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Comparative analysis of microbial diversity in batch reactor with direct interspecies electron transfer system using an effective and inexpensive method mgDNA extraction

Análisis comparativo de la diversidad microbiana usando un método efectivo y económico de extracción de mgDNA en un reactor batch con un sistema de transferencia directa de electrones entre especies

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Abstract

Recently, metagenomic DNA based analysis gained importance in fields such as environmental sciences and bioenergy, where mixed microbial communities embedded in complex matrixes, play a crucial role. Therefore, financially economical, and high quality metagenomic DNA extraction protocols are needed. In this work, a rapid and inexpensive method for high quality DNA extraction from a variety of complex samples was performed; the average DNA yield was 71.65 ng μL^{-1}, with an average purity of 1.68 (A_{260}/A_{280}). The cost of extraction per sample was about 76 % less in comparison with commercial kits and the time needed to obtain the DNA pellets was about 4 hours. The DNA was suitable for 16S rRNA gene amplification by PCR and for next generation sequencing analysis, employing a MiSeq Ilumina platform. A high microbial diversity was detected in this study, and three main groups of bacteria were observed, which were developed according to the effect of the activated carbon had on them. The analysis performed showed a great difference between the samples, highlighting the differences between the microbial communities developed in the activated carbon biofilm and the bacteria detected in the reactor without activated carbon.

Keywords: Metagenomic DNA extraction, carbon biofilm, sludge sample; PCR based 16S rRNA analysis.

Resumen

El análisis basado en ADN metagenómico ha ganado importancia en campos de investigación como las ciencias ambientales y bioenergía, donde un rol crucial lo realizan comunidades microbianas fijadas en matrices complejas. Como consecuencia, son necesarios protocolos de extracción de ADN metagenómico económicamente viables y con alta calidad. En este trabajo, se desarrolló un método rápido y de bajo costo para la extracción de ADN de alta calidad en una variedad de muestras complejas; el rendimiento promedio del ADN fue 71.65 ng μL^{-1}, con una pureza promedio de 1.68 (A_{260}/A_{280}). El costo de extracción por muestra fue 76 % menor en comparación con kits comerciales y el tiempo para obtener los pellets de ADN fue aproximadamente 4 horas. El ADN fue adecuado para la amplificación del gen 16S rRNA por medio de PCR y para análisis de secuenciación de nueva generación, empleando una plataforma MiSeq Ilumina. Asimismo, una gran diversidad microbiana fue detectada, donde predominaron tres grupos de bacterias, los cuáles fueron desarrollados de acuerdo con el efecto del carbón activado sobre ellos. El análisis mostró una gran diferencia entre las muestras, resaltando las diferencias entre las comunidades microbianas desarrolladas en la biopelícula de carbón activado y las bacterias detectadas en el reactor sin carbón activado.

Palabras clave: Extracción de ADN metagenómico, biopelícula de carbón, muestras de lodo, análisis de PCR basado en 16S rRNA.

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1 Introduction

Because the next generation sequencing technologies are available, metagenomic DNA (mgDNA) analysis has become a common tool in environmental and bioenergy sciences for microbial community (Guerrero Barajas *et al*., 2019). Also, mgDNA is used in many PCR based molecular tools, such as gene amplification and pyrosequencing, denaturing gradient gel electrophoresis (PCR-DGGE), restriction fragment length polymorphism (PCR-RFLP), single stranded conformation polymorphism (PCR-SSCP) and quantitative real time PCR (qRT-PCR) (Arunasri *et al*., 2016; Goswami *et al*., 2016; Guermazi-Toumi *et al*., 2019; Guo *et al*., 2013; Kiseleva *et al*., 2015; Venkata Mohan *et al*., 2010). Frequently, the microbial mgDNA of interest is embedded in a complex matrix (i.e. soil, sludge, sediment, biofilm, porous or carbonaceous materials, etc.) immersed in a liquid phase such as natural water streams, culture media, or wastewaters (Dang *et al*., 2017; González-Gamboa *et al*., 2018; González-Muñoz *et al*., 2018; González-Paz *et al*., 2020). Therefore, high concentration and variety of impurities predominate in the samples, the main reason why lengthy procedures, expensive DNA isolation kits and purification columns are required (Canto-Canché *et al*., 2013).

Microbial electrochemical technologies have grown rapidly due to their applications in water bioremediation, energy production, water desalinization and generation of high added-value products (García *et al*., 2019; Santoro *et al*., 2017), reason why microbial community mgDNA analysis from bioelectrodes has become very frequent in the literature (Saratale *et al*., 2017). These microbial communities are usually embedded in polysaccharide rich biofilms, where some biological and electroactive processes take place (Erable *et al*., 2010; Saratale *et al*., 2017). This is also true for anaerobic digestion processes and the microorganisms involved in them (Fagbohungbe *et al*., 2016). The above mentioned biofilms contain organic molecules such as nucleic acids and proteins, and also inorganic substances such as metals and nutrients which are sequestered within the exopolysaccharide matrix (Baishya *et al*., 2020; Flemming & Wingender, 2010). Additionally, the biofilms are supported on different conductive materials (Erable *et al*., 2010), and in many cases, the isolation of the microbial community from the supporting material is barely impossible (Canto-Canché *et al*., 2013). Carbonaceous and porous materials as granular activated carbon, carbon felt, fibers or cloth, layered corrugated carbon, among others, are cheap and conductive supporting materials frequently used in anaerobic reactors (Barua & Dhar, 2017). These materials carry metals which are easily adsorbed on its surface (Erable *et al*., 2010; Guo *et al*., 2013). High chemical oxygen demand (COD), presence of organic acids, metals and other contaminants carried by carbon supporting materials are also frequent (Zhang *et al*., 2018). Therefore, to isolate DNA from complex samples as microbial electrosynthesis (MES) bioelectrodes, anaerobic digesters for bioenergy production and environmental samples used as inoculum, commercial DNA isolation Kits are usually employed (Kiseleva *et al*., 2015; Yang *et al*., 2017), which are expensive in comparison with homemade DNA isolation protocols (Canto-Canché *et al*., 2013). Additionally, in many cases purification columns are required to achieve good quality mgDNA, which are also expensive and produce DNA losses (Canto-Canché *et al*., 2013; Rousseau *et al*., 2016).

The study of the microbial communities developed in anaerobic reactors gives us information about the metabolic processes that are occurring in the reactors, helping to understand the yields and functioning of anaerobic digestion processes under certain conditions (Barua & Dhar, 2017). The microbial diversity in anaerobic digesters comes from the waste origin, inoculum employed and metabolic processes promoted in the reactors (Zhang *et al*., 2018). In recent years, it has been detected that bacteria have the ability to transfer electrons in anaerobic digestion processes of which this capacity is unknown (Park *et al*., 2018). These bacteria use conductive materials to send electrons to electrotrophic archaea (Zhao *et al*., 2016), this makes it necessary to identify them in the biofilms formed in these conductive materials.

In this work, a simple, inexpensive, quick, versatile, and reproducible protocol was developed to obtain high quality mgDNA from different samples: activated sludge from a methanogenic batch reactor for maize processing wastewater treatment and a biofilm embedded in granular activated carbon from the same reactor. The use of this method of DNA extraction allows the identification and study of the bacteria involved in complex anaerobic digestion processes. Additionally, this method offers the opportunity to analyze both the communities developed in the sludge and in the biofilms formed in conductive materials.

2 Methods

2.1 Sample description

The samples were collected in BMP tests carried out in 110 mL serum bottles (reactors) that were capped with rubber septum sleeve stoppers. All the tests were carried out for 30 days at 38 °C with an automatic agitation of 100 rpm. Three experimental conditions in the sludge added to the BMP test were assayed. First, the sludge control (S) consisted in activated sludge of inoculum with a composition reported by Poggi-Varaldo *et al*., 1997. Second, sludge with granular activated carbon (GAC) without previous incubation (S0). Third, sludge with GAC with a previous incubation of 10 days at 38 °C and 100 rpm, to promote the previous growth of a biofilm (S10).

To develop the new protocol, 10 g samples of sludge from the sludge control (S); sludge from second condition (S0); sludge from third condition (S10), as well as biofilms supported on granular activated carbon at the second and third condition (C0 and C10, respectively), were macerated in a sterile mortar and stored at -80 °C in 50 mL sterile tubes with 20 mL of sterile water per sample.

2.2 DNA Extraction for Metagenomic Analysis (protocol)

Incubate the samples at 70 °C for 1 h. Agitate manually every 20 min. Bring under the samples to three freeze-thawing cycles at -80 °C (10 min) and 70 °C (10 min). Centrifuge at 6,940 g (1 min) to get rid of the big solid particles and transfer 300 μL of the supernatant to a 2 mL sterile tube. Add 1 mL of preheated extraction buffer [100 mM Tris-HCl (pH = 8.0), 1% PVP 40, 1.3 M NaCl, 20 mM EDTA (pH = 8.0) and 2.8% CTAB] (65 °C) and 10 μL of Proteinase K (20 μg mL^{-1}). Incubate for 30 min at 65 °C. Agitate manually every 10 min. Add 600 μL of pure chloroform and mix manually during 10 min. Immediately, centrifuge for 10 min at 18,327 g to separate the organic and aqueous phase. Transfer 700 μL of the aqueous phase (supernatant) to a 2 mL sterile tube. Add 55 μL of 7 % CTAB and mix manually for 5 min. Add 600 μL of pure chloroform. Mix the tubes vigorously to form one phase and centrifuge for 10 min at 18,327 g. Transfer the aqueous phase to 1.5 mL sterile tubes. Incubate with chilled isopropanol (1:1 v/v) for 20 min at -20 °C to precipitate the DNA. Centrifuge at 18,327 g for 10 min and discard the supernatant. Wash the DNA pellets with chilled ethanol (70 %). Centrifuge at 18,327 g for 5 min and discard the supernatant. Let the pellets to dry and resuspend in 50 μL of 1X TE buffer (10 mM Tris-HCl (pH = 8.0), 1 mM EDTA (pH = 8.0) and 10 mM NaCl). The DNA extraction were performed in triplicate for each sample, and then the DNA were mixed before performance the sequencing.

2.3 mgDNA quality assessment and quantification

The quantity and quality of extracted DNA was assessed by NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The mgDNA integrity was visualized by 0.8 % agarose gel electrophoresis staining with ethidium bromide under a UV transilluminator; the images were acquired with a GelDoc EZ (Bio-Rad, Singapore, Singapore).

2.4 PCR amplification

To confirm that the metagenomic DNA samples were free of inhibitors, an amplification of the ribosomal 16S was performed. Each reaction (25 μL) contained PCR buffer 1X, $MgCl_2$ 1.5 mM, a mix of dNTP's (0.2 mM), 0.04 U μL^{-1} of *Taq* polymerase, 1 μM forward and reverse primers (16SS forward primer 5'-AGAGTTTGATCCTGGCTC-3' and 16S reverse primer 5'-CGGGAACGTATTTCA-3') (Magnusson *et al*. 2003), and 2 ng μL^{-1} of DNA template. The reaction was performed as follows: initial denaturation at 94 °C during 5 min, 30 cycles of 1 min of denaturation at 94 °C, 30 s of annealing at 59°C and 1 min of extension at 72 °C, and a final extension period of 10 min at 72°C. PCR products were visualized by a 1.5% agarose-TBE 1X gel electrophoresis with ethidium bromide under a UV transilluminator, the image was acquired with a GelDoc EZ system. The 16S rRNA sequences containing variable V3-V4 regions of each DNA sample were amplified by broad-range forward primer Bakt-341F (5'-CCTACGGGNGGCWGCAG-3') and reverse primer Bakt-805R (5'-GACTACHVGGGTATCTAATCC-3'). DNA was sent to Macrogen Inc. (Seoul, South Korea) who performed sequencing on an Illumina MiSeq platform.

2.5 Data analyses

MG-RAST software was used to analyze bacteria and archaeal communities through RDP II databases

(Glass & Meyer, 2011) the abundance data were employed to construct a correlation matrix using Jaccard's coefficients and the unweighted pair group method. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to generate a dendrogram based on Jaccard's similarity coefficient with the sequential agglomerative hierarchical and nested (SAHN) clustering module of NTSYSpc software, version 2.11T (Rohlf, 2004). Principal Coordinate Analysis (PCoA) was also performed to separate the bacterial genus.

3 Results and discussion

The nucleic acid concentration and quality for each sample is presented in Table 1. DNA yield was variable between sample types, with a final DNA concentration range of 53.85 to 133.80 ng μL^{-1}, but in any case, the DNA concentration was enough to perform molecular analysis and the DNA quality at A_{260}/A_{280} ratio was in the range 1.63 to 1.72 in all the mgDNA samples.

The electrophoresis gel for DNA quality assessment and for PCR product visualization obtained from each sample type are presented in Figure 1.

No indicators of significant DNA shredding or degradation was observed and the mgDNA appears to be highly compacted. RNA was not found in most of the samples, even though RNase was not used. All the sample types were successfully amplified when the mgDNA extract was used as template and the expected product of approx. 1400 bp was obtained, indicating that the mgDNA samples are pure enough to perform PCR. Moreover, the samples sequenced by MiSeq Ilumina Platform (Macrogen, Korea) showed good Phred quality scores, Q20 and Q30, where the first is the ratio of bases that have Phred quality score of over 20, and the second, the ratio of bases that have Phred quality score of over 30. All samples exceeded 90% in Q20 and 81% in Q30.

Table 1. Quality and quantity of the mgDNA extracted from seven different complex samples.

Type of sample	**Sample ID**	**mgDNA (ng/μL)**	A_{260}/A_{280}
Activated sludge from a methanogenic batch reactor	S	53.85±1.50	1.63±0.02
	S0	65.40±1.00	1.72±0.03
	S10	133.80±2.50	1.66±0.10
Biofilm supported on activated carbon from a methanogenic batch reactor	C0	62.40±1.86	1.69±0.01
	C10	42.80±1.30	1.71±0.50

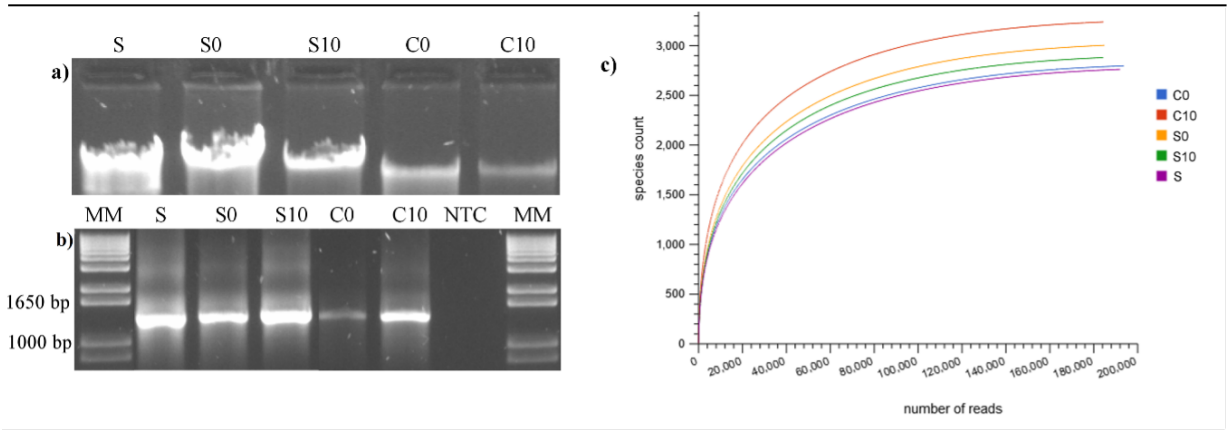

Fig. 1 a) Agarose (0.8 %) gel electrophoresis of the extracted DNA. b) Agarose (1.5 %) gel electrophoresis of PCR products from all the extracted DNA samples c) Rarefaction curves were used to estimated richness in the sample and sampling effort. MM: Molecular marker ladder 1Kb Plus (Invitrogen), NTC: Negative target control, S: Sludge control, S0: sludge reactor with GAC, S10 sludge reactor with GAC were added ten days before undertaking the reactors, C0: granular activated carbon biofilm, C10: granular activated carbon biofilm when GAC were added ten days before undertaking the reactors.

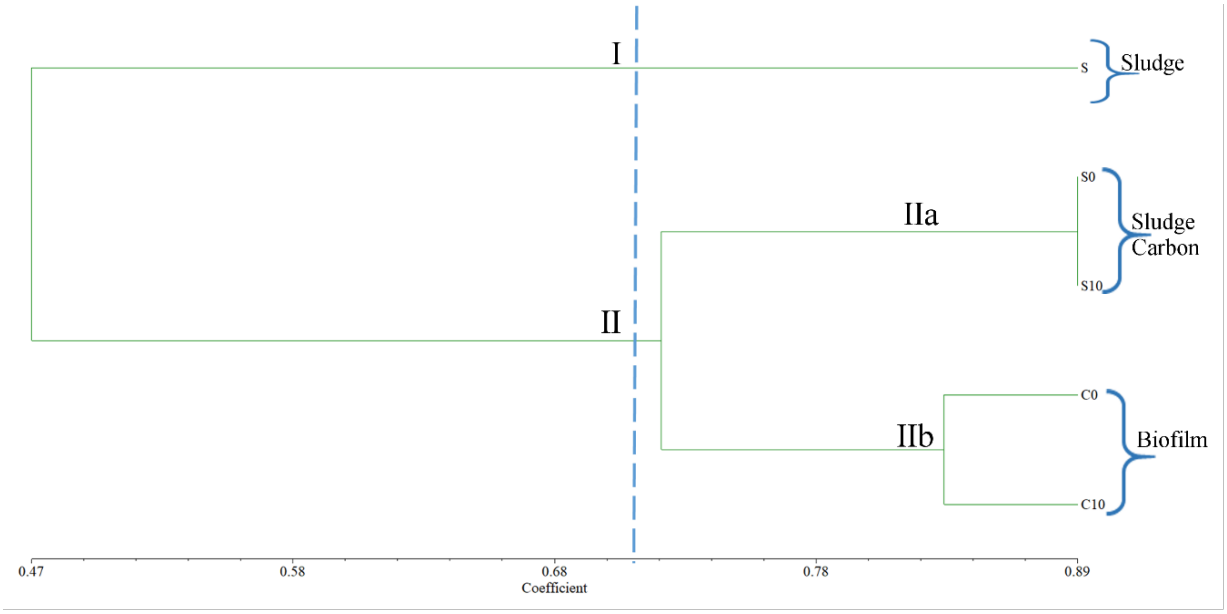

Fig. 2 Analysis based on relative abundance of genera bacteria in the different treatments. Unweighted Pair-Group Arithmetic Average (UPGMA) dendrogram based on Jaccard's coefficient showing the relations between different treatments.

Figure 1c, illustrates the rarefaction curves, both the richness and diversity of microbial communities were estimated by analyzing these curves. It is observed that saturation was reached for all samples, each of them surpassing 2,500 species. This indicates high diversity and richness. The number of readings was over 180,000 in all samples.

Finally, the mgDNA extraction protocol reported here was performed in 4 hours or less, and the cost per sample was of $1.20 USD, a 76% off, in comparison with commercial DNA isolation kits.

3.1 Bacteria communities analysis

The results obtained in the communities analysis, using the new protocol, demonstrates how the carbon directly affects the bacteria development. Figure 2 shows how the samples are clustered, depending on the bacteria detected, as well as their abundance in the analyzed samples. Three main clades are clearly differentiated (I and II) in the cluster analysis of bacteria genera. The sludge sample of the reactor which does not have carbon (S) had the lowest coefficient (0.52) with respect to the rest of the samples. This indicates that S had the least similarity to the rest of the samples. The coefficient of similarity in clade IIa was the highest (0.89). This clade was composed of the sludges of the carbon-containing reactors (S0 and S10). This shows that the bacterial communities associated with anaerobic sludges are affected by carbonaceous materials such as granulated activated carbon (Xu *et al*., 2015; Yang *et al*., 2017). The coefficient of similarity between C0 and C10 was 0.84 (clade IIb). This value was close to that similarity shown in the communities analyzed in samples S0 and S10 (0.89). This indicates that the abundance and genera bacteria are similar in both bacteria developed in carbon biofilms, as well as in communities developed in the same medium with granulated activated carbon. The coefficient clade II had a value of 0.76, distinctly different from the value of clade I in comparison with clades IIa and IIb.

The microbial diversity in the anaerobic processes carried out in the reactors with carbon are different from those performed in the reactor without carbonaceous material. This means that the bacterial communities detected vary considerably. In anaerobic digestion processes the participating bacteria have different metabolic processes by which organic matter is degraded to become methane and carbon dioxide mainly (Wannapokin *et al*., 2018). The carbonaceous materials affect this process by promoting the direct interspecies electron transfer (DIET) through the activated carbon, instead of interspecies hydrogen transfer (IHT) (Liu *et al*., 2012). Exoelectrogenic bacteria send electrons through granulated activated carbon to methanogenic archaea for the formation of methane by reducing carbon dioxide (Rotaru

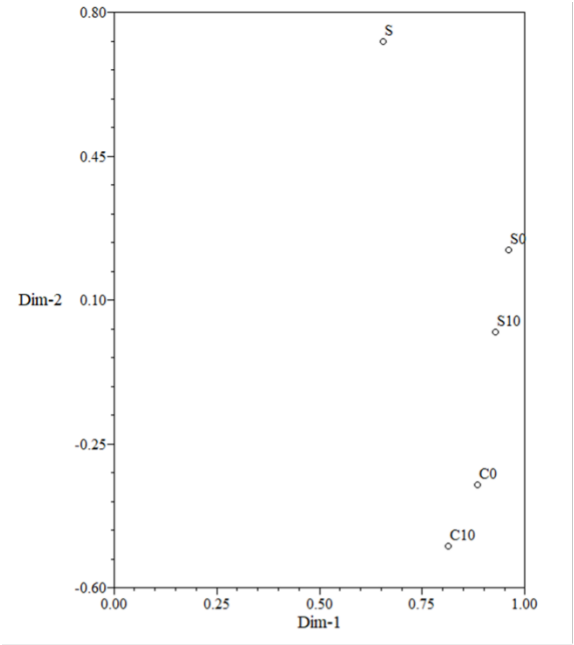

Fig. 3 Two dimensional plot of principal coordinate analysis (PCoA) showing the clustering patterns of samples treatments.

et al., 2014). The variances in the electron transfer via interspecies causes the differences in the bacterial genera detected and their abundances between reactors with granulated activated carbon and those without carbonaceous materials. The development of exoelectrogenic communities in biofilms formed in granulated activated carbon has been demonstrated in several studies (Barua & Dhar, 2017; Zhang *et al*., 2018).

The principal component analysis confirms the differences and similarities shown above (Figure 3). The principal component 1 and 2 shows 73.15 % and 19.02 % respectively of data variance, totaling 92.17 %. On the X axis all samples are located at positive values between 0.67 and 0.96. The lowest value was obtained in S, this sample is located at a greater distance from the others on the X axis. The spatial distribution related to the principal component 2 explains the difference between the communities formed by the different treatments. The biofilms formed in the granulated activated carbon are placed in the most negative values of the Y axis in both reactors. Given that the C10 biofilm was being developed 10 days before C0, it presents greater differences with the rest of the samples, especially with S. Additionally, the carbon surface is completely porous, allowing the development of communities distinct from the rest of the samples (Bertin *et al*., 2004). Sludge samples from the reactors with carbon are located at values close to 0.1 on the Y axis. Due to the influence of communities developed in C10 previously, S10 obtained a value less than S0 on the Y axis. On the other hand, on the most positive side of the Y axis, S is situated presenting the least similarity between the treatments performed.

Two large clades are shown in the bacteria genera dendrogram (Figure 4). The first clade is formed by the bacteria that were detected in greater abundance in the reactors with carbonaceous materials (S0, S10, C0, C10). Bacteria clustered in clade II had greater growth in the S sample than in any other. The correlation coefficient between clades I and II was -0.58, this indicates a greater disparity between the microbial communities developed in samples S0, S10, C0 and C10 and the communities detected in S. Clade I is divided into three main clusters (Ia, Ib and Ic). Bacteria presented in cluster Ia were mainly detected in the biofilm formed in C10. The genera *Geobacter, Bacteroides* and *Syntrophomonas* registered in the Ia cluster have been reported in carbonaceous materials biofilms which were developed in anaerobic reactors (Barua & Dhar, 2017; Zhang *et al*., 2018). These bacteria participate in oxidation reduction processes for the formation of methane by transferring electrons via DIET. The genera *Parabacteroides* and *Porphyromonas* were more abundant in samples S0, S10, C0 and C10 which are clustered in Ib. On the other hand, the bacteria presented in the Ic cluster had a greater abundance in C0. Both *Desulfotomaculum* and *Prolixibacter* have been reported as bacteria with the ability to transport electrons extracellularly (Holmes *et al*., 2007; Plugge *et al*., 2002).

In order to delve into the similarities between the communities detected in the reactors sludges and in the biofilms formed, a principal component analysis was performed. Figure 5 illustrates how bacteria are grouped in the analyzed samples. The principal component 1 and 2 demonstrates 65.76 % and 16.63 % respectively of data variance, totaling 82.39 %. In Figure 5 four main groups are formed. The genera that are spatially distributed in the most negative values of the X axis (green circle) demonstrate an abundance which was significantly higher in S than in any other sample. Conversely, on the positive side of the X axis, three groups are formed (orange, blue and red circles). The bacteria present in these three clusters were not detected in S or their abundance was significantly lower with respect to the other samples.

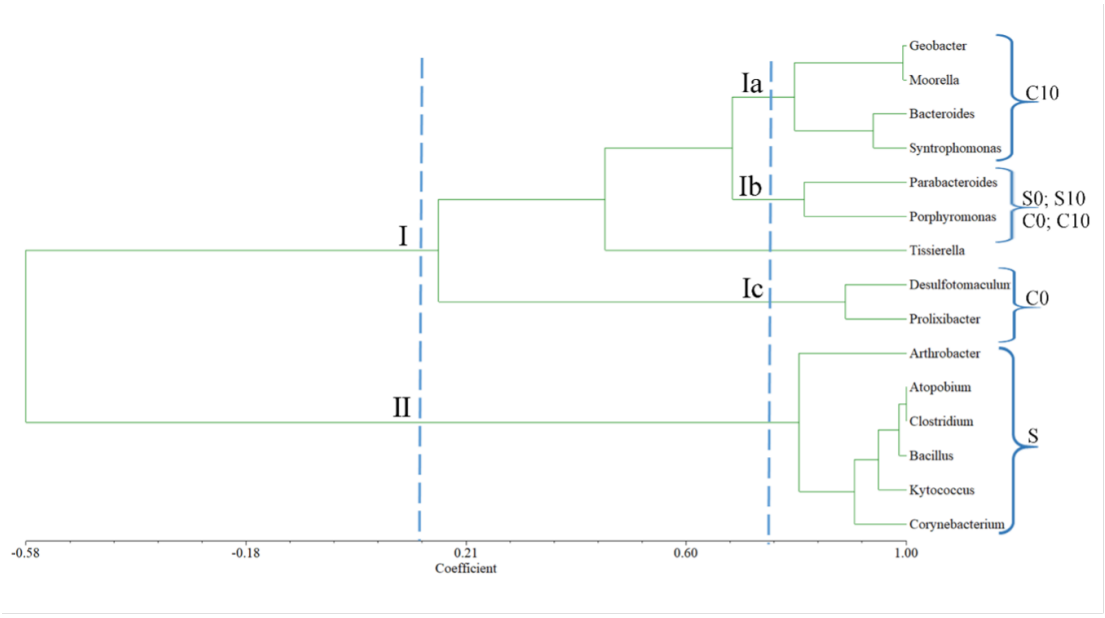

Fig. 4 Abundance relative of genera bacteria. Unweighted Pair-Group Arithmetic Average (UPGMA) dendogram based on Jaccard's coefficient showing the relations between different bacteria genera. S0: sludge reactor with GAC, S10 sludge reactor with GAC were added ten days before undertaking the reactors, C0: granular activated carbon biofilm, C10: granular activated carbon biofilm when GAC were added ten days before undertaking the reactors.

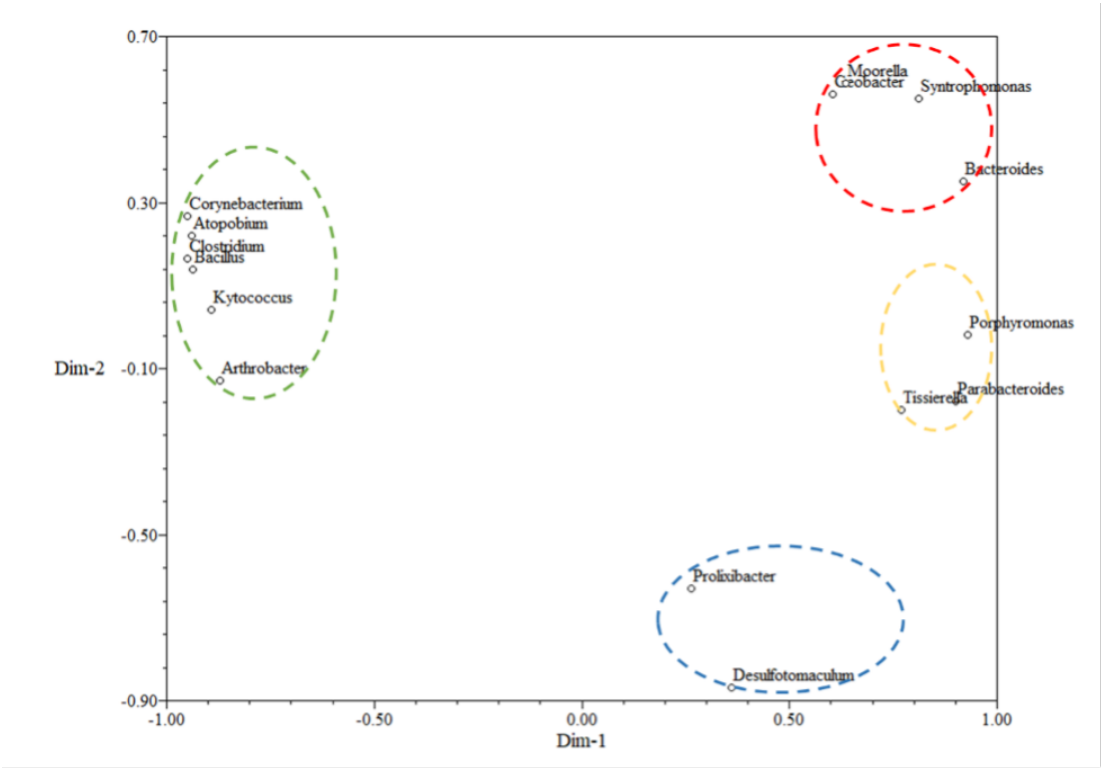

Fig. 5 Two dimensional plot of principal coordinate analysis (PCoA) showing the clustering patterns of genera bacteria.

The spatial arrangement of the bacteria in the principal component 2 illustrates the differences between the biofilm C0 and C10. Bacteria located in the most negative values of the Y axis (blue circle) had a greater abundance in C0. In contrast, the bacteria in the most positive values of the Y axis (red circle) were only detected or had a greater abundance in C10. Finally, the genera within the orange circle at values close to 0 on the Y axis had a similar presence at S0, S10, C0 and C10.

Conclusions

The described mgDNA extraction protocol it is an inexpensive and quick method applicable to a variety of complex samples, with reproducibility and suitability, in quantity and quality, for PCR based analysis and Next Generation Sequencing. This protocol has been successfully applied in the analysis of bacterial communities, both in sludge and in biofilms formed in carbonaceous materials in anaerobic reactors. The presence of carbon promotes the development of exoelectrogenic communities causing a great difference in the similarity with the bacteria developed in the sludge, this is probably due to the stimulation of the alternative electron transport route (DIET) in the carbon biofilm. This method of DNA extraction unlocks boundless opportunities to conduct more thorough and comprehensive studies without the financial hindrance which currently limits many studies.

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Nomenclature

COD	chemical oxygen demand
DIET	direct interspecies electron transfer
GAC	granular activated carbon
IHT	interspecies hydrogen transfer
MES	microbial electrosynthesis
mgDNA	metagenomic DNA
PCR-DGGE	denaturing gradient gel electrophoresis
PCR-RFLP	restriction fragment length polymorphism
PCR-SSCP	single stranded conformation polymorphism
qRT-PCR	quantitative real time PCR

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