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Performance of *Fusarium oxysporum* EKT01/02 isolate in cyanide biodegradation system

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ABSTRACT

This study reports a cyanide resistant and/or tolerant fungus, isolated from the rhizosphere of *Zea mays* contaminated with cyanide-based pesticides. The isolate was characterised using molecular biology. The effect of free cyanide and heavy metals on the growth of isolate in a synthetic gold mine wastewater was examined. The molecular analyses identified the isolate as *Fusarium oxysporum* EKT01/02 (KU985430/KU985431). The isolate had a free cyanide degradation efficiency of 77.6%. The results indicated greater growth impairment in culture containing Arsenic (optical density 1.28 and 1.458) and cyanide (optical density 1.315 and 1.385). Higher growth was observed in all cultures supplemented with extracellular polymeric substance. This study showed that the isolate possesses wide substrate utilisation mechanism that could be deployed in environmental engineering applications.

Keywords: Biodegradation, Cyanide, *Fusarium oxysporum*, ITS, TEF 1-α

1. Introduction

The utilisation of cyanide in the minerals industry is vast despite its toxicity due to its affinity for metals. Therefore, free cyanide (CN^-) and metal complex cyanides are released into the environment due to the insufficient treatment of wastewater generated from such metal recovery operations. The danger to humans and the ecological system is thus prominent; as such, there are several reports that indicate the impact of pollutants such as CN^- and their contribution to the global burden of diseases [1, 2]. Several processes have been used to degrade cyanide namely; photo-decomposition, volatilisation, oxidation and biodegradation [3]. Chemical methods such as alkaline chlorination and hydrogen peroxide oxidation are commonly used for the treatment of cyanide-contaminated wastewater owing to their suitability for achieving low levels of CN^- and weak acid dissociable (CN-WAD) cyanides as by-products. However, these methods have high operational costs, with unfavourable by-products, which further deteriorates the environment [1].

Biological degradation processes have increasingly gained popularity due to their cost effectiveness, robustness and environmental benignity, with a great deal of research being conducted in this area [4]. Besides the biological cyanide degradation system at the Homestake Mine, (Dakota USA), a biological process named the Activated Sludge Tailings Effluent Remediation (ASTER™) technology, is being used for the safe handling of effluent containing CN^- and thiocyanate (SCN^-) in South Africa [5, 6]. The processes have been widely reported to be dominated by bacterial and fungal organisms, which facilitate the biodegradation process. Fungal species such as *Aspergillus* sp. and *Fusarium* sp. and bacterial species such as *Bacillus* sp. and *Pseudomonas* sp., amongst others, have been found to utilise cyanide as a nitrogen source and/or carbon source, which generally results in the production of ammonium-nitrogen $(NH_4^+ - N)$[7-10].

Here, we report the potential of *Fusarium oxysporum* (*F. oxysporum*) in biodegradation of cyanide containing wastewater. Extracellular polymeric substance (EPS) produced by the isolate was used to support the growth of the isolate in gold mine wastewater containing cyanide and heavy metals. Impact of cyanide on the architecture of the isolate and the suitability of the isolate for heavy metals and free cyanide biodegradation was investigated.

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2. Experimental

2.1. Fungal Isolation and Identification

Fungi were isolated from the rhizosphere of *Zea mays* (*Z. mays*) contaminated with CN^--based pesticides. The rhizosphere of *Z. mays* was sprinkled into a Potato Dextrose Agar (PDA) plate and maintained at room temperature for between five and seven days depending on isolate's growth. The isolates were sub-cultured repeatedly on PDA plates in order to obtain pure colonies, subsequent to incubation for five days at 25 ± 1℃, alternating exposure to light and darkness at 12 h interval for optimum growth [11]. The fungus isolate was identified both morphologically and by structural ribosomal deoxyribonucleic acid (rDNA) sequencing analysis. The genomic DNA was extracted using a PowerBiofilm DNA kit (MOBIO Laboratories, Inc., CA-USA) according to the manufacturer's instructions. The Polymerase Chain Reaction (PCR) amplification and sequencing was done using the universal primers ITS1/ITS4 (ITS1: ITS 'TCCGTAGGTGAACCTGCGG' and ITS4: ITS 'TCCTCCGCTTATTGATATGC') including EF1F/EF1R (EF1F: 'ATGGGTAAGGARGACAAGAC' and EF1R: 'GGARGTACCAGTS ATCATGTT'), as described earlier [12]. The QIAquick PCR purification kit (Qiagen, Hilden, Germany) was used to purify the amplicons. EKT01 represents the amplicons from TEF 1-α gene while EKT02 denotes those of ITS gene. Sequences were run on a CLC Main Workbench 7 and blasted against the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database. Alignment of 16 nucleotide sequences from the NCBI database and obtained sequences was done using the MAFFT online server (MAFFT version 7, http://mafft.cbrc.jp/alignment/server/). The aligned sequences were imported into the Molecular Evolutionary Genetics Analysis (MEGA) software version 6 [13], which was used for sequence analyses. The evolutionary history was done for the individual dataset obtained. The neighbour joining (NJ) method based on the Kimura 2-parameter model with 1,000 bootstrapped data sets was used [14]. All trees are drawn to scale, with positions < 95% site coverage eliminated.

2.2. Extraction of Extracellular Polymeric Substance (EPS) from the Isolate

The isolate was incubated in two different media: initially in nutrient broth and subsequently in gold mine wastewater containing CN^- and heavy metals as shown in [15] with glucose as a carbon source for 48 h at 140 rpm, 28℃ and pH of 10. After incubation, the culture was centrifuged at 10,000 rpm for 10 min at 4℃ in an Avanti® J-E centrifuge (Beckman Coulter, Inc., USA.). To the recovered supernatant, cold ethanol (96% v/v, Merck, USA) at 4℃ was added using a ratio of 1:2, i.e. supernatant: ethanol, and incubated overnight at 4℃. The mixture was swirled and centrifuged at 10,000 rpm for 10 min at 4℃. The EPS was rinsed twice in sterile distilled water (SDW) and dialysed in SDW overnight at 4℃. The recovered EPS was lyophilised using Duran® desiccator.

2.3. Cyanide Removal and Isolate Growth Assay

The isolate was inoculated into two sets of reactors (250 mL Erlenmeyer flasks) containing nutrient broth in a rotary shaker (ZHICHENG® model ZHWY-200D, Shanghai, China) at 25℃, pH of 10 and 140 rpm. After 24 h, CN^- (KCN) was added to one of the reactors to make 100 mg CN^-/L while the other set was free of CN^-. The uninoculated reactor served as control. Samples (2 mL) were periodically withdrawn from the reactor and analysed for free cyanide biodegraded, residual ammonium-nitrogen and nitrate-nitrogen using Merck® cyanide (CN^-) (09701), ammonium (NH_4^+-N) (00683) and nitrate (NO_3-N) (14773) test kits respectively, in a NOVA 60 spectroquant. The procedures were in triplicate. Mass balance Eq. (1) and (2) were used to estimate free cyanide biodegraded, taking into account free cyanide volatilised.

$$CN_B^- = CN_I^- - CN_R^- - CN_V^- \quad (1)$$

$$CN_V^- = CN_I^- - CN_{FC}^- \quad (2)$$

Where CN_B^- is the free cyanide biodegraded; CN_I^- is the initial free cyanide in the media; CN_R^- is the residual free cyanide after incubation period; CN_V^- is the free cyanide volatilised during culture incubation; CN_{FC}^- is the final free cyanide in the control media. All measurements are in mg CN/L. The free cyanide removal efficiency (biodegradation efficiency) was determined using Eq. (3)

$$Biodegradation\ \ Efficiency = \frac{CN_B^-}{CN_{FC}^-} \times 100 \quad (3)$$

After 120 h, biofilm were harvested and visualized on a scanning electron microscopy (SEM: FEI Nova NanoSEM 230) with a field emission gun using the method described in Huddy et al (2015). Furthermore, heavy metal salts of $CuSO_4$, Na_2HAsO_4, $PbBr_2$, $Fe_2(SO_4)_3$, $ZnSO_4$, and CN^- in the form of KCN (Merck, USA), each at 1 mM which was higher than effluent concentration reported by Acheampong (2013), were prepared in two sets of 50 mL nutrient broth, and inoculated with a loopful of the isolate and incubated at 28℃ for 24 h. Nutrient broth containing the isolate without CN^- and heavy metal served as a control. Then, 1 mg of the isolate's EPS was added to a set of inoculated nutrient broth and further incubated at 28℃ overnight. The microbial growth was observed in a microplate reader; SpectraMax® M2 (Molecular Devices, California, USA) based on optical density at a wavelength of 300 nm. All treatments were in triplicate.

3. Results and Discussion

The consensus nucleotide sequence amplicons of 703 base pair (bp) and 465 bp were obtained for TEF 1-α and ITS genes from the isolate respectively. The sequences were deposited in NCBI database and assigned the following accession numbers: KU985430 for EKT01 and KU985431 for EKT02. The phylogenetic trees for individual sequences showed that the isolates belong to *Fusarium* sp. with bootstrap support for both ITS genes and TEF 1-α gene. EKT01 and EKT02 are most closely related to *F. oxysporum* isolate FUS1 and *F. oxysporum* strain SK 1649, respectively. The tree with the highest Log Likelihood is shown in Fig. 1 for each dataset.

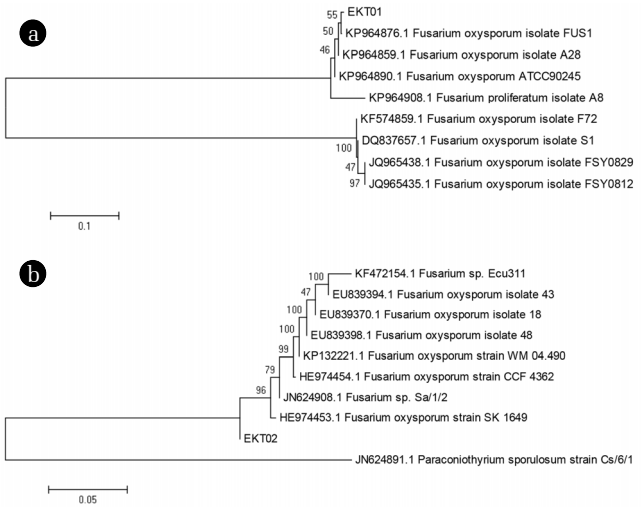

Fig. 1. Neighbour joining trees of (a) TEF 1-α genes and (b) ITS genes.

Previous reports on *F. oxysporum* f. sp. *cubense* showed that molecular genetic techniques could be used to determine a phylogenetic relationship [16]. The tree analysis of TEF 1-α was very similar to that of Taylor *et al.* (2016) and Bogale *et al.* [17]. The EPS recovered from isolate incubated on nutrient broth was miniscule (2 mg) compared with 40 mg from cultures supplemented with wastewater containing CN^- and heavy metals. This suggests that the EPS production system was a consequence of adaptation to the toxic environment which facilitated the isolate's ability to sustain its metabolic activity. The growth of the isolate was impaired in cultures supplemented with heavy metals, with the exception of Fe^{3+} cultures which showed to be highly tolerant of the contaminant at 1 mM. Arsenic (As) and CN^- showed a greater inhibitory effect on the microbial growth of the isolate both with and without EPS. The supplementation of the EPS improved the growth of the isolate, see Fig. 2(a).

This indicated the ability of the isolate to protect itself against variations in toxicant concentration, an admirable trait suited for mining wastewater bioremediation. The inhibitory effect of As and CN^- may be attributed to their tendencies to impede ATP production thereby disrupting oxidative phosphorylation [18].

Furthermore, the isolate showed degradation efficiency of 77.6% within five days from an initial free cyanide concentration of 100 mg CN^-/L (see Fig. 2(b)). The free cyanide loss owing to volatilisation was less than 10% with residual ammonium-nitrogen and nitrate-nitrogen being 30.1 mg/L and 0.2 mg/L, respectively. The accumulation of ammonium suggested a hydrolytic mechanism utilisation in the cyanide biodegradation process by the isolate [19, 20]. The micrographs showed that the microbial biomass is embedded in EPS which plays a major role in the structural integrity of the biofilm formed; hence, its resistance to toxicants (see Fig. 3).

Similarly, the biomass from the culture without CN^- appeared physically distinct from that exposed to CN^-. The solute well is bigger with more gums which improves nutrient uptake in biofilms with CN^- as the biomass adjusted to the inhibitory effect of the CN^- and heavy metals.

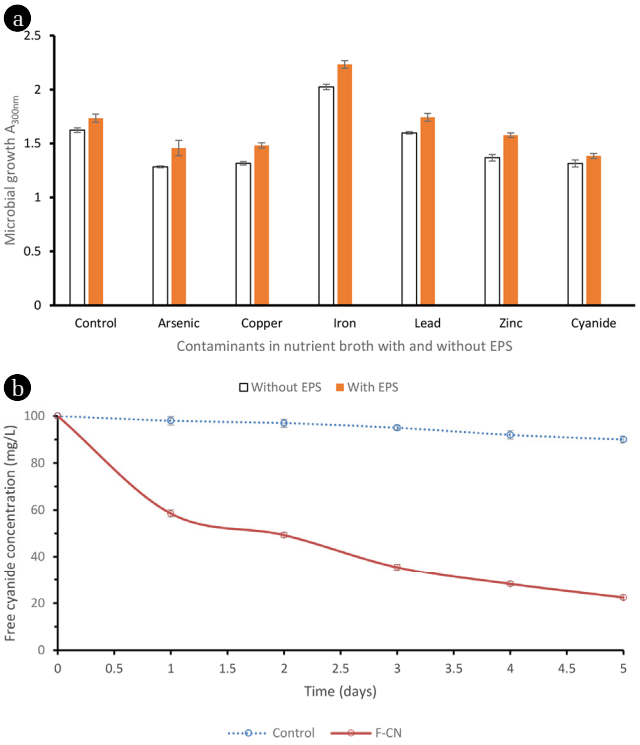

Fig. 2. (a) Isolate growth in heavy metals and free cyanide and (b) free cyanide degradation profile of the isolate.

Generally, microorganisms have different tolerance levels for heavy metals. Previous studies have indicated *Fusarium* sp. can tolerate most of these heavy metals up to 1,000 mg/L [21-23]. The concentration of the contaminants used in this study was higher compared with the report of Acheampong *et al.* [15], thus this isolate could be explored to bioremediate cyanide containing gold mine wastewater. Also, previous biochemical analysis of the isolate showed that it was dominated by aminopeptidase an indication of its suitability to survive nitrogen limitation conditions [12, 24]. For instance, Leucine arylamidase, a type of aminopeptidase produced by this fungus isolate is a cellular enzyme that is common in bacteria and yeast, which helps to liberate amino acids from high molecular compounds [25, 26]. In addition, most heavy metal tolerant microorganisms have been shown to express these enzymes in their quest for survival in polluted environments [21, 27]. The aminopeptidases produced by this isolate was an indicator of a wide range of substrate utilisation capabilities. Previously, Anuradha *et al.* (2010) have established the ability of *Fusarium* sp. to utilise numerous carbohydrates for multi-enzyme production which was validated by other researchers [28-30].

There are limited reports on fungal biofilms besides that of *Zygomycetes* sp. [31], *Aspergillus* sp. [32, 33] and *Fusarium* sp. [2, 6, 34] which indicated that inhibitors such as CN^-, SCN^- and fungicides have adverse effect on the architecture of the *Fusarium* sp. The bigger solute wells within the biofilms of the isolate suggested minimal impact of the CN^- on the structural characteristics of the biofilm formed by the *F. oxysporum* isolate. The presence of CN^- within the biomass walls is a key factor in cellular respiration.

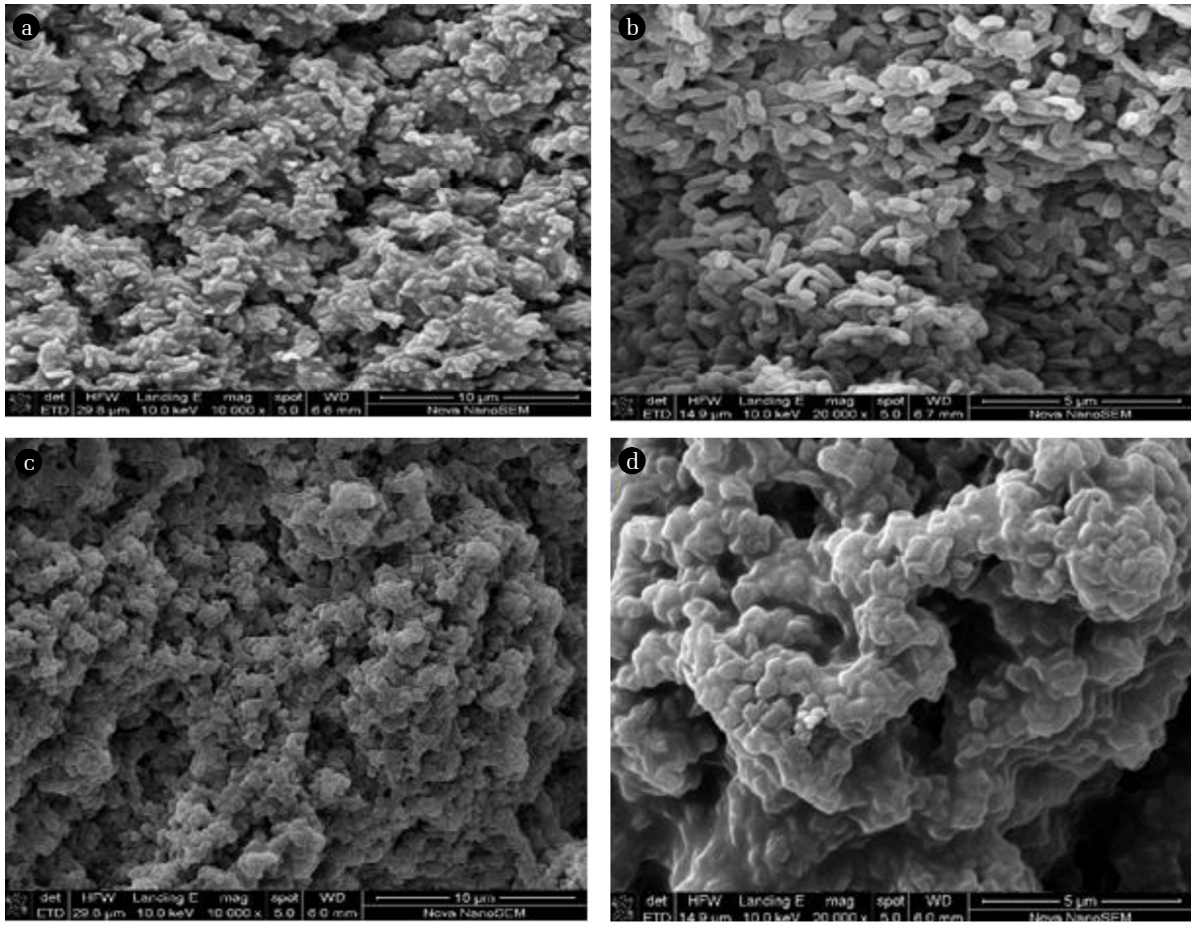

Fig. 3. Scanning electron micrographs of *Fusarium oxysporum* biofilm from cultures without cyanide (a-b) and those with cyanide (c-d).

4. Conclusions

This study showed the ability of *F. oxysporum* EKT01/02, which was isolated from the rhizosphere of *Z. mays* contaminated with cyanide-based pesticide, and was able to degrade free cyanide with 77.6% degradation efficiency after 5 d. The isolate was able to grow in the presence of both cyanide and heavy metals which makes it a suitable candidate for biological treatment of wastewater on a large scale. Inaddition, the growth of the isolate was aided by the production of EPS, an information that is invaluable for the design of microbial wastewater treatment plant. However, it is recommended that studies on simultaneous biodegradation of metal-complex cyanide and free cyanide, including genes and degradation enzymes involved be investigated for a holistic environmental engineering applications.

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