Microbial communities associated with the co-metabolism of free cyanide and thiocyanate under alkaline conditions

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Abstract

This study focused on the identification of free cyanide (CDO) and thiocyanate (TDO) degrading microbial communities using a culture-dependent and independent approach. Culturable microbial species were isolated from the CDOs ($n = 13$) and TDOs ($n = 18$). The CDOs were largely dominated by *Bacillus* sp. while the TDOs were dominated by *Bacillus* sp., *Klebsiella oxytoca*, *Providencia* sp. and *Pseudomonas* sp. However, 16S rRNA amplicon gene-sequencing revealed the complexity and diversity of the microbial communities in contrast to the organisms that were detected using culture-dependent technique. Overall, the organisms were mainly dominated by *Myroides odoratimimus* and *Proteus* sp. at 37.82 and 30.5% for CDOs, and 35.26 and 17.58% for TDOs, respectively. The co-culturing of the CDOs and TDOs resulted in biochemical changes of key metabolic enzymes, and this resulted in the complete degradation of CN^- and SCN^- simultaneously; a phenomenon which has not been witnessed, especially under alkaline conditions. Current ongoing studies are focused on the application of these organisms for the biodegradation of CN^- and SCN^- in a continuous system, under changing operational parameters, to assess their effectiveness in the biodegradation of CN^- and SCN^- .

Keywords Biodegradation · Cyanide · 16S rDNA · Microbial communities · Thiocyanate · VITEK[®] Compact

Introduction

Cyanide and its associated complexes are one of the major environmental contaminants which have contributed significantly to environmental degradation, and are mainly produced from metallurgical processes. In such processes, cyanide is used as a preferred lixiviant for the recovery of precious (Pt, Ag, Au) and base metals (Cu, Ni, Zn, etc.) from sulphide ores, in a process known as the cyanidation process (Habashi 1966, 1967). After cyanidation, the solid and liquid fractions are separated and stored in storage ponds for the natural attenuation of cyanide and related complexes. The liquid fraction generated from the cyanidation process normally contains elevated concentrations of free cyanide

(CN^-), thiocyanate (SCN^-) and metal-complexed cyanides in the form of weak acid dissociable (CN_{WAD}^-) and strong acid dissociable cyanides (CN_{SAD}^-) (Akciil 2002; Akciil and Mudder 2003). In most cases, SCN^- and CN^- are normally observed to be the major contaminants found in cyanidation wastewaters. The co-existence of these chemical constituents has been observed to be pernicious to the environment. Therefore, there is a need for the development of environmentally benign processes that will effectively treat SCN^- and CN^- containing wastewaters; a requirement which has been enforced by the International Cyanide Management Code (ICMC) (www.cyanide-code.org) (Akciil 2010). Additionally, the discharge of cyanide containing wastewaters to drainage systems, which ultimately end-up in wastewater treatment plants (WWTP), has been reported to have deleterious effects on the microorganisms that are normally employed in such systems as they are sensitive to the presence of CN^- (Han et al. 2013; Kim et al. 2011). This, in-turn, results in the failure of such WWTP.

Chemical treatment methods are currently utilised for the treatment of CN^- due to the rapid degradation rates achieved by these methods. However, chemical techniques are unable to effectively treat cyanide complexes

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and result in the uncontrollable formation of toxic and biologically persistent chemical compounds, which add to environmental deterioration (Baxter and Cummings 2006). Biotechnological approaches for the treatment of cyanide-containing wastewaters, have gained popularity over the years due to the robustness, cost-effectiveness and environmental friendliness of this process. Furthermore, microbial-mediated cyanide degradation does not produce degradation products which are hazardous to the environment, but rather produce products which serve as sources of carbon and nitrogen to the employed organisms (Mekuto et al. 2015). This is achieved through diverse enzymatic reactions that the cyanide degrading organisms possess, which include hydrolytic, oxidative, reductive and substitution/transfer pathways (Ebbs 2004; Gupta et al. 2010).

The first microbial-mediated commercial cyanide degradation system was reported at the Homestake Gold mine; a process that was dominated by *Pseudomonas paucimobilis* which was able to treat 4 million gallons of cyanide containing wastewater per day. This process remained successful and robust throughout the operational years until mine closure (Whitlock 1990). This demonstrated the effectiveness of utilising microorganisms in the treatment of cyanide-containing wastewaters. However, this process was hampered by the utilisation of traditional autotrophic nitrifying and denitrifying organisms downstream of the cyanide degradation system, and this led to process failures due to the susceptibility of such organisms to CN^- (Akciil 2003). In addition, there was a lack of a thorough understanding of the microbial constituents contributing to that process. The characterisation of the organisms that contribute to the process is imperative, as it dictates the performance of the system and, in cases where the system fails, the characterisation would enable the determination of the organisms that would be necessary for re-inoculation within the system, to regain successful process performance (Mekuto et al. 2016b). Furthermore, there is a lack of information on the co-metabolism of CN^- and SCN^- by microbial consortiums, especially under alkaline conditions. In addition, the information on the biochemical properties of microbial community co-cultures is limited. Therefore, this study was aimed at (1) the identification and characterisation of free cyanide and thiocyanate degrading organisms using a culture-dependent and independent approach and (2) evaluation of the co-metabolism of CN^- and SCN^- by the organisms under alkaline conditions, which has not been investigated. Culture dependent and independent approaches were investigated to compare the effectiveness and/or inadequacy of the tested techniques for the identification of the microbial communities involved in CN^- and SCN^- biodegradation.

Methods

Microorganisms

Cyanide-degrading organisms (CDOs)

The CDOs were previously isolated from electroplating wastewater and were previously reported to be dominated by *Bacillus* sp. (Mekuto et al. 2013). However, these organisms were observed to be unable to degrade thiocyanate (data not shown); hence, this necessitated the isolation of thiocyanate-degrading organisms. Identity confirmation of the CDOs was performed using 16S rDNA amplicon gene sequencing.

Thiocyanate-degrading organisms (TDOs)

The TDOs were isolated by gravimetric sampling, a bio-aerosol entrapment technique, utilizing synthetic wastewater solution (500 mL) containing (g/L): K_2HPO_4 (3.4), KH_2PO_4 (4.3), glucose (0.01), SCN^- (0.2) and CN^- (0.2), at a pH of 10 (± 0.05). In this technique, the solution was exposed for 2 months to allow airborne microorganisms to settle on the media outside the laboratory. After the 2 month period, the culture was transferred, maintained and agitated (200 rpm) in a 1 L stirred tank bioreactor, operated at a 48 h hydraulic retention time using the aforementioned media. Aeration was set at 400 mL/min and the reactor was operated at room temperature (21–25 °C). A fraction of the biofilm containing media was filter sterilised in a 0.22 μm Millipore membrane and thereafter, the filter was washed with sterile distilled water. This was done to isolate culturable organisms from the enrichment culture. Serial dilutions on sterile distilled water were performed subsequent to plating on nutrient agar plates that contained 100 mg SCN^-/L . The plates were incubated at 30 °C for a period of 120 h. The colonies were selected based on their morphological appearance and purified by re-streaking on freshly prepared media containing 100 mg SCN^-/L . Gram reaction of the isolates was performed to confirm the purity of the isolates and the organisms were subsequently cryopreserved in 40% (v/v) glycerol solution and stored at -80°C .

Batch biodegradation experiments

Free cyanide and thiocyanate biodegradation experiments

Free cyanide biodegradation experiments were performed in 250 mL airtight multiport flasks with a working volume of 100 mL. These flasks were utilised to minimise CN^- volatilisation. Minimal media (MM) was utilised as growth media and the chemical composition of this media has been described

elsewhere (Mekuto et al. 2016c). The pH of the media was set at 9.9. The CDO and TDO were inoculated separately, on MM containing 250 mg CN⁻/L and 250 mg SCN⁻/L, respectively, at 10% (v/v) from previously grown cultures. The flasks were incubated in an orbital shaker at 30 °C and 180 rpm. Samples were collected at various time intervals and centrifuged at 10,000g for 5 min prior to the analysis of CN⁻, SCN⁻, NH₄⁺-N, NO₃⁻-N, NO₂⁻-N and SO₄²⁻-S. Uninoculated flasks served as a control. A triplicate set of experiments were used to obtain averaged data. The biodegradation efficiency was calculated according to Eq. 1:

$$\text{Biological removal efficiency (BRE) (\%)} = \frac{C_i - C_f}{C_i} \times 100, \quad (1)$$

where C_i and C_f represents the initial and final concentrations of free cyanide and thiocyanate.

However, the volatilisation of free cyanide had to be accounted for, therefore, Eq. 1 was modified to Eq. 2 to determine the biodegradation efficiency of free cyanide supplemented experiments:

$$\text{Biological removal efficiency (BRE) (\%)} = \frac{\text{CN}_B^-}{\text{CN}_S^-} \times 100 \quad (2)$$

Where CN_B⁻ is the biologically degraded cyanide (mg CN⁻/L) and CN_S⁻ is the initial free cyanide (mg CN⁻/L), CN_B⁻ was determined according to the mass balance equation formulated by Mekuto et al. (2016c).

Co-metabolism of free cyanide and thiocyanate experiments

This study was conducted in 250 mL airtight multiport flasks with a working volume of 100 mL. Previously grown CDOs and TDOs were mixed and inoculated on MM supplemented with both CN⁻ and SCN⁻ in the same media. This was done to evaluate the co-metabolism of CN⁻ and SCN⁻. The pH of the media was set at 9.9 and the concentrations were set at 250 mg/L for both free cyanide and thiocyanate. The incubation and sampling procedure was performed as described in Sect. “Cyanide-degrading organisms (CDOs)”.

Statistical analysis

The experimental error was calculated as the standard error of mean using the standard deviation obtained from the multiple sets of data ($n = 3$), as demonstrated on Eq. 3:

$$\text{SEM} = \frac{\text{Standard deviation}}{\sqrt{\text{number of samples tested}}} \quad (3)$$

Identification of organisms using a culture-dependent approach

VITEK® 2 Compact approach

The culturable CDOs and TDOs were cultured on nutrient agar for a period of 16 h at 30 °C prior to analysis. The isolates were then inoculated on the VITEK GN (Gram negative) and GP (Gram positive) colorimetric strips using the VITEK® 2 Compact system (BioMérieux, France). Microbial suspensions were adjusted to a McFarland standard of 0.50–0.63 in 0.5% (w/v) sodium chloride using a Densi-Lameter (BioMérieux, France). The VITEK® 2 Compact system detects biochemical reactions at 15 min intervals and the generated data was analysed using the VITEK 2 software version VT-R03.1, according to the manufacturer's instructions.

Genetic approach

Identification of the culturable organisms was performed using the genomic DNA (gDNA) extraction technique, followed by Polymerase Chain Reaction (PCR), using the universal bacterial primers targeting the 16S rDNA gene. The gDNA was extracted using a commercial DNA extraction kit (Promega, Madison, Wisconsin, USA), according to manufacturer's instructions. The presence of the gDNA was assessed using a 1% (w/v) molecular grade agarose gel containing 0.5 µg/mL ethidium bromide (EtBr), using 1X Tris–acetate–ethylenediamine tetra-acetic acid (TAE) electrophoresis buffer at 100 V for 1 h. PCR was performed using a GeneAmp PCR 9700 System (Applied Biosystems, USA). Target DNA was amplified using PCR in a total reaction volume of 25 µL containing 5 µL (± 50 ng/µL) of the purified gDNA, 0.5 µL of 10 µM of the forward and reverse primers, 12.5 µL of a 2X KapaTaq Readymix solution (KapaBiosystems, South Africa) and made up using sterile distilled water. Bacterial specific primers used were the forward 8F primer 5'-AGAGTTTGATCCTGG CTCAG-3' and reverse primer 1492R 5'-GGTTACCTT GTTACGACTT-3'. The amplification process included an initial denaturing step at 94 °C for 10 min, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The reaction was completed with a final extension period of 7 min at 72 °C followed by cooling and storage at 4 °C. PCR amplicons (2 µL) were electrophoretically analysed on a 1% (w/v) molecular grade agarose gel that was stained with ethidium bromide, using 1X TAE electrophoresis buffer at 100 V for 1 h. The sequences were blasted against the NCBI GenBank database (www.ncbi.nlm.nih.gov).

Identification using a culture-independent approach

16S rDNA amplicon gene sequencing

Total gDNA was extracted directly from the CDOs and TDOs, using commercially available extraction kits (Promega, Madison, Wisconsin, USA), according to the manufacturer's instructions. The presence of the genomic DNA was assessed as per Sect. "Genetic approach". The purified DNA was PCR amplified using the 16S rRNA forward bacterial primers 27F-16S-5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primers 518R-16S-5'-ATTACCGCGTCTGCTGG-3' (Satokari et al. 2001) that targeted the V1 and V3 regions of the 16S rRNA. The PCR amplicons were sent for sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa), a commercial NGS service provider. Briefly, the PCR amplicons were gel purified, end repaired and illumina[®] specific adapter sequence were ligated to each amplicon. Following quantification, the samples were individually indexed, followed by a purification step. Amplicons were then sequenced using the illumina[®] MiSeq-2000, using a MiSeq V3 (600 cycle) kit. 20 Mb of the data (2 × 300 bp long paired end reads) were produced for each sample. The Basic Local Alignment Search Tool (BLAST)-based data analysis was performed using an Inqaba in-house developed data analysis pipeline.

Scanning electron microscope

Microbial samples were concentrated by centrifugation at 10,000g for 5 min at 4 °C followed by fixation in 2.5% glutaraldehyde overnight at 4 °C. The glutaraldehyde solution was discarded and the fixed organisms were washed twice with phosphate buffer, followed by microbial dehydration in an alcohol series of 50, 70 and 100% (v/v) ethanol for a period of 12 h at 4 °C. The samples were dried using hexamethyldisilazane (HMDS) solution and mounted on a stub coated with carbon prior to visualisation in a scanning electron microscope (Nova NanoSEM 230).

Evaluation of biochemical reactions

The biochemical properties of the CDOs, TDOs and a mixed culture of both CDOs and TDOs (referred to as CDO + TDO) were evaluated using the VITEK[®] 2 Compact system. The cells were grown for 16 to 18 h at 30 °C in MM and thereafter, recovered by centrifugation at 10,000g for 5 min at 4 °C. The cultures were inoculated into GN, GP, YST (Yeast or moulds) and BCL (Bacilli) colorimetric cards. Microbial suspensions were adjusted to a McFarland standard of 0.50–0.63 (GN and GP) and 1.8–2.2 (YST and BCL), in 0.5% (w/v) sodium chloride using a DensiLameter.

This was done to assess the biochemical reaction changes prior and post co-culturing of the CDOs and TDOs.

Analytical methods

Free cyanide was determined using the barbituric acid–pyridine method (Lambert et al. 1975), while ammonium nitrogen was detected using the Berthelot reaction (Patton and Crouch 1977). Sulphates were determined turbidimetrically using barium sulphate (Kolmert et al. 2000). The nitrites were detected using the method developed by Rider and Mellon (1946) and thiocyanate was detected using the ferric method (Katayama et al. 1992). Nitrate nitrogen was determined using Merck nitrate (14773) (Merck, Germany), as per manufacturer's instructions.

Results and discussion

Culture-dependent approach

The culturable CDOs and TDOs were isolated from cyanide and thiocyanate-laden habitats, and were identified comparatively using the Vitek[®] 2 Compact system and a DNA-based approach. The Vitek[®] 2 Compact system assigned the thirteen culturable CDOs to *Bacillus* sp. with a probability of 99% (see Table 1). The CDOs were further confirmed using a DNA-based approach, confirming the *Bacillus* sp. identity that the Vitek[®] 2 Compact system detected. Similarly, identification of the TDOs using the Vitek[®] 2 Compact system achieved similar results compared to the DNA-based approach (see Table 2). This observation demonstrated the accuracy of the Vitek[®] 2 Compact system for

Table 1 Identification of the CDO using the Vitek[®] 2 Compact system and PCR-based technique

Sample	Vitek [®] 2 Compact	PCR	Accession number
A	<i>Bacillus pumilus</i>	<i>Bacillus pumilus/safensis</i>	–
B	<i>Bacillus pumilus</i>	<i>Bacillus licheniformis</i>	–
C	Unidentified	<i>Bacillus pumilus/safensis</i>	–
D	Unidentified	<i>Bacillus pumilus/safensis</i>	–
E	<i>Bacillus pumilus</i>	<i>Bacillus pumilus/safensis</i>	–
F	<i>Bacillus</i> sp.	<i>Bacillus licheniformis</i>	–
G	<i>Bacillus pumilus</i>	<i>Bacillus pumilus/safensis</i>	–
H	<i>Bacillus pumilus</i>	<i>Bacillus pumilus/safensis</i>	–
I	<i>Bacillus pumilus</i>	<i>Bacillus pumilus/safensis</i>	–
J	<i>Bacillus</i> sp.	<i>Bacillus pumilus/safensis</i>	–
K	<i>Bacillus</i> sp.	<i>Bacillus subtilis</i>	–
L	Unidentified	<i>Bacillus pumilus/safensis</i>	–
M	Unidentified	<i>Bacillus pumilus/safensis</i>	–

Table 2 Identification of the TDO using the Vitek® 2 Compact system and PCR-based technique

Sample	Vitek® 2 Compact	PCR	Accession number
1	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas aeruginosa</i>	KR011154
2	Unidentified organism	MS	–
3	<i>Bacillus pumilus</i>	<i>Alcaligenes faecalis</i>	KX185712
4	<i>Achromobacter xylosoxidans</i>	MS	–
5	<i>Providencia rettgeri</i>	<i>Providencia rettgeri</i>	–
6	<i>Klebsiella oxytoca</i>	<i>Klebsiella oxytoca</i>	KT767971
7	<i>Klebsiella pneumoniae/oxytoca</i>	MS	–
8	<i>Klebsiella pneumoniae/oxytoca</i>	<i>Klebsiella oxytoca</i>	KT825748
9	<i>Bacillus cereus/thuringiensis/mycoides</i>	<i>Bacillus cereus/subtilis</i>	FJ696633/JF706263
10	<i>Acinetobacter</i> sp.	<i>Bacillus pumilus/safensis</i>	HQ003450/KM054689
11	<i>Raoultella ornithinolytica</i>	<i>Enterobacter</i> sp.	KJ806392
12	<i>Brucella melitensis</i>	MS	–
13	<i>Providencia rettgeri</i>	<i>Providencia vermicola/rettgeri</i>	KJ909024/KJ909021
14	<i>Sphingomonas paucimobilis</i>	<i>Pseudomonas stutzeri</i>	KX344913
15	<i>Providencia rettgeri</i>	<i>Providencia vermicola/rettgeri</i>	NR_042415/KC456564
16	<i>Sphingomonas paucimobilis/Acinetobacter haemolyticus</i>	<i>Bacillus pumilus/safensis</i>	KR780583/KU921072
17	<i>Bacillus cereus/thuringiensis/mycoides</i>	<i>Bacillus cereus/subtilis</i>	DQ289055/JX188065
18	<i>Providencia rettgeri</i>	<i>Providencia vermicola/rettgeri</i>	KC456564/NR_042415

MS Mixed sample

identification of culturable organisms which are available on the system's database. This observation was confirmed in a separate study, where an organism with an atypical phenotype (mucous morphology) was identified successfully via the DNA-based approach and Vitek® 2 Compact system as *Escherichia coli*, whereas the MALDI-TOF technique characterised the organism as *Citrobacter* sp. (Książczyk et al. 2016). Furthermore, the Vitek® 2 system also allows for the rapid detection of antibiotic or antimicrobial resistance, especially from pathogenic organisms (Barry et al. 2003); an important microbiological characteristic. Due to the limitations associated with phenotypic techniques, some of the isolated organisms were not accurately identified by the Vitek® 2 Compact system, i.e. sample 2, 4, 7, 10, 11, 12, 14 and 16. The inaccuracy in the detection of the aforementioned organisms might be due the limitations on the Vitek® 2 Compact system database and the similarities of the biochemical properties of organisms, thus resulting in inaccurate diagnosis.

Phenotypic techniques are generally characterised by their inability to differentiate between related or fastidious organisms (Amor et al. 2007; Houpikian and Raoult 2002). This phenomenon was observed by Schröttner et al. (2014), where the Vitek® 2 system reliably identified pathogenic species belonging to *Myroides* genus but was unable to differentiate between *Myroides odoratus* and *Myroides odoratimimus*. The database of the Vitek® 2 Compact system may lack the existence of novel organisms; hence, resulting in the mis-identification of organisms, whereas DNA-based

techniques detect novel organisms that were not previously identified, using constantly updated databases and search engines (Amor et al. 2007; Emerson et al. 2008; Houpikian and Raoult 2002). However, phenotypic techniques are necessary for the preliminary identification of organisms, especially through the Vitek® 2 Compact system, which provides physiological traits of the organisms.

Culture-independent approach

The culturable organisms represent a minute representation (1%) of the total microbial populations (Amann et al. 1995) in a particular ecological setting. Since the identification of microbial communities via the culturable technique has been reported to misrepresent the microbial composition of a particular habitat, a culture-independent approach was undertaken to fully elucidate the microbial composition within the CDOs and TDOs, using 16S rRNA amplicon gene sequencing. This technique revealed the complexity of the microbial diversity within the CDOs and TDOs. The CDOs were dominated by bacteria (98.63%) (Fig. 1a), with the detected organisms that belong to *Proteobacteria* (46.25%), *Bacteroidetes* (44.79%), including unidentified organisms (8.35%) (Fig. 1b), with their related family profiles (Fig. 1c). Similarly, the TDOs were dominated by bacteria (98.56%) (Fig. 1d), with the detected organisms that belong to *Proteobacteria* (46.94%), *Bacteroidetes* (42.41%) and unidentified organisms (9.96%) (Fig. 1e), with their respective families (Fig. 1f). The structure of the CDOs was dominated by

Fig. 1 A microbial characterization profile demonstrating, CDOs taxonomy (a), phylum (b) and family of the inoculum (c), and TDOs taxonomy (d), phylum (e) and family of the inoculum (f)

congested, slimy, rod-shaped organisms (Fig. 2a), while the TDOs contained scattered, rod-shaped organisms (Fig. 2b).

The bacterial composition of the CDOs and TDOs was determined to be diverse and complex (see Mekuto et al. 2017) and were majorly dominated by *Myroides*

odoratimimus and *Proteus* sp. at 37.82 and 30.5% for CDOs; including 35.26 and 17.58% for TDOs, respectively (see Fig. 3a, b). The dominance of these organisms within the CDOs and TDOs was hypothesised to be opportunistic since there are no reports on their ability

Fig. 2 Scanning electron microscope images demonstrating the structure of the a CDOs and b TDOs

Fig. 3 The microbial composition profiles of the identified CDOs (a) and TDOs (b)

to degrade CN^- and SCN^- . Recent research has reported on the pathogenicity of these organisms, where they were associated with urinary tract infections, meningitis, pneumonia and other diseases (Coker et al. 2000; Maraki et al. 2012; Schröttner et al. 2014). This observation prompted an investigation, which is currently on-going, on the detection of pathogenic genes within these cultures. The gathered information would enable a thorough understanding on the extent of the consortium's pathogenicity. Hence, this would enable for the development of safety measures. Organisms belonging to *Ralstonia*, *Thiobacillus*, *Bacillus*, *Pseudomonas*, *Klebsiella*, *Burkholderia* and other organisms have been reported to possess CN^- and SCN^- biodegradation capabilities were detected within the CDOs and TDOs (see Mekuto et al. 2017). These organisms and their biodegradative functions were evaluated in numerous studies and are summarised elsewhere (Mekuto et al. 2016b). Recent culture-independent studies on the ASTERTM process organisms revealed the complexity and diversity of the ASTERTM process organisms (Huddy et al. 2015) compared to the culture-dependent approach which was reported earlier (van Buuren et al. 2011). The study detected the presence of *Ralstonia*, *Thiobacillus*, *Bacillus*, *Pseudomonas* and *Sphingomonas* sp.; organisms which were detected in the current study. Kantor et al. (2015) further investigated the microbial composition and metabolic functions of the ASTERTM process organisms and demonstrated that the bulk fraction of the ASTERTM organisms were autotrophic, dominated by *Thiobacillus* species which comprised of operons which harboured functions that were not previously reported. Therefore, the detected microbial species in this study would aid in a better understanding of the microbial composition found in CN^- and SCN^- containing wastewaters and would likely contribute to a pool of identified organisms possessing the capacity

to degrade CN^- and SCN^- , which will ultimately result in improved process performance.

Biochemical reactions

The changes in the biochemical properties of the CDOs and TDOs prior and post co-culturing were ascertained via the Vitek[®] 2 Compact System. The CDOs and TDOs were inoculated on GN, GP, YST and BCL colorimetric Vitek[®] 2 Compact System cards, separately and as mixed cultures. The data demonstrated negative phenotypic changes with respect to α -glucosidase and β -xylosidase activities (Table S2), and Amygdaline assimilation (Table S3), Pyruvate assimilation and L-Pyrrolydonyl-arylamidase (Table S4), after co-culturing. The metamorphoses in the biochemical properties of the consortiums when co-cultured is unclear. However, this phenomenon may be attributed mainly to competition and contaminant toxicity. Although there was an observed biochemical change in key metabolic enzymes, the simultaneous biodegradative capacity of the consortiums' co-culture increased, demonstrating the efficacy of co-culturing.

Free cyanide and thiocyanate biodegradation experiments

In the present study, the biodegradation of free cyanide by the CDO was evaluated in batch cultures, where the initial free cyanide concentration was set at 250 mg CN^-/L . The CDO completely degraded free cyanide, achieving a BRE of 97.3% (see Fig. 4a) but were unable to degrade SCN^- (see Fig. 4c). The inability of the CDOs to degrade SCN^- is unclear, although microorganisms that possess thiocyanate biodegradation potential such as *Burkholderia cepacia* (Adjei and Ohta 1999), *Pseudomonas*

Fig. 4 Biodegradation of free cyanide and thiocyanate by **a** cyanide degrading organisms (CDO) **b** thiocyanate degrading organisms (TDO) and **c** by CDO and TDO. Error bars represent deviations

putida (Chapatwala et al. 1998) and other organisms were detected within the CDOs. However, electroplating wastewater does not contain SCN^- (Mekuto et al. 2013) and this might have contributed to the CDOs not being able to utilise SCN^- . The biodegradation of CN^- was accompanied by the production of ammonium-nitrogen. The ammonium concentration increased between 0 and 26 h to a concentration of 152 mg $\text{NH}_4^+\text{-N/L}$ and thereafter, the concentration decreased to 0.7 mg $\text{NH}_4^+\text{-N/L}$. Similarly, the ability of the TDOs to degrade SCN^- was assessed under alkaline conditions and these organisms achieved a degradation efficiency exceeding 99.9% (see Fig. 4b). In comparison to the CDOs, the TDOs were also observed to have CN^- degradation potential as they were able to degrade CN^- , achieving a BRE of 89% (see Fig. 4c). The ability of the TDOs to utilise CN^- is attributed to the isolation technique of the organisms which enabled for the detection of CN^- degrading organisms within the consortium, thus resulting in its ability to degrade CN^- . Similarly, the degradation of SCN^- resulted in the presence and accumulation of ammonium nitrogen and sulphates within the medium. The ammonium nitrogen reached 115 mg $\text{NH}_4^+\text{-N/L}$ after 51 h and thereafter decreased, suggesting ammonium utilisation by the TDO. The sulphates

accumulated throughout the experimental run, with the maximum concentration of 90 mg $\text{SO}_4^{2-}\text{-S/L}$ after 100 h.

Co-metabolism of free cyanide and thiocyanate

The co-metabolism of CN^- and SCN^- by a mixed culture of the CDOs and TDOs (ratio 1:1) was assessed in batch cultures, under alkaline conditions. This was done to evaluate the relationship between the utilised microbial communities, where complete degradation of CN^- and SCN^- was achieved within 170 h (see Fig. 5). The degradation of CN^- and SCN^- occurred simultaneously, demonstrating the importance of co-culturing for effective treatment of CN^-/SCN^- wastewaters. This phenomenon was witnessed in a co-culture of *Ralstonia* sp. and *Klebsiella pneumoniae* for the biodegradation of thiocyanate (Chaudhari and Kodam 2010), where these organisms achieved a degradation rate of 500 mg $\text{SCN}^-/\text{L h}$ from an initial concentration of 2500 mg SCN^-/L . Furthermore, successful co-metabolism of high CN^- concentrations (up to 2000 mg CN^-/L) by a mixed culture of *Trichoderma* sp. and *Fusarium* sp. was achieved in a separate study (Ezzi and Lynch 2005), while accelerated co-metabolism of SCN^- and CN^- under alkaline conditions was achieved by Mekuto

Fig. 5 Co-metabolism of free cyanide and thiocyanate by the CDO and TDO co-cultures. Error bars represent deviations

et al. (2016a). These studies revealed the significance of co-culturing. The degradation process was followed by the production of ammonium- and nitrate-nitrogen, with maximum concentrations of 246.5 mg $\text{NH}_4^+\text{-N/L}$ and 114 mg $\text{NO}_3^-\text{-N/L}$, respectively. The produced ammonium and nitrates were utilised by the organisms after 72 h (for $\text{NH}_4^+\text{-N}$) and 100 h (for $\text{NO}_3^-\text{-N}$).

The utilisation of the produced ammonium nitrogen by the CDO and TDO has demonstrated the effectiveness of these organisms in the overall treatment of cyanide containing wastewaters, including the degradation of by/end products. This phenomenon was observed by Mekuto et al. (2016c), where *Pseudomonas aeruginosa* STK 03 was able to conduct heterotrophic nitrification under cyanide containing conditions. Similarly, Mpongwana et al. (2016) observed heterotrophic nitrification and aerobic denitrification by *Enterobacter*, *Yersinia* and *Serratia* sp. in the presence of CN^- . Microbial species such as *Alcaligenes faecalis*, which were detected in the microbial cultures, were previously reported to possess heterotrophic nitrification and aerobic denitrification potential (Papen et al. 1989; Joo et al. 2005). In addition, denitrifying organisms such as *Alicyclophilus denitrificans* (Mechichi et al. 2003), *Thiobacillus denitrificans* (Yu et al. 2015), *Pseudomonas denitrificans* (Kornaros and Lyberatos 1998) and other organisms were detected from the CDOs and TDOs. These organisms were previously reported to possess denitrification capabilities. The ability of these organisms to conduct nitrification and denitrification aerobically and heterotrophically under cyanide-laden conditions, signifies the robustness and effectiveness of these organisms, a necessary characteristic for microorganisms that could be utilised for the treatment of cyanidation wastewater as proposed by Mekuto et al. (2016b).

Conclusion

The reported research data demonstrated the biasness associated with the culture-dependent techniques, for the identification of microbial communities associated with co-metabolism of CN^- and SCN^- . This is due to the inability of most microbial communities to grow on solid media, resulting in inaccurate characterization of the microbial communities. However, culture-independent techniques revealed diverse microbial communities that were majorly dominated by *Myroides odoratimimus* and *Proteus* sp. In addition, the biochemical properties of the CDOs and TDOs changed when these communities were co-cultured, which led to the rapid co-metabolism of CN^- and SCN^- . The co-culturing increased the biodegradative capacity of the consortium; a phenomenon which has never been reported before. However, an investigation on the pathogenicity of the microbial communities needs to be undertaken to assess the extent of the pathogenicity of the consortium. In addition, future research should focus on the metabolomics of the consortiums after co-culturing, to elucidate the root cause of the change in the biochemistry of the co-culture. Also, an investigation on the utilisation of the produced ammonium by these organisms should be investigated to assess their nitrification and denitrification abilities.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest associated with this work.

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