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Authors: L.C. Razanamahandry, C.T. Onwordi, W. Saban, A.K.H. Bashir, L. Mekuto, E. Malenga, E. Manikandan, E. Fosso-Kankeu, M. Maaza, S.K.O Ntwampe

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Performance of Various Cyanide Degrading Bacteria on the Biodegradation of Free Cyanide in Water

L.C. Razanamahandry [1,2,*], C.T. Onwordi [3], W. Saban [1,2], A.K.H. Bashir [1,2] , L. Mekuto [4], E. Malenga[5], E. Manikandan [1,2,6], E. Fosso-Kankeu [5], M. Maaza [1,2] and S.K.O Ntwampe [7,8]

[1]UNESCO UNISA Africa Chair in Nanoscience's/Nanotechnology Laboratories (U2AC2N), College of Graduate Studies, University of South Africa (UNISA), Muckleneuk Ridge, P.O. Box 392, Pretoria, South Africa

[2]Nanosciences African network (NANOAFNET), Materials Research Group (MRG), iThemba LABS-National Research Foundation (NRF), 1 Old Faure Road, 7129, P.O. Box 722, Somerset West, Western Cape Province, Cape Town, South Africa

[3]University of Western Cape, Environmental and Nano Sciences, Department of Chemistry, Faculty of Natural Sciences, Bellville, Private mail Bag X17, Cape Town, 7535

[4]University of Johannesburg, Department of Chemical Engineering, Johannesburg, South Africa

[5] Water Pollution Monitoring and Remediation Initiatives Research Group, School of Chemical and Minerals Engineering, North-West University, Private Bag X1290, Potchefstroom, 2520, South Africa

[6]Thiruvalluvar University, Department of Physics, TUCAS Campus, Thennangur-604408, Vellore, India

[7]Bioresource Engineering Research Group (*BioERG*), Cape Peninsula University of Technology, P.O. Box 652, Cape Town 8000, South Africa

[8]Department of Chemical Engineering, Faculty of Engineering and the Built Environment, Cape Peninsula University of Technology, PO Box 1906, Bellville, 7535, Cape Town, South Africa

*Corresponding author: clrazanamahandry@tlabs.ac.za / tantely1989@gmail.com / Telephone: (+27) 61 948 6210

HIGHLIGHTS

- Cyanide degrading bacteria (CDB) from various sources were chosen
- CDB ability to degrade FCN in liquid medium was tested and its performance was compared
- Deposit materials in decontaminated liquid medium were characterised
- Possible reuse of deposit materials was revealed

Abstract

This study reports on the biodegradation of free cyanide (FCN) by cyanide degrading bacteria (CDB) that were isolated from mining wastewater and thiocyanate containing wastewater. The performance of these isolates was compared to cryopreserved CDBs that were used in previous studies. The performance of the isolates to degrade FCN was studied in batch cultures. It was observed that the CDB from the thiocyanate wastewater showed higher biodegradation rates (2.114 g $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-1}$) compared to the isolates from the mining wastewater. The isolates from the cryopreserved CDBs and from the mining wastewater achieved a biodegradation rate of 1.285 g $CN^- L^{-1}.O.D_{600nm}^{-1}.h^{-1}$ and 1.209 g $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-1}$, respectively. This study demonstrated that the source of the organisms plays a significant role on FCN biodegradation.

Keywords: Bioremediation; Cyanide degrading bacteria; Free cyanide; Mining wastewater; Microorganism.

1. Introduction

Free cyanide (FCN) ion is one of the most toxic forms of ions from all cyanide compounds [1]. FCN is also the main by-product that results from metallurgical processes in which cyanide compounds are used for precious metal recovery [2].

Effluents containing FCN should be treated before they are released into the environment to avoid detrimental effects on the receiving environment, such as the contamination of fresh water sources and soil [3], which would culminate in deleterious human health outcomes such as: metabolic acidosis, seizures, bradycardia, and a lack of response to oxygen treatment [4]. Physical, chemical and biological treatments are the different treatment methods available for FCN removal from wastewater [5]. Nevertheless, biological methods are the most applied since this technology is inexpensive and does not contribute to hazardous by-products accumulation, which are witnessed in other treatment methods [6]. Microorganisms that are used to degrade FCN, have an ability to break the triple bond between the carbon and nitrogen atoms (-C≡N) in FCN and use these elements as either a carbon or/and nitrogen source for their metabolism [7]. Microorganisms use enzymes to accelerate the bond destruction of FCN [8], with the biodegradation by-products formed being dependent on the enzyme that the microorganisms used and the conditions of the environment in which the biocatalysis occurs, including whether the physicochemical conditions [9]. Several pathways are available to biodegrade FCN but the hydrolytic pathway is the most commonly used process [10].

Enzymes for hydrolytic conversion of FCN include nitrogenases, cyanide hydratases, nitrile hydratases, thiocyanate hydrolases, nitrilases, and cyanidases [11] among many others. Depending on these enzymes, various by-products from the FCN biodegradation could be

formed such as: ammonium nitrogen (NH_4^+-N), ammonia (NH_3), formate ($HCOO^-$), formamide ($HCNOH_2$), carbonyl sulphide (COS), sulphite (SO_3^{2-}) and hydrogen sulphide (H_2S) [12–16].

However, the environmental conditions, herein referred to as external factors, could affect the enzyme activity from the microorganisms known for FCN conversion [17]. These external factors include pH and temperature, making it difficult to choose suitable microorganisms to be inoculated in bioreactors used to biodegrade FCN [18]. However, microorganisms, depending on their species, have an ability to acclimate and directly evolved to new environmental conditions during their latency phase [19]. Huertas et al. [20] have reported a FCN biodegradation latency phase of 20 h for *Pseudomonas pseudoalcaligenes* CECT5344 isolated from river water. Generally, most of FCN biodegradation technologies use different microorganisms sourced from the wastewater containing the pollutant of interest to engineer a suitable consortium. Mekuto et al. [12] have studied FCN biodegradation in FCN contaminated wastewater by using a consortium of different *Bacillus sp.* and found a similar trend of FCN biodegradation when compared to other studies. However, there are limited studies that have focused on FCN biodegradation using microorganisms which were isolated from different cyanide contaminated sources.

Therefore, the aim of this study is to evaluate the FCN biodegradation performance of bacteria derived from various sources. This paper also supports a decision on the choice of the suitable source of the CDB for FCN biodegradation for large-scale operations.

2. Materials and method

2.1. Materials Sources

Microorganisms (n=5) were tested for their performance to degrade FCN. These microorganisms were collected from different sources, i.e. thiocyanate wastewater and mining wastewater.

Three (n = 3) bacterial strains of *Pseudomonas aeruginosa* (C_1, C_2 and C_3) conserved at -80°C were obtained from the *BioERG* laboratories at the Department of Biotechnology, CPUT, South Africa, were subcultured in Tryptone Soy broth with respect to resuscitate the cryopreserved organisms and incubated at 37°C for 24 h.

Two (n= 2) types of bacterial consortium, from mining wastewater (C_m) and wastewater containing thiocyanate (C_t) respectively, were collected from a mining company in South Africa.

The thiocyanate wastewater was also mixed with wastewater from a previous study in which the effluent was treated [21].

2.2. CDB Isolation

In this study, two different media were tested i.e. one with nutrient supplementation (AN) in a form of the addition of macro and micro-nutrients, and one without nutrient supplementation (SN). This is was done to evaluate the need for nutrient supplementation or not, since this nutrient addition can add to operational costs of the treatment processes. The SN medium was enriched using Tryptic Soy Agar (Merck, South Africa) and 2 g CN^- L^{-1} from KCN (Merck, South Africa). AN medium had the same composition as the SN medium, with supplemental nutrients , i.e. macro and micro-nutrients, as highlighted in [3]. The SN and AN medium were autoclaved at 120 ºC for 15 min prior to cooling and thereafter poured on agar plates under sterile conditions. A volume (200 µL) of the mining wastewater and thiocyanate wastewater was spread plated onto agar plates constituted by either SN or AN medium, then incubated at 37 °C for 7 days to assess microbial growth. The visible colonies on agar plates were subcultured and grown in Tryptone Soy Broth (TSB) at 37 °C overnight. Tryptone soy broth (TSB) was chosen to achieve the aerobic conditions required for these experiments. TSB promotes the growth of aerobic bacteria than those which are strictly anaerobic [28].

Microorganism samples to be visualized under Scanning Electron Microscopy (SEM) were prepared according to [21] but hexamethyldisilazane (HDMS) solution was replaced by silicon tetrachloride solution. Microbial cultures grown in TSB solution were centrifuged at 10 000 *g* for 5 min and fixed in 2.5 % glutaraldehyde for 24 h at 4°C. The glutaraldehyde solution was discarded and the microbial pellets were washed twice by using a phosphate buffer (pH 7) before dehydration in an ethanol series of 50%, 70% and 100% during a 12 h period at 4°C. Thereafter, silicon tetrachloride solution was used for drying the samples and the final microorganisms pellets were visualised using a scanning electron microscope.

2.3. SEM analysis method

The morphological analysis of microorganisms pellets were carried out using the SEM TESCAN VEGA3 a Czech Republic model equipped with a tungsten filament used as an electron source operating in Nano space. For the present study, the sample was carbon coated prior analysis to improve the conductivity. The surface morphology was acquired using backscatter and secondary detectors, using the high voltage of 20 kV at different magnifications using Vega software. The compositional analysis was determined on selected area and spots using INCA software from Oxford. The images are given with a scale bar of 10 micrometers and 100 nanometers.

2.4. FCN Biodegradation Tests

The observable colonies from the agar plates containing 2 g CN^-L^{-1} were streaked out and grown in Tryptone Soy broth (Merck, Germany) and thereafter incubated at 37 °C overnight. The isolates were subcultured and tested for their ability to biodegrade free cyanide as KCN (Sigma Aldrich, Germany), in solutions containing 1, 2 and 3 g CN^-L^{-1}subsequent to use in mining wastewater biodegradation studies. A volume (1 mL) of the bacterial isolates was added in 99 mL of the KCN solution and in 99 mL of the mining wastewater; an inoculum concentration equivalent to 1%(v/v). The pH was set at 8.5 and the temperature was kept at 25°C, with a constant incubator rotation speed of 120 rpm. FCN, NH_4^+ concentrations and bacterial growth

were quantified every 2 h over a 24 h period. Photometric methods were used to measure the FCN and NH_4^+ concentrations according to the analytical methods reported in Mekuto et al. [22], in which Merck tests kits 09701 and 00683 were used to measure FCN and NH_4^+ concentrations using Merck Spectroquant Nova 60 instrument, respectively. Bacteria density was quantified at a wavelength of 600 nm using spectrophotometer (JENWAY 7305 series). At the end of the biodegradation tests, i.e. when the FCN concentration was below the detection limit (<0.010 mg CN^-L^{-1}), a small volume of the solution was recovered from each test subsequent to drying at 60°C for 30 min, then the dried samples were sent for X-Ray diffraction (XRD) analyses (Model Bruker AXS D8), using a copper anode radiation with a wavelength λ = 1.5406 Å.

2.5. CDB Performance Evaluation

FCN biodegradation performance (P_{FCN}) for each CDB was calculated according the following equation Eq. (1):

$$P_{FCN} = [FCN_i] \times 1/BD_i \times 1/t \qquad \text{Eq. (1)}$$

Where:

$[FCN_i]$: Initial FCN concentration (g. L^{-1}),

BD_i: Initial Bacterial Density ($O.D._{600nm}$), and

t: degradation duration (h).

3. Results and discussion

3.1. FCN Biodegradation

FCN biodegradation by various sources of the CDBs have shown a similar trend as presented by the FCN profiles in Figure.1.

Fig.1 (a) shows that the $O.D._{600nm}$ of the CDB decreased during the first 4 h and increased to the optimal value before decreasing again. The initial $O.D._{600nm}$ of the CDB was 0.055; 0.048; 0.050; and 0.035 for C_1, C_2, C_3, C_m and C_t, respectively. After the first 4 h, the $O.D._{600nm}$ values increased from 6×10^{-3} to 5×10^{-2}; 4×10^{-3} to 10^{-2}.; 5×10^{-3} to 2×10^{-2}; 5×10^{-3} to 10^{-2}; and 8×10^{-3} to 7×10^{-2} for C_1, C_2, C_3, C_m and C_t, respectively. All CDBs tested were able to grow on the medium containing various FCN concentrations. Nevertheless, from the medium with an FCN concentration of 3 g CN^-L^{-1}, the CDB growth was minimal, achieving maximum $O.D._{600nm}$ values of 0.007; 0.008; 0.008; 0.01 and 0.014 for C_1, C_2, C_3, C_m and C_t, respectively. FCN biodegradation rates of 99% were obtained for all tests after 28 h, 37 h and 72 h in 1 g, 2 g and 3 g CN^-L^{-1} solutions, respectively.

In addition, NH_4^+ was produced during the FCN biodegradation tests as by-product. The optimal NH_4^+ concentration varied in function of the CDB types (0.12 mgL^{-1} for C_1, 0.08 mgL^{-1} for C_2; 0.10 mgL^{-1} for C_3, 0.05 mgL^{-1} for C_m, and 0.16 mgL^{-1} for C_t). The NH_4^+ concentration produced was initially very high than its final concentration at the end of the tests. NH_4^+ was used by the CDB for their growth as highlighted in [23]; as nitrifying CDB could also convert NH_4^+ to nitrate and nitrite.

Biodegradation of the FCN leads to NH_4^+ formation as reported by previous studies on FCN biodegradation [24][25]. The formation of NH_4^+ implies that FCN biodegradation occurred through hydrolytic pathway[5].

FCN biodegradation in mining wastewater was achieved in a short period of less than 10 h, i.e. 5 h for C_t (Fig.1 b) and in 6 h for C_1, C_2, C_3 and C_m. The exponential phase of the bacterial growth was observed after 1 h of contact time between CDB and the mining wastewater. Then, CDB growth declined proportionally with the decrease in the pollutants (NH_4^+ and FCN). However, at higher CN^- concentrations (Fig 1a), the highest amount of FCN biodegradation by-product namely NH_4^+ was recorded. Ct bacterium could use the NH_4^+ by-product as a nitrogen

source for survival even when the FCN was depleted [26]. In Fig 1b the NH_4^+ concentration was lower than in Fig 1a. In addition, NH_4^+ declined rapidly which subsequently culminated in the Ct bacterium cells declining as the contaminant was also being depleted as shown in Fig 1b. Nutrients were no longer available in Fig 1b when FCN and NH_4^+ were depleted. However, in Fig 1a, NH_4^+ was still available at low concentration when FCN was depleted. Therefore, the Ct bacterium could survive better in KCN wastewater containing 3g CN^-L^{-1} than in mining wastewater containing 0.37g CN^-L^{-1}, as mining wastewater also contain a high concentration of other toxicants.

The CDB and FCN substrate equilibrium time was 2 h and 8 h for KCN solution and the mining wastewater, respectively.

Each CDB had its own unique behaviour and performance to degrade FCN. Figure 2 presents the topographic surface of the biomass from different CDBs used after growing in the medium containing FCN. Two different surfaces were observed for all biomass such as the background surface and the front surface. The background surface formed by the irregular holes represents the biomass support. Similar surface characteristics were observed for all CDB biomass. Prevalent mucilaginous exudes consisting polymeric substances were observed on the surface of C_t biomass with such exudes being minute for Cm and subsequently the C_2 biomass. C_1 and C_3 biomasses had minimal mucilaginous exudes. Mucilaginous exudes consisting polymeric substances sometimes are represent as a result of the excessive nutrient and/or toxicant (FCN) presence, and can be accumulated in the biomass of the CDB for its protection [5]. As such, C_t embedded the highest toxicant (FCN) concentration than C_m and C_2, while C_1 and C_3 had the lowest ability to uptake FCN, anomalies attributed to the mucilaginous exudes and their characteristics to influence mass transfer processes within a biofilm (biomass).

Besides the by-product, i.e. NH_4^+, some crystallographic materials have been detected in the mixture of FCN and CDB solution, shown as deposits as illustrated in Figure 3.

Three kinds of crystallographic materials including Nacholite ($NaHCO_3$), Kalicinite ($KHCO_3$) and Sylvite (KCl) were produced. The first two products have a monoclinic facial and the third one has a face-centered cubic. The by-product depends on the enzyme used by the CDB to degrade the FCN [9].

In addition, Figure 3 shows the absence of the CN compound as a complex. CN chemical compounds were determined to have been consumed and/or converted by the CDB since the CDBs can utilise the CN in their metabolism as reported in [9].

Also, FCN biodegradation by-product peaks are an inverse to the initial FCN concentration being observed in solution; albeit, CDBs biodegradation performance was relatively low at high FCN concentration in solution.

From the XRD profiles, the KCl disappearance at FCN concentration of 3 g CN^-L^{-1} was evident. This was hypothesised to be as the resultant toxicity of the FCN that enabled the non-formation of the by-product at higher concentrations of FCN as reported by Kuyucak and Akcil [27].

2.1. CDB Performance

CDB potential to degrade FCN is presented in Figure 4. The isolate labelled as C_t had the highest P_{FCN} value (i.e. 1.905 g $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-1}$ for the KCN solution and 2.114 $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-1}$ for the mining wastewater). Isolates C_2, C_3 and C_1 had similar P_{FCN} value of 1.190 g $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-1}$; 1.176 g $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-1}$ and 1.102 g $CN^-.L^{-1}.O.D_{600nm}^{-1}.h^{-}$

^{1} for the KCN solution and 1.285 g $CN^{-}.L^{-1}.O.D_{600nm}{}^{-1}.h^{-1}$; 1.233 g $CN^{-}.L^{-1}.O.D_{600nm}{}^{-1}.h^{-1}$ and 1.121 g $CN^{-}.L^{-1}.O.D_{600nm}{}^{-1}.h^{-1}$ for the mining wastewater, respectively. Isolate C_m had the lowest P_{FCN} value of 1.06 g $CN^{-}.L^{-1}.O.D_{600nm}{}^{-1}.h^{-1}$ in the KCN solution and 1.209 g $CN^{-}.L^{-1}.O.D_{600nm}{}^{-1}.h^{-1}$ for the mining wastewater.

These results confirmed the information resulting from the observation of SEM images according to which the biomass surface of C_t contained the highest exudes compared to C_m and C_2. Biomass surface of C_1 and C_3, that had the lowest P_{FCN} value, contained the lowest gums. Only the biomass $C_{m,}$ which had very low P_{FCN} had shown more exudes than C_1, C_2 and C_3.

The P_{FCN} values for all CDB were higher for the lowest initial FCN concentration compared to the highest initial FCN concentration. FCN concentration of 3 $gCN^{-}L^{-1}$ was the highest FCN concentration biodegraded by all CDB types used. Overall, the initial concentration of FCN could affect its biodegradation [19]; albeit, for this study, FCN in the mining wastewater used was easily biodegraded by all CDBs. Mining wastewater is rich in other trace nutrients such as metals and with relatively low initial FCN concentration (0.37 g $CN^{-}L^{-1}$), the ability of the CDBs to effectively removed the pollutant from the wastewater.

In addition, both KCN and the mining wastewater can be substrates; albeit, with some microbial inhibition can be observed even when CDB is considered as being effective for the CN^{-} degradation. Compounds containing CN^{-} could be used by CDB as both carbon and nitrogen source for their metabolism [2,3]. Therefore, the substrates inhibition was limited with the environmental conditions studied favouring the CDB (C1, C2, C3, Cm and Ct) growth.

4. Conclusion

CDB from different sources were tested for the biodegradation of free cyanide (FCN). CDBs

that were isolated from the thiocyanate solution have shown the potential to degrade FCN at a higher degradation rate (P_{FCN} = 2.114 g CN^- $.L^{-1}.O.D_{600nm}{}^{-1}.h^{-1}$) as confirmed by the SEM images exhibiting high exudes embedded within the C_t biomass. Besides NH_4^+, various crystallographic by-products were also detected after the FCN biodegradation process. Further research will determine the main enzymes used by the CDBs, in order to explain the reaction mechanisms that ensued during FCN biodegradation and evaluate whether they can be recovered for further applications.

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Figure captions

Fig. 1: FCN Biodegradation by Ct: (a) in KCN solution synthesised containing 3g $CN^-L^{-1}$and (b) in Mining wastewater containing 0.37g CN^-L^{-1}

Fig. 2: SEM images: (a) C_1, (b) C_2, (c) C_3, (d) C_m and (e) C_t

Fig. 3: XRD patterns of FCN solution deposit

Fig. 4: FCN biodegradation performance (P_{FCN}) by various CDB

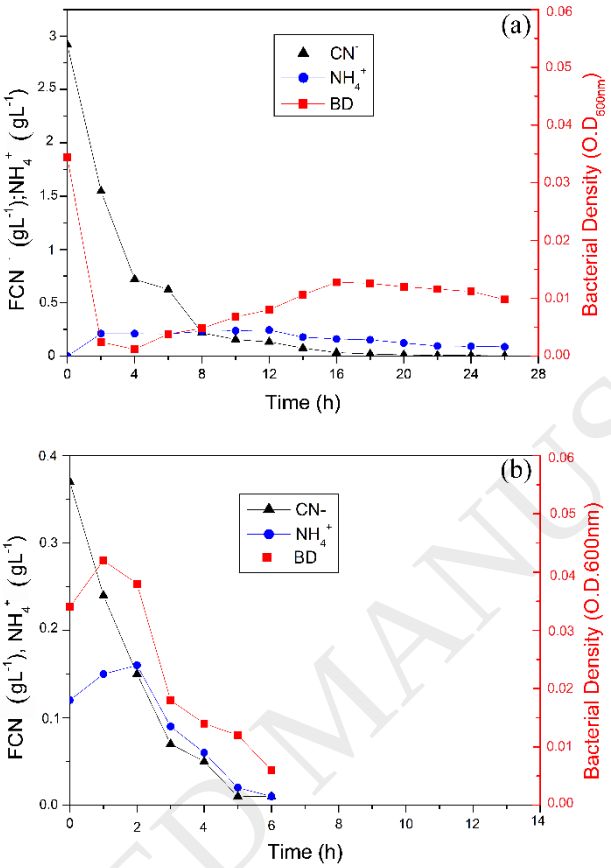

Fig. 1

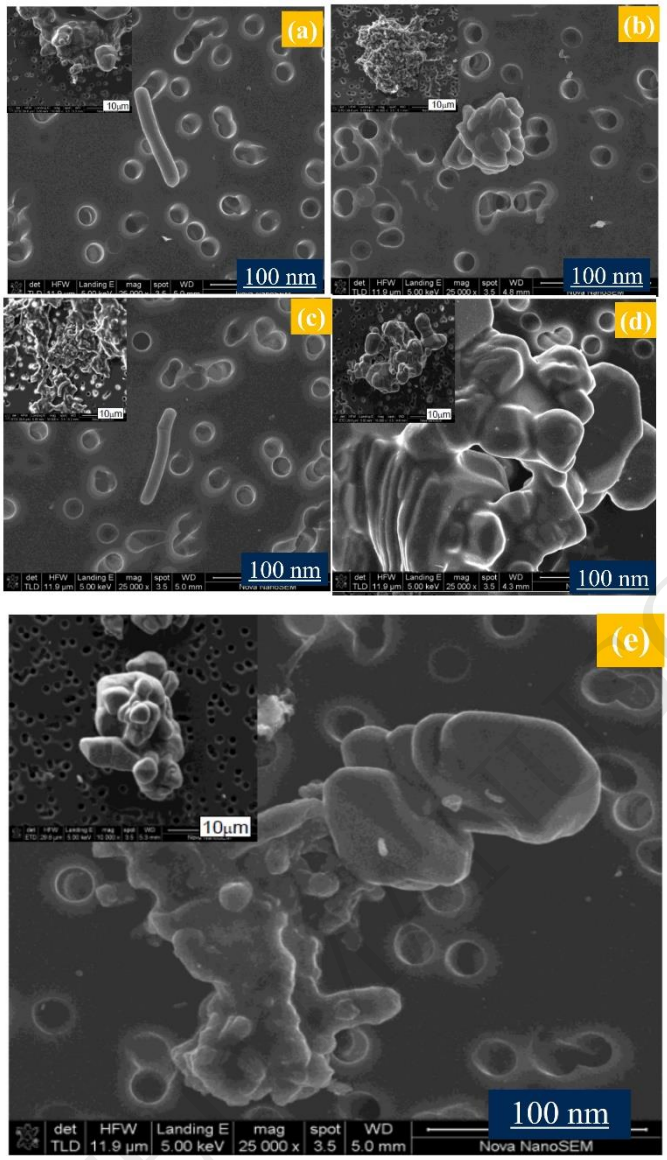

Fig. 2

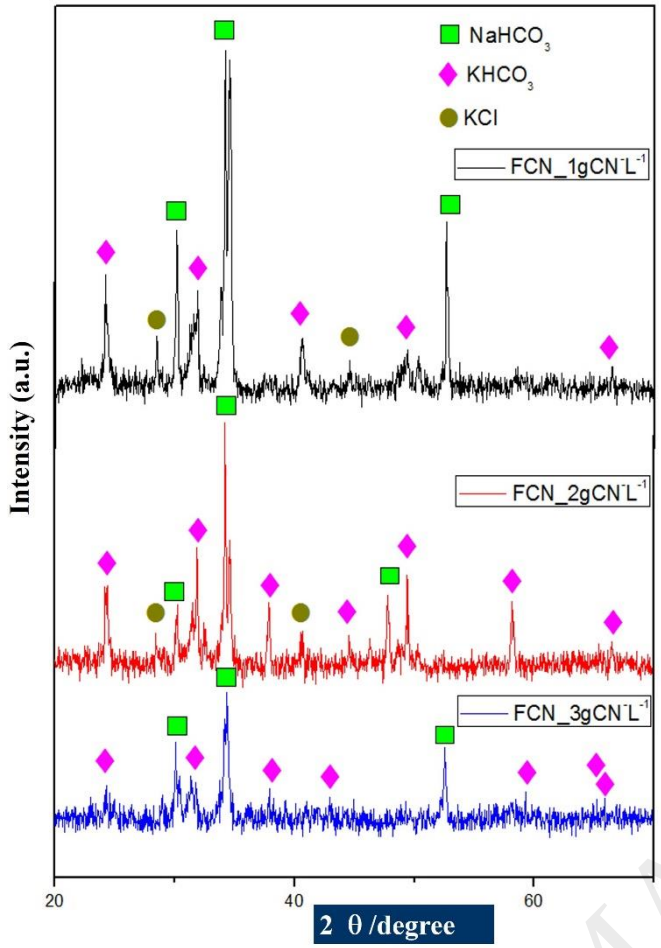

Fig. 3

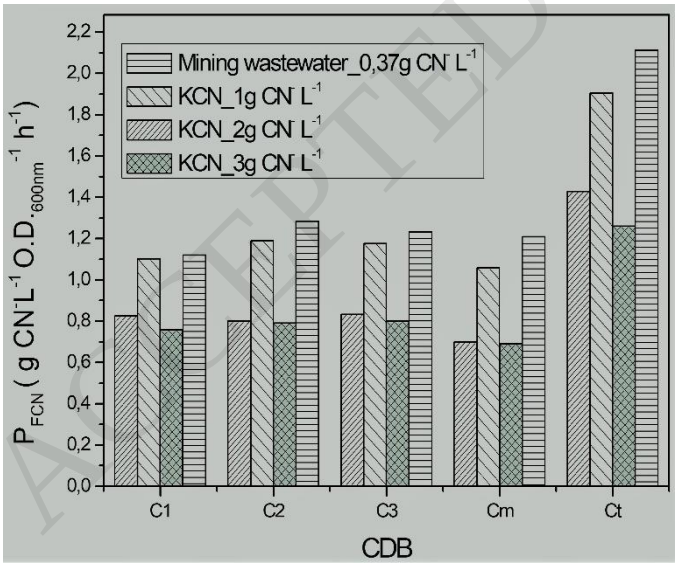

Fig. 4