such compounds would inhibit microbial activity, resulting in poor mineral oxidation (Makhotla et al., 2010, Van Buuren et al., 2011). The inability to recycle the water has negative implications on water conservation and re-use, especially in arid regions.

The widely utilised processes throughout the world for remediating cyanide-contaminated waters include: 1) alkaline chlorination, 2) hydrogen peroxide, and 3) sulphur dioxide oxidation (INCO process)(Gupta et al., 2010, Akcil, 2003, Kuyucak and Akcil, 2013). However, the use of these methods over the years has declined, owing to high capital investment and the production of toxic end products which require further treatment, thus increasing operational costs of the processes (Akcil et al., 2003, Akcil and Mudder, 2003). The development and operation of a biological treatment process for cyanide containing wastewater at the Homestake Mine in 1984 elicited interest from researchers on the biodegradation of cyanide. The process utilised microbial species such as bacteria and fungi, including algae which possess different enzymes that are responsible for cyanide catalysis (Gupta et al., 2010). The biological processes involved are environmentally friendly as they do not produce toxic compounds (Dash et al., 2009). Attention has focused on aerobic biodegradation rather than anaerobic biodegradation, owing to the latter's having a cyanide threshold of 2 mg CN^-/L, while the former has a threshold of 200 mg CN^-/L (Kuyucak and Akcil, 2013) with the main emphasis being given on bacterial cyanide biodegradation due to their faster growth rates. However, cyanide and metal-cyanide complexes biodegradation (Patil and Paknikar, 1999) have been observed by use of fungal (Ezzi and Lynch, 2005) and algal species (Gurbuz et al., 2009). Biological degradation of thiocyanate in a rotating biological contactor (RBC), where the thiocyanate degradation efficiency exceeded 99%, with subsequent recycling of the process water to the mineral bioleaching circuit, has been studied elsewhere (Stott et al., 2001). Furthermore, this study demonstrated the effectiveness of using a microbial consortium involved in the degradation of thiocyanate and, more importantly, the ability to recycle the treated water to the bioleaching stage without reducing the efficacy of the microbial community involved.

However, research studies have focused on the utilisation of refined carbohydrates to support microbial communities within biodegradation processes, thus increasing the operational costs of these processes. However, these costs can be reduced by utilising agro-waste feedstock for the microbial communities within the biodegradation processes. The utilisation of such waste would drastically reduce the costs associated with the process, while promoting process efficiency through biostimulation and bioaugmentation, as the agro-waste contains necessary nutrients in the form of carbon, nitrogen and phosphorus, including micro-nutrients. Cyanide biodegradation efficiency of 99% using *Citrus sinensis* waste as a supplement has been reported (Santos et al., 2013), while different agro-wastes were assessed as potential supplements for the continuous bioremediation of cyanide (Ntwampe and Santos, 2013).

Research on the biological degradation of free cyanide, the most toxic form of cyanide, and subsequent utilisation of by products by the same microbial consortia in continuous systems has not been thoroughly explored. Additionally, there is limited information on free cyanide biodegradation at concentrations above the

reported 200 mg CN^-/L threshold, in a continuous system. The microbial consortia used in this study were also evaluated for the utilisation of cyanide biodegradation by-products, i.e. ammonium and nitrates, subsequent to cyanide biodegradation. Therefore, it was imperative to study free cyanide biodegradation up to a free cyanide concentration of 500 mg CN^-/L, while evaluating the ability of the cyanide degrading consortia for the successive utilisation of biodegradation by-products in a continuous system. In this study, whey waste was used exclusively as a bioaugmentation feedstock for the biodegradation of cyanide in batch and continuous systems. Furthermore, as cyanide biodegradation can be affected by physico-chemical parameters such as pH, temperature and the availability of a growth-limiting substrate (Adjei and Ohta, 2000), it was therefore necessary to optimise these parameters. Consequently, the objective of this study was to optimise the physico-chemical conditions for cyanide biodegradation in wastewater using cyanide-degrading bacteria (CDB) mobilised in a continuous system containing dual-stage packed-bed bioreactors (PBB) combined with a pneumatic bioreactor system (PBS) in series.

2 Materials and methods

2.1 Microorganisms and inoculum preparation

Bacterial species that were previously isolated in electroplating wastewater and observed to be dominated by species belonging to the *Bacillus* genus were used in this study (Mekuto et al., 2013). The inoculum was prepared by mixing the 13 isolated strains in a phosphate- buffered medium in a 250 mL airtight multiport Erlenmeyer flask fitted with a sampling syringe with a maximum volume of 100 mL. This was done to minimise cyanide volatilisation. This was solely supplemented with whey waste (8 g/L) and grown for 48 h in a shaking incubator at 37°C at a speed of 180 rpm. The whey waste that was utilised in this study was obtained from a dairy producing company that is situated in Cape Town, South Africa. The inoculum concentration for the optimisation study was set at 10 % (v/v) for the batch cultures. The inoculum was equivalent to a cell concentration of 6.2×10^7 cells/mL.

2.2 Response surface methodology (RSM): central composite design experiments

RSM is a combination of mathematical and statistical methods for the design of experiments to evaluate valuable parameters which affect a process, with the main objective of optimising the process. In this study, a 14-run experimental plan which included the independent variables (temperature, pH and whey concentration) was generated using central composite design (CCD) at three different levels; low (-1), medium (0) and high (+1) – see Table 1. The experimental design used in this study is tabulated in Table 2, with the corresponding responses. The experiment was in 250 mL multiport airtight shaker flasks (to prevent volatilisation of cyanide as HCN gas) with a working volume of 100 mL, for 96 h. The flasks were inoculated with 10 % (v/v) inoculum size from a pre-grown culture described in section 2.1, while the uninoculated flasks served as a control at a set cyanide concentration of 500 mg CN^-/L. The pH of the media was adjusted using 1M NaOH or 1M HCl. All the runs were conducted in duplicate and the average of cyanide biodegradation obtained from the data was taken as the dependent variable or response (Y). Design-Expert® software (version 6.0.8, Stat-Ease Inc., Minneapolis, USA) was used to calculate and analyse the second-order polynomial coefficients. The behaviour of the system can be described using the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad [1]$$

where Y, β_0, β_i, $\beta_{ii}$and β_{ij} respresent response variable, interception coefficient, coefficient of linear effect, coefficient of quadratic coefficient, and interaction coefficient, respectively. X_i and X_j are input variables that influence the response (Y), while ε represents the random error.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included Fisher's F-test (overall model significance), its associated probability $p(F)$, correlation coefficient R, and determination coefficient R^2, which measures the goodness of fit for the regression model.

Table 1: The various media components included in CCD experiments and their corresponding high, medium and low concentration levels

Variables	Code	High levels (+1)	Medium levels (0)	Low levels (-1)
pH	A	11.00	10.25	9.50
Substrate concentration (g/L)	B	15.00	12.50	10.00
Temperature (°C)	C	40.00	32.50	25.00

α = 1.682

Table 2: Central composite design using 3 variables and the corresponding response

Run	A	B	C	F-CN biodegradation (mg/L)
1	-1	-1	+1	60.5
2	-1	+1	+1	143
3	+1	-1	-1	86.5
4	+1	-1	+1	14
5	0	0	0	189
6	0	0	+α	68
7	+1	+1	-1	69.5
8	+α	0	0	50.499
9	-1	+1	-1	98
10	+1	+1	+1	89
11	0	0	-α	95.5
12	0	-α	0	54
13	0	+α	0	212.996

14	-1	-1	-1	94
15	-α	0	0	184

A, B and C represent the coded level of variables while α represents the axial point with coded level of 1.682

2.3 Bioreactor construction and operation

A dual-stage packed-bed bioreactor with a pneumatic bioreactor was constructed according to the schematic diagram shown in Figure 1. The dual-stage packed-bed reactor served as primary cyanide biodegradation process, while the pneumatic bioreactor served primarily as a simultaneous nitrification and aerobic denitrification bioreactor. The PBS was constructed using polyvinyl chloride and had maximum volume of 1.5 litres where the working volume was set at 1 litre. The internal diameter of the reactor was 100 mm and was covered with an airtight lid and sampling port was located on the side of the reactor. The airtight lid was introduced to counteract ammonium stripping thus allowing the stripped ammonium to re-dissolve in the solution for further removal by the microbial species. The sparger at the bottom of the reactor was a 0.22µm stainless steel filter.

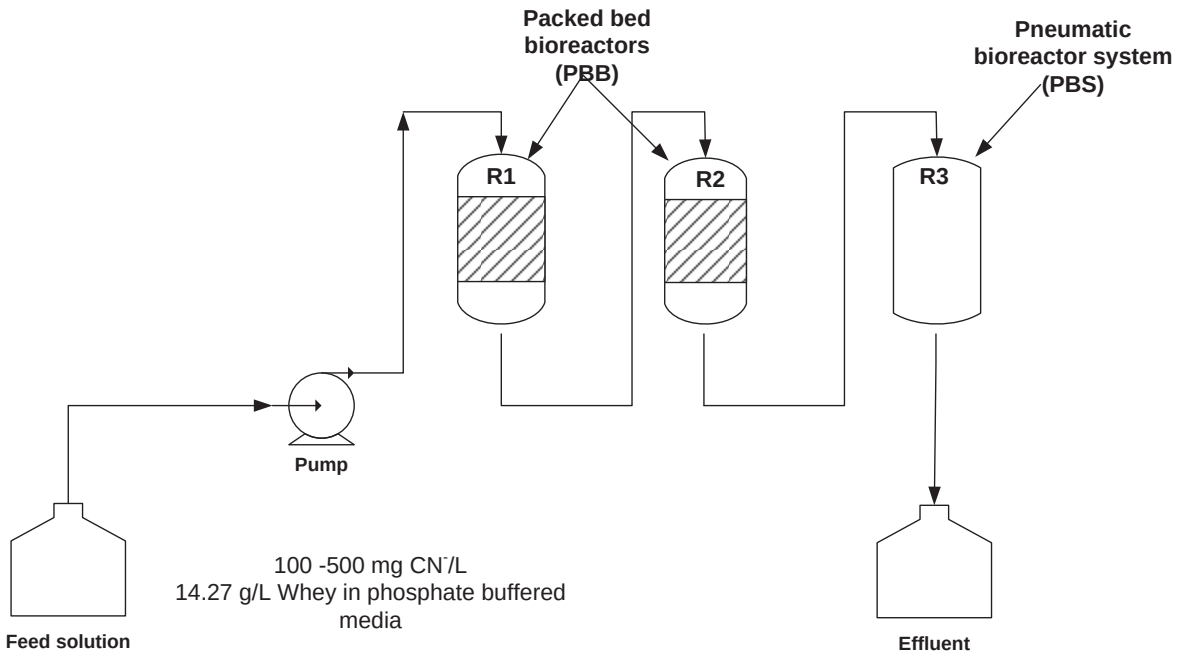

Figure 1: A schematic diagram dual stage packed bed bioreactor (PBB) with a pneumatic bioreactor system (PBS) for the continuous biodegradation of free cyanide and subsequent nitrification and aerobic denitrification of ammonium and nitrates, respectively (Schematic diagram was developed using Microsoft Vision 2010).

All the materials that were utilised in this experiment were sterilised at 121 °C for 20 minutes. The system was operated at the optimised temperature of 33.6 °C, at pH 9.88 where the cyanide concentration was gradually increased from 100 to 500 mg CN^-/L for a period of approximately 80 days. Each packed-bed bioreactor had a storage system for sampling purposes. The system had airtight sampling ports to prevent volatilisation of cyanide and the samples were obtained using 20 mL BT syringes with a Luer Lock tip. Each PBB had 44 g of

agglomerated granite rock (size = <2 mm) packing. The agglomeration was achieved by adding 10 mL of the phosphate media. To assist with uniform liquid distribution, sterile marbles were placed on top and beneath the agglomerated granite rocks. The PBBs were inoculated by flooding the system with CDB-based inoculum which was aseptically added to the packed bed and left for 18 h to allow the microorganisms to attach to the rocks. The solution was slowly drained before commencing with irrigation. The feed solution was supplied from the top of the PBB at a flow rate of 260 mL/day using the Watson Marlow 520S peristaltic pump, in a phosphate-buffered medium (pH 9.88). The system was not aerated, and therefore the microbial species utilised the dissolved oxygen in the wastewater to fulfil their oxygen requirements. A recycle stream was not used in the system.

2.4 Microbial detachment from granite packing

To assess microbial growth as the F-CN concentration increases at different stages of microbial attachment, a modified detachment procedure was used to detach microbial cells from the granite (Govender et al., 2013). Sterile phosphate buffer (pH 7.0) was used instead of the autotrophic basal salt (ABS) for detachment. The method differentiates between the interstitial, weakly-attached and strongly-attached cells. Packing samples weighing 2 g were placed into a sterile, 100 mL conical flask, followed by several (n = 3) washing steps. Each washing step involved adding 2 mL of the phosphate buffer solution, followed by vortexing for 2 minutes (except for the first step where the flask was swirled smoothly), followed by centrifugation (800 *g*) for 1 minute, decanting the supernatant and discarding the settled residue. In the last step, Tween 20 (0.4 % v/v) was added to the phosphate buffer to detach strongly-attached microorganisms from the packing. Interstitial phase cells were obtained from the first washing step, with cells from the second step regarded as weakly-attached cells, while those obtained in the last step were regarded as strongly-attached cells. Subsequent to cell detachment, microbial population was quantified by a cell count obtained from each of the washing steps.

2.5 Analytical methods

Merck ammonium (NH_4^+) (00683), cyanide (CN^-) (09701) and nitrate (14773) test kits were used to quantify the concentration of free cyanide, ammonium, and nitrates in a Merck Spectroquant Nova 60 instrument. Briefly, the cyanide test kit works on the on the reaction of cyanide with chloramine-T and pyridine-barbituric acid. The ammonia test kit works on the Berthelot reaction between ammonia, chlorine and phenolic compounds to form indophenol dyes. The nitrate test kit makes use of concentrated sulphuric acid in the presence of a benzoic acid derivative. The pH was measured using a Crison Basic20 pH meter which was calibrated daily. The microbial population was quantified using the Thoma counting chamber under a microscope at 100x magnification.

3 Results and discussion

3.1 Response surface methodology: central composite design

The central composite design was employed to study the interactions among the independent variables and also determine their optimal levels. The statistical model, based on the sequential model sum of squares and lack-of-

fit tests, explained the fitness of mean and reduced quadratic model for cyanide biodegradation (Table 3). A model (Eq. 1) was obtained that could relate to cyanide biodegradation quantified as measured output to the independent input variables. Analysis of variance (ANOVA) was used to assess the significance of each variable in the model. Multiple regression analysis was used to analyse the data and thus the polynomial equation derived from the regression analysis was:

$$Y = 189.78 - 26.44A + 30.16B - 6.43C - 30.49A^2$$
$$-24.75B^2 - 43.04C^2 - 3.56AB - 8.06AC + 21.31BC \quad [2]$$

where A, B and C are coded values for pH, whey concentration and temperature respectively. By considering the coefficients with significant effects (p<0.05) as shown in Table 3, Eq. 2 was reduced to:

$$Y = 189.78 - 26.44A + 30.16B - 30.49A^2 - 24.75B^2 - 43.04C^2 + 21.31BC \quad [3]$$

Using ANOVA, the adequacy of the model was assessed through the use of Fisher's statistical analysis and the results are shown in Table 3. The model *F* value of 11.75 implied that the model used was significant. In this case, the R^2 (0.914) denoted a good correlation between the experimental and the predicted values. The *P* values obtained denoted the significance of the coefficients in the model, highlighting interactions between the variables. The *P* values suggest that among the three variables studied, A (pH) and B (whey concentration) showed that they had a significant impact on the primary output – cyanide biodegradation. Adequate precision measures the signal to noise ratio with a desirable ratio of 4. The adequate precision ratio of 10.417 obtained in this study indicates an adequate signal. The coefficient of variance (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is normally denoted by a high CV value. The high CV value of 20.72 underscored the precision and reliability of the model. The calculated squared correlation coefficient (R^2 = 0.914) was good for the model. It is important to confirm the adequacy of the model to ensure that it gives a sufficient approximation of the actual test. The residuals from the least squares fit play an important role in judging model adequacy, and this can be done by constructing a normal probability of the plot of the residuals as shown in Figure 2. The normality assumption was satisfactory as the residual plot approximated along a straight line.

Table 3: Analysis of variance (ANOVA) for the quadratic model

Source	Sum of squares	DF	Mean square	*F* value	Prob > *F*
Model	68298.22	9	7588.69	11.75	0.0003
A	9543.65	1	9543.65	14.78	0.0032
B	12423.08	1	12423.08	19.24	0.0014
C	563.82	1	563.82	0.87	0.3721
A^2	13398.15	1	13398.15	20.75	0.0011

B^2	8825.13	1	8825.13	13.66	0.0041
C^2	26698.45	1	26698.45	41.34	< 0.0001
AB	101.53	1	101.53	0.16	0.7001
AC	520.03	1	520.03	0.81	0.3906
BC	3633.78	1	3633.78	5.63	0.0391
Residual	6458.34	10	645.83	-	-
Lack of fit	6458.34	5	1291.67	-	-

$R^2 = 0.914$

Table 4: Observed and predicted responses obtained using CCD

Run no.	**Observed (mg/L)**	**Predicted (mg/L)**
1	60.5	64.54
2	143	174.61
3	86.5	74.27
4	14	2.67
5	189	189.78
6	68	57.24
7	69.5	84.84
8	50.5	59.08
9	98	128.71
10	89	98.49
11	95.5	78.85
12	54	69.07
13	213	170.51
14	94	103.89
15	184	148

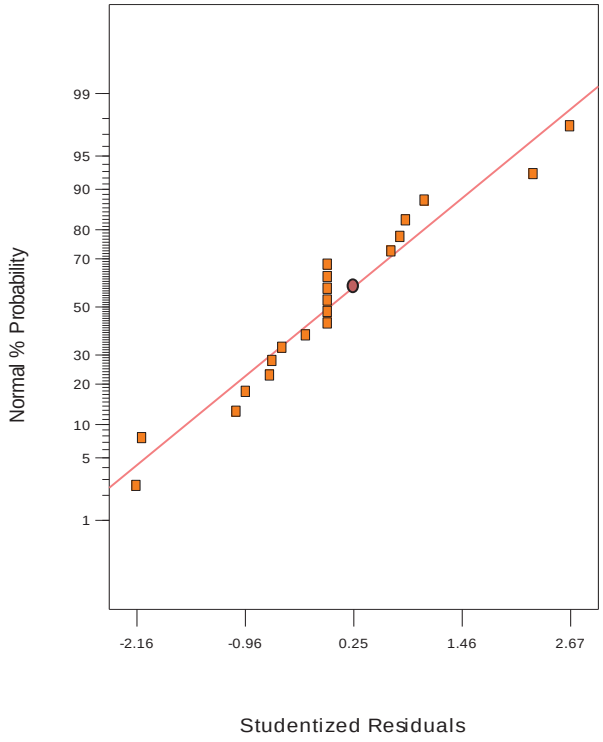

Figure 2: Normal probability plot of the studentised residuals

The observed and predicted results based on the generated quadratic model (Eq. 3) are shown in Table 4. The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. Figure 3 shows the 3D graphs of the interactions between cyanide biodegradation and the independent variables, that is, pH, temperature and whey concentration. Based on this, a contour plot was generated for a pair combination of pH and whey (Fig. 3a), temperature and whey (Fig. 3b) and temperature and pH (Fig. 3c).

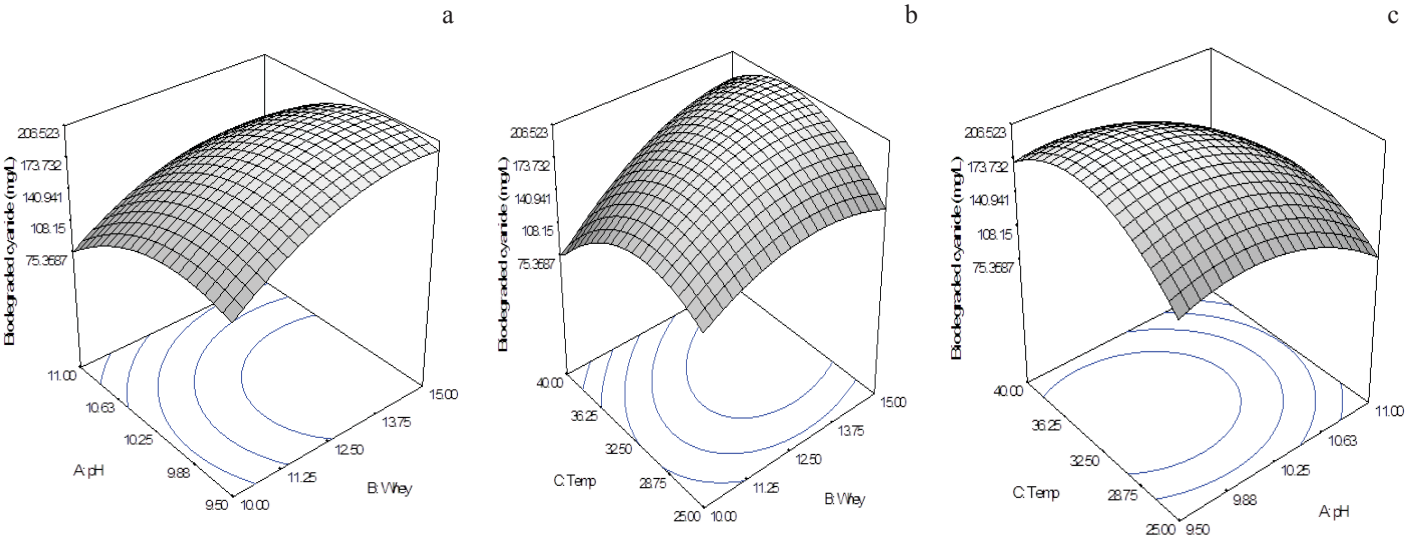

Figure 3: Response surface graphs showing the interaction of whey concentration and pH (a), whey concentration and temperature (b), and pH and temperature (c) on cyanide degradability

Design-Expert® software allows for numeric optimisation of the assessed variables, where the input variables can either be set at maximum, minimum, target or within range. In this study, the response was set to maximise cyanide biodegradation and the resultant optimal responses for pH, temperature and whey concentration were found to be 9.88, 33.6 °C and 14.27 g/L, respectively, with a desirability of 0.97, with a maximum cyanide biodegradation of 206.526 mg CN^-/L (excluding cyanide volatilisation) from an initial concentration of 500 mg CN^-/L over an incubation period of 96 hours being achievable. A similar study (Wu et al., 2013) observed that the isolated *Bacillus* species were more efficient at a temperature and pH of 31 °C and 10.3 respectively, an analysis obtained using a response surface methodology with results similar to those obtained in this study.

3.2 Biodegradation of cyanide in a dual-stage PBB

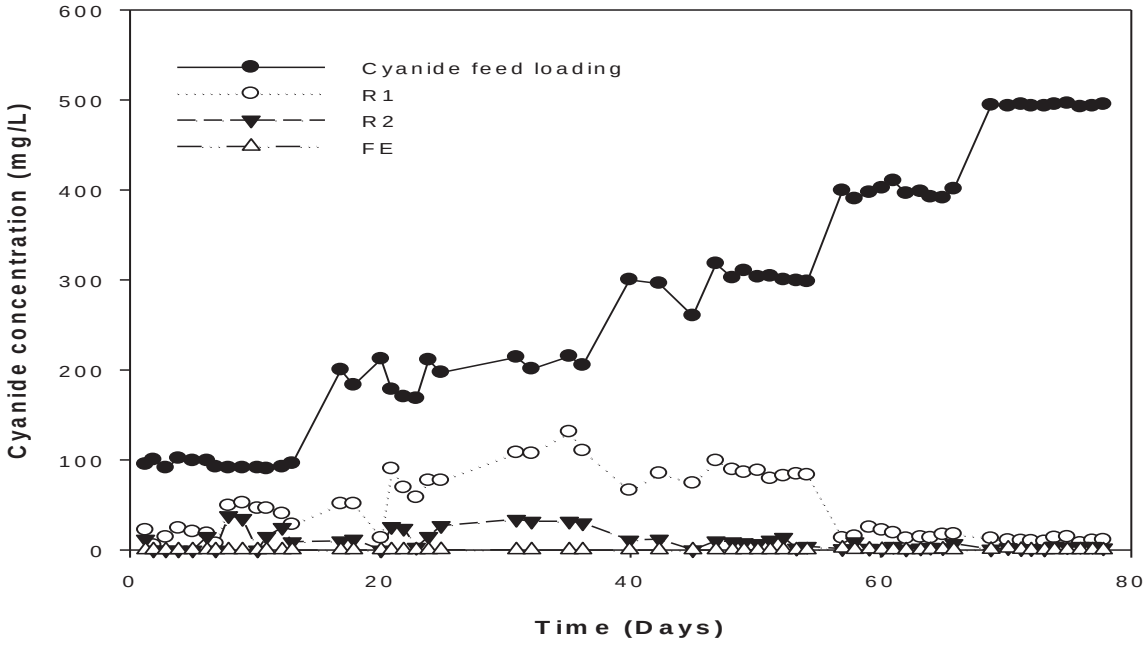

Figure 4: Residual free cyanide concentration as a function of time and feed concentration

The PBBs operated at the optimised temperature (33.60 °C) and pH (9.88) was continuously fed with 100 mg CN^-/L during the start-up period. The residual cyanide concentration from the reactors 1 (R1), 2 (R2) and 3 (R3) was analysed, with the average biodegradation efficiency observed as 72% (R1), 86% (R2), and 100% (R3), respectively. The feed CN^- concentration was subsequently increased to 200 mg CN^-/L (days 17 to 36), where the average biodegradation efficiency was found to be 61% (R1), 90% (R2) and 99% (R3), respectively. This cyanide feed concentration was maintained for a period of 10 days before increasing the F-CN concentration to 300 mg CN^-/L for an additional 14 days. The degradation efficiency increased in all the reactors, suggesting the biofilm was stable. The average degradation efficiency when the feed was maintained at 300 mg CN^-/L was

72% (R1), 97% (R2) and 99.8% (R3). During this period, significant biofilm growth on the reactor walls and on the granite rocks in the PBSs was observed. The biomass retention within the reactors led to higher effective cell concentration and thus higher degradation efficiencies. When the concentration was increased to 400 mg CN^-/L, the biofilm within the PBSs had already stabilised, which led to the complete biodegradation of cyanide with similar results observed when the concentration was increased to 500 mg CN^-/L. The increased biodegradation rates coincided with the development of large quantities of biofilm mass within the reactors, suggesting that the biodegradation of cyanide was a function of biomass retention rather than the activation of a particular metabolic pathway in the biofilms. From the data, it was observed that the microbial community were not inhibited at cyanide feed concentration of 500 mg CN^-/L, a contradiction to the maximum threshold of 200 mg CN^-/L observed for an active biological process that was evaluated by Kuyucak and Akcil (2013).

Akcil *et al* (2003) evaluated the ability of the *Pseudomonas* species in degrading weak acid dissociable cyanide (CN_{WAD}) at a maximum cyanide concentration of 400 mg CN_{WAD}/L, an efficiency of approximately 90% while Huertas *et al.* (2010) evaluated *Pseudomonas pseudoalcaligenes* CECT5344 for the biodegradation of 45 mg CN^-/L and observed a degradation efficiency of approximately 100%. However, in both of these studies aeration was utilised. It has been reported that air introduction in cyanide degradation studies results in cyanide volatilisation and therefore the results that was reported as being due to biological degradation might be due to cyanide volatilisation and biodegradation.

3.3 Biofilm attachment on the PBB packing

a b

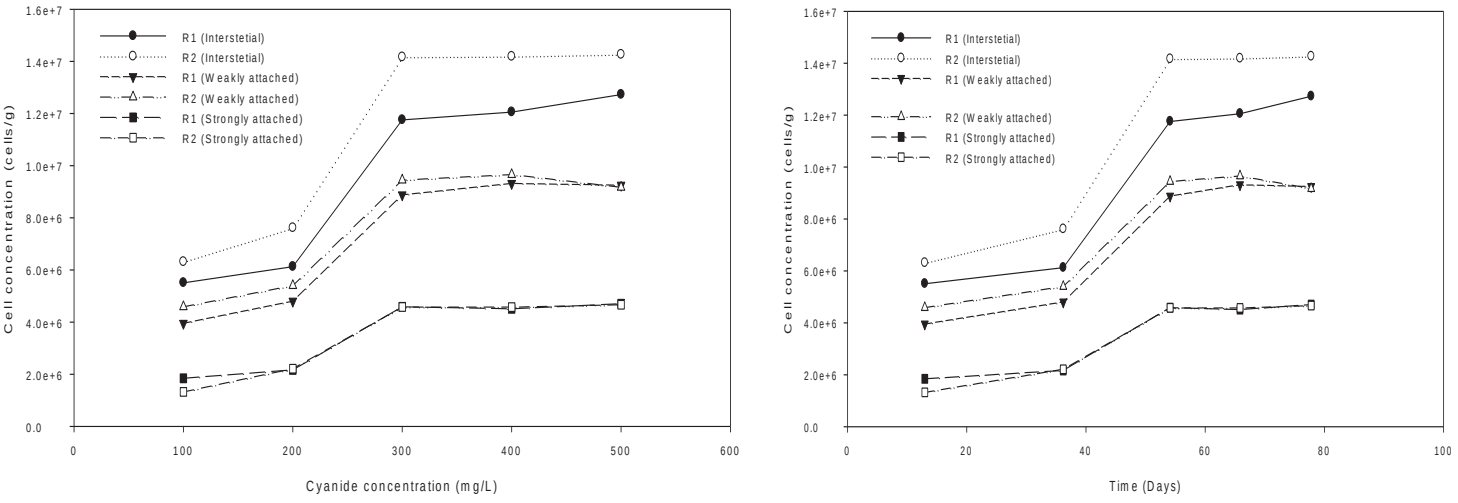

Figure 5: Microbial concentration in the three detachment stages, viz. interstitial, weakly attached and strongly attached cells, as a function of (a) cyanide concentration and (b) time

It was proposed that during biofilm formation, the microbial population attaches to surfaces to form two distinctive phases, that is, reversible and firm or irreversible attachment (Van Loosdrecht et al., 1990). The microbial cell numbers associated with the three detachment stages (interstitial, weakly attached and strongly attached) were quantified intermittently before the cyanide concentration was increased (Fig. 5). The microbial growth within the reactors followed a logistic growth pattern with three distinctive growth phases observed, viz. lag, exponential and stationary phases. The microbial species showed a similar growth pattern in all the bioreactors. From day 0 to 36, when the cyanide concentration was set between 100 to 200 mg CN^-/L, the microbial species showed a lag phase period (R1 and R2); thereafter the microbial population increased exponentially prior to a cyanide concentration increase to 300 mg CN^-/L. The microbial species reached the stationary phase between days 54 and 78. The maximum cell concentration observed as interstitial, weakly attached and strongly attached for R1 and R2 (primary units for cyanide biodegradation), were 1.28 x 10^7 cells/g, 9.30 x 10^6 cells/g, 4.70 x 10^6 cells/g and 1.42 x 10^7 cells/g, 9.45 x 10^6 cells/g and 4.67 x 10^6 cells/g, respectively.

From this information, it was observed that the microbial population attached to the packing were not inhibited by an increase in the cyanide concentration; overall, the microbial population were able to tolerate, thus degrade the cyanide, even at elevated cyanide concentration of 500 mg CN^-/L (Fig. 4), although with reduced nitrification and denitrification capabilities, thus showing the suitability of the microbial consortia employed in this study. The reactor design and nutrient source employed in this study are easily accessible and can be successfully utilised to biostimulate and bioaugment the cyanide-ammonia-nitrate biodegradation process designed.

3.4 Simultaneous nitrification and aerobic denitrification in a continuous PBB and PBS

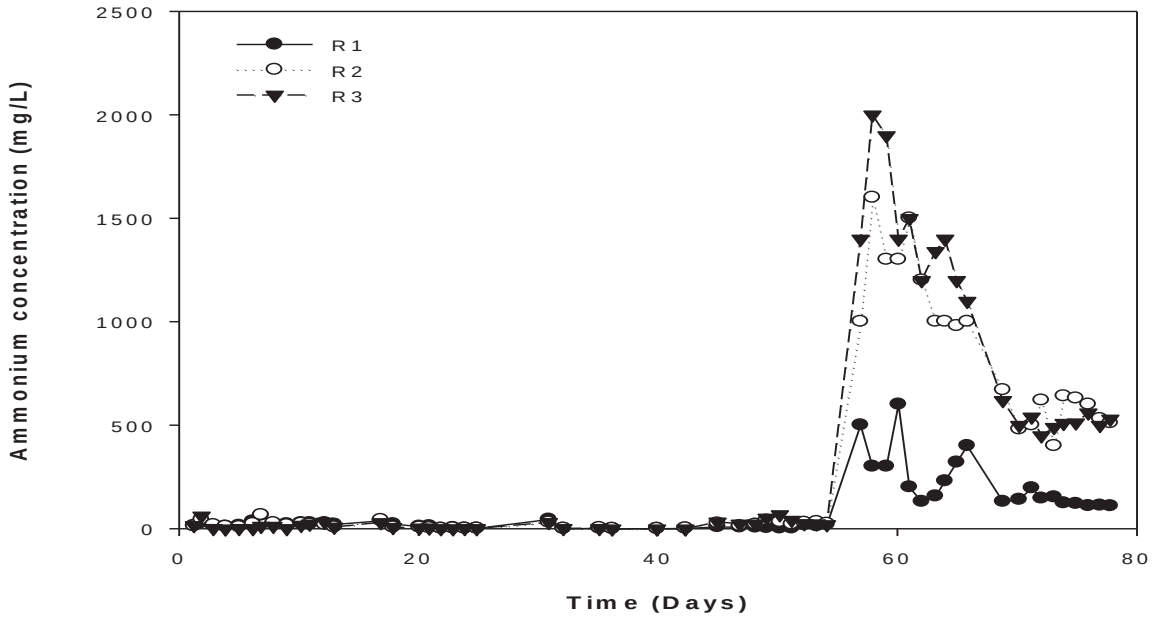

Figure 6: Ammonium concentration profile produced from cyanide biodegradation as a function time

Reactor 3 (R3), a pneumatic bioreactor (PBS), served as a nitrification and aerobic denitrification bioreactor where the conversion of ammonium and nitrates formed from cyanide biodegradation took place. The volume of R3 was 0.28 L, with a working volume of 0.25 L, and hydraulic retention time (HRT) of 24 h. When cyanide concentration was 100 mg CN^-/L, the average residual ammonium concentration from the effluent of each bioreactor was 25.2 (R1), 22.6 (R2) and 14.4 (R3) mg NH_4^+-N/L, respectively. After a period of 13 days, the cyanide concentration was increased to 200 mg CN^-/L; the average residual ammonium concentration remained between 7.1 to 11.1 mg NH_4^+-N/L in effluent from the remediation system – see Fig. 6. It was expected that as the cyanide concentration increased, the rate of ammonium formation would also increase. Therefore, when the concentration of cyanide was increased to 300 mg CN^-/L and further to 400 mg CN^-/L, the average residual ammonium concentration increased to 21.8 to 28 mg NH_4^+-N/L initially, a manageable concentration for nitrifying bacteria; however, at 400 mg CN^-/L, the accumulation of ammonium in all the reactors was observed, with average residual ammonium concentration from R1, R2 and R3 being observed between 313.6 to 1506 mg NH_4^+-N/L. Similarly, the residual concentration was high when the cyanide concentration was set to 500 mg CN^-/L. The average residual ammonium concentration was 133.8 (R1), 558(R2) and 521(R3) mg NH_4^+-N/L, respectively. The maximum ammonium concentration in the reactors was observed around day 57 where the ammonium concentration in R1, R2 and R3 was found to be 600, 1600 and 2000 mg NH_4^+-N/L. These results demonstrate that as the cyanide concentration increased, the rate of ammonium formation also increased as the biofilm within the system had stabilised, thus accelerating cyanide biodegradation to form ammonium with reduced nitrification and denitrification. When the cyanide concentration was set at 100 to 300 mg CN^-/L, the microbial species were able to nitrify the formed ammonium, but when the concentration was increased from 400 to 500 mg CN^-/L, nitrification was minimal.

Nitrification and aerobic denitrification by *Bacillus* species has been reported in numerous studies (Kim et al., 2005, Yang et al., 2011, Zhang et al., 2012). These studies have demonstrated that *Bacillus* species are able to nitrify and aerobically denitrify as observed in this study. Based on the reported nitrification ability of the *Bacillus* species employed in this study, it is suggested that an increase in the HRT in R3 from 48 to 72 h could have resulted in complete oxidation of ammonium and nitrates. This demonstrates the importance of optimising variables that affect cyanide biodegradation and also the optimisation of the nitrification process including denitrification downstream of the cyanide biodegradation processes.

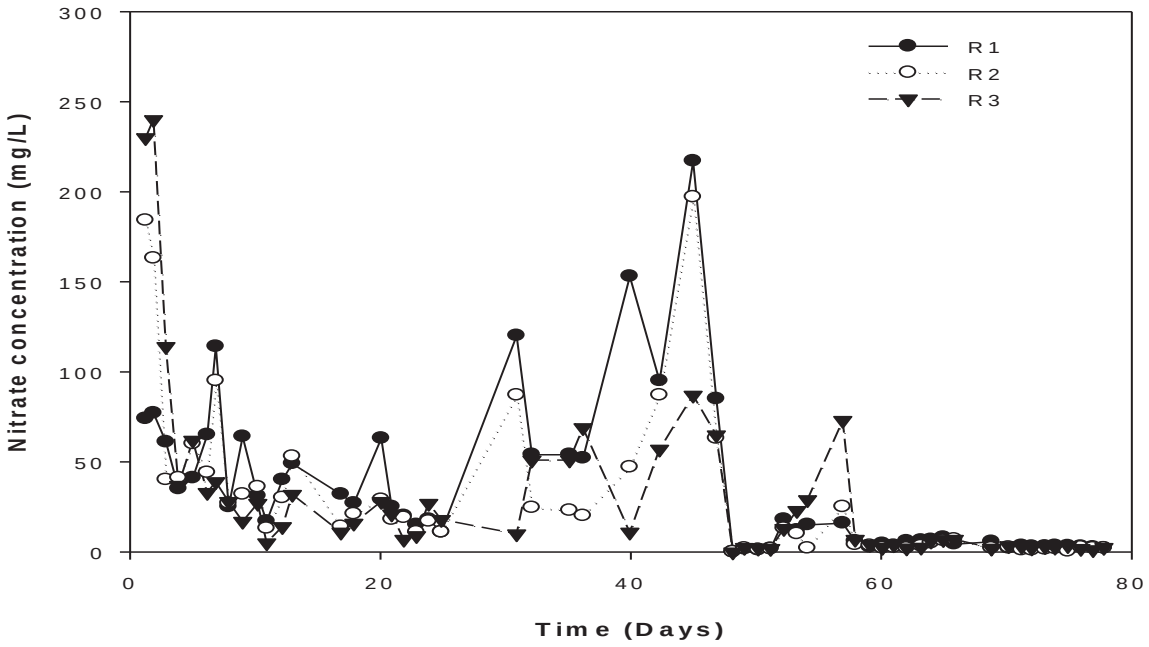

Figure 7: Nitrate concentration profile produced from the nitrification stage as a function of time

During the start-up period, the nitrate concentration in all the reactors, including the final effluent, was high, with observed concentrations of 74 (R1), 184 (R2) and 230 (R3) mg NO_3^--N/L, respectively; however, by day 24, the nitrate concentration had rapidly decreased in all the reactors, indicating aerobic denitrification by the cyanide-degrading bacteria (Fig. 7). These results demonstrated that the microbial species were able to simultaneously utilise the ammonium and the formed nitrates. This phenomenon was observed by Chen et al. (2012) where the *Rhodococcus sp* CPZ24 was able to simultaneously utilise the ammonium and nitrates. Furthermore, the *Bacillus* species have been reported to be able to accomplish nitrification and aerobic denitrification heterotrophically (Kim et al., 2005, Yang et al., 2011, Zhang et al., 2012). As the period of operation was increased to day 50 with subsequent increases in cyanide loading, the maximum nitrate concentration observed on day 42 was 217 (R1), 197 (R2) and 87 (R3) mg NO_3^-/L, respectively. From day 57 to day 78, the nitrates present in the effluent of each bioreactor ranged from 1 to 6.5 mg NO_3^--N/L; this did not correspond to increased denitrification rates, but failure of nitrification as a result of high cyanide concentration. It was observed that nitrification and aerobic denitrification within the PBB reactors was visible.

4 Conclusion

The alkaliphillic bacterial consortia dominated by *Bacillus* species, which were employed in this study, were determined to be cyanide degraders, nitrifiers and aerobic denitrifiers. Using response surface methodology for optimisation of the cyanide biodegradation process, the optimum pH, whey-waste concentration and temperature were found to be 9.88, 14.27 g/L and 33.6 °C, respectively. Using the optimised data from batch cultures, a continuous process for the biodegradation of cyanide using a packed-bed system was designed. It was observed that the microbial species degraded cyanide successfully with an overall degradation efficiency of

>99.9%, irrespective of the cyanide loading concentration. This was also confirmed by the stability of the detached microbial species at various cyanide concentrations (100 to 500 mg CN^-/L), as the microbial population did not show any decline in cell numbers after the cyanide concentration was increased. Furthermore, the organisms were able to carry out nitrification and aerobic denitrification, although nitrification was minimal when the cyanide concentration was increased from 400 to 500 mg CN^-/L. However, nitrification and denitrification rates can be improved by increasing the residence time of the nitrification and aerobic denitrification stage to optimise the efficiency of the bioprocess. This study demonstrated that the microbial consortia used were effective at degrading high cyanide concentrations with respect to the reported 200 mg CN^-/L threshold and were also able to utilise the biodegradation by-products in a continuous system while using whey waste as feedstock to sustain microbial growth. It is however recommended that; 1) a multi-stage system for nitrification-aerobic denitrification be used for effective nitrification and denitrification, 2) to also optimise the bioreactors' residence time such that the wastewater meets the required discharge standards, 3) assess the applicability of these microbial species on the biodegradation of metal-complexed cyanides and thiocyanate from the mining extraction operations coupled with the biosorption of the released metals.

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6 Conflict of interest

There is no conflict of interest associated with this work.

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