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Biocatalyst developed with recovered iron-rich minerals enhances the biotransformation of SARS-CoV-2 antiviral drugs in anaerobic bioreactors

Francisco J. Mares-Carbajal, M. Carolina Espinosa-Arzate, Luis A. Ramírez-Montoya, Aurora M. Pat-Espadas, J. Ernesto Ramírez, J. René Rangel-Méndez, Juan A. Ascacio-Valdes, Cristóbal N. Aguilar, Petia Mijaylova, Germán Buitrón, Francisco J. Cervantes

The biotransformation of the SARS-CoV-2 antiviral drugs, ribavirin and tenofovir, was studied in methanogenic bioreactors. The role of iron-rich minerals, recovered from a metallurgic effluent, on the biotransformation process was also assessed. Enrichment of anaerobic sludge with recovered minerals promoted superior removal efficiency for both antivirals (97.4 % and 94.7 % for ribavirin and tenofovir, respectively) as compared to the control bioreactor lacking minerals, which achieved 58.5 % and 37.9 % removal for the same drugs, respectively. Further analysis conducted by liquid chromatography coupled to mass spectroscopy revealed several metabolites derived from the biotransformation of both antivirals. Interestingly, tracer analysis with 13CH4 revealed that anaerobic methane oxidation coupled to Fe(III) reduction occurred in the enriched bioreactor, which was reflected in a lower content of methane in the biogas produced from this system, as compared to the control bioreactor. This treatment proposal is suitable within the circular economy concept, in which recovered metals from an industrial wastewater are applied in bioreactors to create a biocatalyst for promoting the biotransformation of emerging pollutants. This strategy may be appropriate for the anaerobic treatment of wastewaters originated from hospitals, as well as from the pharmaceutical and chemical sectors.

1. Introduction

Pandemic outbreaks, such as those originated by influenza and SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) viruses, constitute a serious threat to public health, which have also caused profound social and economic impacts [1]. While these viral diseases can be treated with vaccines, there is an increased demand of antiviral drugs because of inaccessibility of the corresponding injections at required time and place. In fact, large amounts of antiviral agents have been consumed to control the spread of SARS-CoV-2 since the first report of patients with coronavirus disease at the end of 2019 (COVID 19) [2,3]. For instance, ribavirin and tenofovir were prescribed and consumed by approximately 270 million people worldwide since the beginning of COVID 19 spread. This demand led to an environmental load of 80 and 240 tons for ribavirin and tenofovir, respectively, considering the daily dose and release during the pandemic period [4,5].

Once these antiviral drugs are consumed by infected patients, they are excreted in urine and feces, thus ending in sewage systems where they need to be degraded [6,7]. However, these pharmaceuticals are barely degraded in conventional wastewater treatment plants (WWTP)

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* Corresponding author.
E-mail address: fcvantesc@iingen.unam.mx (F.J. Cervantes).
Moreover, advanced treatment processes (e.g., ozonation), which have shown efficient removal [9–11], are highly expensive due to the high dose of chemicals required to achieve complete mineralization. There are scarce reports on the biodegradation of antivirals under aerobic conditions, while they typically remain unaltered in anaerobic systems due to their recalcitrant structure [7,8,12].

Metallic catalysts, such as Pd, Pt, Mo, Zn, and Cu, have previously been shown to enhance the anaerobic biodegradation of recalcitrant pollutants, such asazo dyes [13], nitroaromatics [14], trichloroethylene [15], as well as pharmaceuticals like iopromide [16] and diatrizoate [17] in anaerobic treatment processes due to their catalytic input. However, they are extremely costly to consider their application at full-scale. Thus, recovery of minerals (containing catalytic elements) from industrial wastewaters for their subsequent application in anaerobic bioreactors to boost the biodegradation of pharmaceuticals could be an attractive scheme to revalue these elements for an environmental purpose [18]. The aim of the present study was to test the catalytic input of iron-rich minerals, recovered from a metallurgic effluent [19], on the biotransformation of antiviral agents in upflow anaerobic sludge bed (UASB) reactors. Two antivirals that have been shown to be effective against SARS-CoV-2, tenofovir and ribavirin, which are also frequently applied to treat other viral diseases [1,20], were selected as model contaminants. These drugs have previously been reported to be poorly degraded under aerobic conditions [7,8,12], while their biodegradation has not been reported under anaerobic conditions. There are adverse effects documented on their supply, such as renal failure, nephrogenic diabetes, nephrotoxicity, and hyperlactatemia [21,22]. Furthermore, environmental concerns associated with these antiviral agents include toxicity toward daphnids, fishes, and other aquatic species [23,24]. Additionally, recent studies revealed that transformation products derived from their degradation by an advanced oxidation process exhibit higher toxicity on Zebrafish embryos than the parent compound [11]. Therefore, efficient treatment technologies for their complete mineralization are required to prevent environmental and public health issues associated with their release via wastewaters.

2. Materials and methods

2.1. Inoculum and materials

Methanogenic sludge was obtained from a full-scale anaerobic digester treating effluents from a wheat flour factory (Querétaro, Mexico). This inoculum showed a content of volatile solids (VS) of 6.3 g/L. The anaerobic sludge was maintained at 4 °C before use. Tenofovir and ribavirin were purchased from Sigma-Aldrich (>99 % purity). Iron-rich minerals used in the present study were recovered from a stainless-steel industrial effluent [19]. These minerals were recovered by a precipitation process, which was sustained by the alkalinity produced from a denitrifying bioreactor, which removed the nitrate present in this industrial effluent [19]. The recovered material was pulverized in a mortar and dried before characterization and used.

2.2. Characterization of iron-rich minerals

After pulverizing and drying the recovered minerals, they were characterized by X-ray diffraction (XRD) in a Rigaku equipment (Model UV) provided with an ultra-fast detector (DteX) and operated under the following conditions: measurement range (2θ) from 5 to 80°, velocity at 2°/min with sampling every 0.02 s. Characterization was complemented by scanning electron microscopy coupled to energy dispersive X-ray spectroscopy (SEM-EDS) analysis performed in a Hitachi equipment (model SU-8230) with an applied voltage of 5 kV. The EDS equipment was from the same supplier (model XFLASH 6160), which was operated at 6 kV. In order to detect additional components, acid extraction from these minerals was conducted with a mixture of nitric acid (15 M) and hydrochloric acid (12 M) at a ratio of 1:3 (vol/vol), then the extract was filtered (0.45 μm) and analyzed by inductively coupled plasma (ICP, Varian model 730ES) coupled to optical emission spectroscopy (ICP-OES) under the following conditions: power, 1 kW; plasma flow, 15 L/min; nebulizer flow, 0.75 L/min. Calibration standard 6, solution A high purity, corresponding to EPA standard method 200.7 was used for the calibration curve.

2.3. Biotransformation of antiviral agents in UASB reactors

Two UASB reactors with a working volume of 400 mL were installed, inoculated with 150 mL of anaerobic sludge (equivalent to 375 g/L), and operated at room temperature (22 ± 1 °C) with a hydraulic residence time (HRT) of 1 day. One reactor was inoculated with unamended sludge (control), while the other one was inoculated with the same sludge, but previously mixed with the recovered minerals (95 % sludge/5 % minerals, wt/wt). For preparing the sludge/minerals mixture, the sludge portion was suspended in the medium described below in a glass container. Gentle, magnetic stirring was then applied, and the portion of minerals was gradually added until obtaining a homogenous material before inoculating the enriched bioreactor. Preliminary batch incubations showed that this portion of minerals did not inhibit the methanogenic activity of this anaerobic consortium (Supporting Information (SI), Fig. S1). Both reactors were fed with the following medium (g/L): glucose (2), NaHCO3 (5), K2HPO4 (0.25), MgCl2⋅6H2O (0.2), NH4Cl (0.1), CaCl2⋅2H2O (0.01) and 1 mL/L of trace elements, which composition has previously been reported [16]. Both reactors were supplied with this medium until steady state conditions were reached (after ~2 weeks of operation). Afterwards, ribavirin and tenofovir were individually added in both reactors at a concentration of 50 μg/L by introducing these compounds to the composition of the feeding medium. The performance of the bioreactors was evaluated in terms of the removal of chemical oxygen demand (COD) and the studied antivirals; moreover, the composition of the produced biogas was also monitored as well as the pH value. Additionally, transformation products (TPs) derived from the biotransformation of both antivirals were identified by high-performance liquid chromatography coupled to mass spectroscopy (HPLC-MS) as described below.

2.4. Adsorption isotherms

The contribution of adsorption processes on the removal of both antiviral agents in the UASB reactors was also assessed. For this purpose, adsorption tests were performed using sterilized biomass taken from both bioreactors. Experiments were conducted using 3.75 g of biomass and 10 mL of the previously described medium, which were placed in amber serum bottles and then hermetically sealed. Biomass portions were previously washed several times with the basal medium described above (without glucose). Adsorption isotherms were conducted at the pH prevailing in the bioreactors (7.4 and 7.3 for ribavirin and tenofovir, respectively). Before the adsorption test, three sterilization cycles were applied to bioassays in a vertical autoclave at 121 °C for 15 min to annihilate any biological activity. This sterilization procedure has previously been used for assessing abiotic processes driving the removal of organic contaminants [25,26]. After sterilization, 1 mL of antiviral solution of different concentrations (0.1, 0.5, 1, 5, 10 and 20 mg/L) was injected to the serum bottles and incubated for 72 h at room temperature to assess the adsorption capacity of biomass collected from both bioreactors. Afterwards, portions of 0.5 mL were collected from each experimental unit and centrifugated at 15500 rpm for 20 min in an Eppendorf Centrifuge Minispin Plus and the final concentration of the antiviral agents was measured as described below. All experiments were executed by triplicate under anaerobic conditions. Finally, different adsorption models, including Freundlich, Langmuir, and Redlich-Peterson, were tested for fitting the experimental data. Langmuir model appeared as the best model describing the experimental data with $R^2 = 0.99$. 
2.5 Methanotrophic activity test

To verify if iron-rich minerals could promote methanotrophic activity in the enriched bioreactor, tracer analysis was conducted in batch sludge incubations with labelled methane (\(^{13}\)CH\(_4\), Sigma-Aldrich 99 % \(^{13}\)C). Serological flasks (120 mL) were inoculated with 1 mL of homogenized sludge taken from the bioreactor enriched with the metals recovered from a metallurgical effluent. This inoculation procedure was conducted inside of an anaerobic chamber under a N\(_2\) atmosphere (LABCONCO, Precise Controlled Atmosphere model, Kansas City, MO). Once inoculated, 60 mL of N\(_2\)-flushed basal medium were dispensed on the vials, which were then sealed with air-tight rubber stoppers and aluminum caps and taken outside the anaerobic chamber. The composition of the basal medium was as follows (g/L): NH\(_4\)Cl (0.3), K\(_2\)HPO\(_4\) (0.2), MgCl\(_2\)-6H\(_2\)O (0.03), CaCl\(_2\) (0.1), and 1 mL/L of trace elements, which composition has previously been reported [27]. Anoxic conditions were established by flushing the headspace with N\(_2\) (Ultra High Purity; Praxair, Mexico) for 5 min. Experimental treatments include incubations with only sludge, sludge enriched with chemically synthesized ferricydrite (4 mM), sludge spiked with \(^{13}\)CH\(_4\) (4 mL), and sludge provided with both ferricydrite and \(^{13}\)CH\(_4\). Additionally, abiotic controls (amended with the same concentrations of ferricydrite and \(^{13}\)CH\(_4\)) were also prepared by autoclaving the sealed microcosms three times at 120 °C for 21 min and by the subsequent addition of 10 % v/v of anhydrous chloroform. The methanotrophic activity was monitored by quantifying the \(^{13}\)CO\(_2\) and ferrous iron (Fe\(^{2+}\)) produced during the incubation period as described below. Fig. S2 in SI shows a schematic diagram of the experimental setup to clarify the overall strategy of the study.

2.6 Analytical techniques

Methane concentration in produced biogas from the bioreactors was measured in a gas chromatograph (SRI Instruments Model 8610C, Champaign, IL, USA) equipped with a thermal conductivity detector (TCD) and two steel columns (2 m in length; 0.79 mm in diameter). The injector, column and detector temperatures were 90, 110 and 150 °C, respectively. N\(_2\) was used as the carrier gas at a flow rate of 20 mL/min.

The production of \(^{13}\)CO\(_2\) from sludge incubation to assess the methanotrophic activity linked to ferric iron reduction was measured by gas chromatography coupled to mass spectroscopy (GC-MS). For this purpose, 1 mL of slurry taken from incubations was acidified with HCl (3 N) in 20-mL anoxic tubes (Thermo Scientific, USA) equipped with a thermal conductivity detector (TCD) and two steel columns (2 m in length; 0.79 mm in diameter). The injection, column and detector temperatures were 90, 110 and 150 °C, respectively. N\(_2\) was used as the carrier gas at a flow rate of 20 mL/min.

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2.7.1 Characterisation of ores

The minerals considered in the present study as catalytic material for promoting the biotransformation of antiviral agents were recovered from a stainless-steel industrial wastewater [19]. XRD analysis detected four different components in this residue, including FeO(OH), (PO\(_4\)\(_2\)\(_9\)\(_2\)\(_8\)\(_2\)\(_2\))\(_2\) and barite ((MgFe\(_2\))(PO\(_4\)\(_2\)\(_9\)\(_2\)\(_8\)\(_2\)\(_2\)\(_2\)). Additionally, abiotic controls (amended with the same concentrations of ferricydrite and \(^{13}\)CH\(_4\)) were also prepared by autoclaving the sealed microcosms three times at 120 °C for 21 min and by the subsequent addition of 10 % v/v of anhydrous chloroform. The methanotrophic activity was monitored by quantifying the \(^{13}\)CO\(_2\) and ferrous iron (Fe\(^{2+}\)) produced during the incubation period as described below. Fig. S2 in SI shows a schematic diagram of the experimental setup to clarify the overall strategy of the study.

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The discrepancy with the stoichiometry could be due to a fraction of produced \(^{13}\)CO\(_2\), which could have been precipitated with components of the minerals present in this sludge; a scenario which was shown to occur in wetland sediments [28]. The slight production of \(^{13}\)CO\(_2\) in controls lacking \(^{13}\)CH\(_4\) could be explained by endogenous activity and considering that \(^{13}\)C represents 1.1 % of the natural abundance of carbon.

Further experiments conducted with the antiviral agent, tenofovir,
showed that the catalytic input of minerals promoted a much higher removal of this drug (94.7 %) in the enriched bioreactor as compared to the unamended control (only 37.9 % removal under steady-state conditions) (Fig. 2 and Table 1). Nevertheless, a slight decrease on COD removal efficiency was observed in the enriched bioreactor (87.8 %) as compared to the unamended control (95.7 %). These findings imply that long-term exposure to the metals by the amended sludge triggered some inhibitory effects (Table 1), even though preliminary incubations did not detect any inhibition at the concentration applied in the bioreactor (Fig. S1). Biogas produced from the enriched bioreactor showed lower methane content (48.5 %) as compared to the unamended control (68.5 %), which is consistent with the results obtained during the biotransformation of ribavirin.

3.3. Adsorption of antiviral agents on anaerobic biomass

Sterilized sludge incubations were performed in batch assays to assess the contribution of adsorption phenomena on the removal of ribavirin and tenofovir in both bioreactors. Fig. S4 in SI shows the adsorption isotherms of these antivirals on biomass collected from both UASB reactors. According to Langmuir model, which reports a correlation coefficient higher than 0.99 (SI, Table S2), the maximum adsorption capacity ($q_m$) of biomass enriched with minerals for tenofovir was 0.1048 mg/g. This value is very distant from the capacity reported for the same contaminant with adsorbing materials, such as activated carbon ($q_m = 145.194$ mg/g) [31]. Moreover, the same biomass showed much lower adsorption capacity for ribavirin ($q_m = 0.0025$ mg/g). Meanwhile, unamended biomass collected from the control bioreactor showed $q_m$ values of 0.0013 mg/g and 0.0054 mg/g for ribavirin and tenofovir, respectively (SI, Table S2). Further calculations, considering these $q_m$ values, suggested that adsorption was responsible for 17 % and 31 % of ribavirin removal in the unamended control and in the enriched bioreactor, respectively, while this process would have contributed with 25 % and 42 % of removal for tenofovir in the same systems. Nevertheless, these assessments should be carefully considered. First, adsorption capacities are not equivalent in batch and continuous conditions.
processes since mass transfer limitations and axial dispersion affect the amount of adsorbed contaminant, leading to a significant decrease on the efficiency [32]. In fact, several reports have indicated that the adsorption capacity of an array of materials for distinct pollutants decreases up to 5-fold in continuous columns as compared to the capacity observed in batch assays [32]. Second, the anaerobic sludge systems implemented in the present work were not well packed since there was a mobility of granular biomass during their operation. Also, the pattern of a fixed bed reactor (breakthrough curves) indicates a gradual decrease on the adsorption capacity throughout the operational period [33]; thus, not all the adsorption capacity is expected to prevail during the whole operational period. Finally, adsorption isotherms were conducted with autoclaved biomass, which was disintegrated during the sterilization process. It has previously been reported that the adsorption capacity of anaerobic granular sludge for organic contaminants significantly increased when using autoclaved, disintegrated biomass [34]. Therefore, while there certainly is a contribution of adsorption phenomena on the removal of the studied antivirals in the UASB reactors, the adsorption capacity would be much lower than that calculated from batch isotherms.

3.4. Identification of TPs from the biotransformation of ribavirin and tenofovir

TPs originated from the biotransformation of tenofovir and ribavirin were identified in both UASB reactors. All mass spectra were compared with those obtained from original samples of tenofovir or ribavirin, dissolved in basal medium, to distinguish the produced TPs under the established experimental conditions (SI, Figs. S5 and S6).

Identified TPs from tenofovir biotransformation are shown in Fig. 4. It could be expected that deamination of its adenine nucleus (as evidenced by TP3, m/z 254) and degradation of the ([propan-2-yl]oxy) phosphonic acid side chain may drive structural instability by microbial degradation under the experimental conditions prevailing [35]. Additionally, the cleavage of the N—C bond between the adenine nucleus and the phosphonic acid side chain could lead to the formation of TP4 (m/z 163), TP5 (m/z 156), and TP6 (m/z 168). Similar structure to proposed TP5 has previously been suggested as a TP of tenofovir under hydrolytic conditions [35]. Other structures could be intermediaries in the pathway; for instance, TP1 (m/z 254) and TP2 (m/z 240) represent transformations occurring in the phosphonic acid side chain that eventually could lead to the production of TP3 (m/z 163). These fractions may eventually undergo further degradation. TP7 was the smallest identified m/z, which is suggested to be a product from adenine microbial degradation as it has been reported in some anaerobic strains [36].

Regarding the TPs detected during the biotransformation of ribavirin, Fig. S7 in SI illustrates the identified structures. Initial steps of biotransformation are suggested to lead to the formation of TP1, TP2 and TP3, which might have derived from the removal of the amide group side chain. TP3 (m/z 197) could also be obtained as a TP from the
dehydrogenation on the hydroxyl groups as previously reported in an ozonation system [9]. Further degradation could derive in TP4 (m/z 158), which structure is expected after C–N bond cleavage of the previous TP3 (m/z 197). Similar structures to TP4 have previously been proposed as possible degradation pathway of ribavirin in wastewater treatment facilities [37]. The reaction implies the split of ribavirin into a triazole ring and an oxygen-containing five-membered heterocyclic ring [38]. An additional fraction was also identified as TP5 and could have been formed as a final degradation product. Similar structures have been proposed by different transformation pathway for ribavirin in an advanced oxidation process [9].

3.5. Role of iron-rich minerals on the biotransformation of antivirals in anaerobic bioreactors

The recovered minerals applied as a catalyst to promote the biotransformation of ribavirin and tenofovir in the UASB reactors, contain different forms of Fe(III), such as iron oxyhydroxide (FeO(OH)), ferrihydrite (Fe(OH)₃), and magnetite (Fe₃O₄) [19]. Furthermore, ferrous iron was concomitantly produced during the operation of the enriched bioreactor (Fig. 2), although a fraction was precipitated as vivianite and barite (SI, Fig. S3). Thus, it is suggested that Fe(III) might have been served as terminal electron acceptor driving the biotransformation of ribavirin and tenofovir. Several previous reports have documented the capacity of Fe(III)-reducing microorganisms to degrade different recalcitrant pollutants, such as hydrocarbons, phenols, chlorinated solvents, among others [39-43].

An additional mechanism, which might have been at stake during the biotransformation of the studied antivirals in the presence of iron-rich minerals, is the production of reactive oxygen species (ROS). Indeed, microbial production of hydroxyl radical (HO·) has recently been reported to be driven by iron redox transformation under anoxic conditions [44]. HO· is considered as one of the most powerful ROS promoting the transformation of several organic pollutants in Fenton reactions [45], which might have played an important role on disrupting the structure of ribavirin and tenofovir, leading to their faster biotransformation in the enriched UASB reactor as compared to the unamended control reactor. Moreover, the redox conversion of other catalytic elements detected in the minerals applied in the enriched bioreactor, such as Ni, Pd, Zr, Mo, and Co, may be also involved in the production of ROS, which ultimately contributed to the superior biotransformation of the antiviral agents observed in the enriched bioreactor. Similar results have previously been documented; for instance, by applying Pd-enriched biomass for the biotransformation of iopromide in UASB reactors [16].

The recovery of minerals from industrial wastes for their application as catalysts for the degradation of emerging contaminants is a sustainable approach, which agrees with the concept of circular economy. This treatment concept could be suitable for enhancing the biotransformation of emerging pollutants present in effluents derived from hospitals, as well as from the pharmaceutical and chemical sectors. However, attention should be placed on monitoring eventual leaching of toxic heavy metals during the application of these minerals for the biodegradation of emerging contaminants in bioreactors. In the present study, heavy metals, such as Cr, Cu, Mn, Ni, and Pb, were not detected or their concentration was below the established limits by the Mexican legislation for treated wastewater (Table S3). However, the amount of applied minerals for these purposes could be decreased to prevent environmental risks and to avoid long-term inhibition on anaerobic consortia in bioreactors.

4. Conclusions

Iron-rich minerals, recovered from a stainless-steel industrial wastewater, were applied in anaerobic bioreactors to create a biocatalyst for enhancing the biotransformation of the antiviral agents, ribavirin and tenofovir, which have recently been supplied against SARSCoV-2. Fe(III) reduction occurred in the enriched bioreactor driving much higher removal efficiency for both antiviral drugs as compared to the unamended bioreactor. Adsorption isotherms conducted with sterilized biomass suggested that adsorption was responsible for a minor fraction of the removed pharmaceuticals. Moreover, further analysis by HPLC-MS confirmed the biotransformation of both antiviral agents to several transformation products. Finally, anaerobic methane oxidation coupled to Fe(III) reduction also occurred in the enhanced system, which led to a lower content of methane in the biogas produced as compared to the unamended bioreactor. This biocatalyst concept may be appropriate for the removal of emerging contaminants from wastewaters originated from hospitals, as well as from the pharmaceutical and chemical sectors.
CRediT authorship contribution statement

Francisco J. Mares-Carbajal: Data curation, Writing–original draft preparation, Investigation, Validation, M. Carolina Espinosa-Arzate: Investigation, Validation, Methodology. Luis A. Ramírez-Montoya: Investigation, Validation, Methodology. Aurora M. Pat-Espadas: J. Ernesto Ramírez: J. René Rangel-Mendez: Juan A. Ascacio-Valdes: Investigation, Validation, Methodology. Cristobal N. Aguilar: Investigation, Validation, Methodology. Petia Mijaylova: Supervision, Validation, Writing–reviewing. German Buitrón: Supervision, Validation, Writing–reviewing. Francisco J. Cervantes: Writing–Original draft preparation, Conceptualization, Validation, Supervision, Funding acquisition, Writing–reviewing and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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