UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
FACULTAD DE CIENCIAS QUÍMICAS

IMMOBILIZATION OF REDOX MEDIATOR FOR ANAEROBIC BIOTRANSFORMATION OF CONGO RED DYE

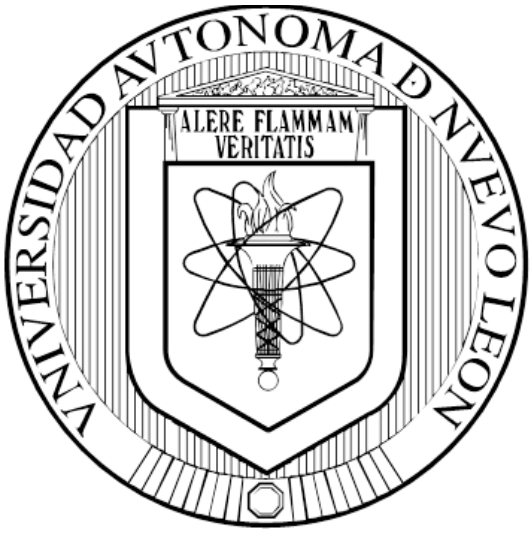
POR

DANIEL SIMÓN OLIVO ALANÍS

COMO REQUISITO PARCIAL PARA OBTENER EL GRADO DE MAESTRÍA EN CIENCIAS CON ORIENTACIÓN EN PROCESOS SUSTENTABLES

NOVIEMBRE, 2015

UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN
FACULTAD DE CIENCIAS QUÍMICAS



IMMOBILIZATION OF REDOX MEDIATOR FOR ANAEROBIC BIOTRANSFORMATION OF CONGO RED DYE

BY

DANIEL SIMÓN OLIVO ALANÍS

AS PARTIAL REQUIREMENT TO OBTAIN THE DEGREE OF MASTER DEGREE OF SCIENCES WITH ORIENTATION IN SUSTAINABLE PROCESSES

NOVEMBER, 2015

IMMOBILIZATION OF REDOX MEDIATOR FOR ANAEROBIC BIOTRANSFORMATION OF CONGO RED DYE

Dissertation Review committee:

Dr. Refugio Bernardo García Reyes
Advisor

Dr. Luis Humberto Álvarez Valencia
Co-Advisor

Dr. Alcione García González
Reviewer

Dr. María Teresa Garza González
Reviewer

Dr. Angel Martínez Hernández
Reviewer

ABSTRACT

Daniel Simón Olivo Alanís
Universidad Autónoma de Nuevo León
Facultad de Ciencias Químicas
Dissertation title: IMMOBILIZATION OF REDOX MEDIATOR FOR ANAEROBIC BIOTRANSFORMATION OF CONGO RED DYE

Number of pages: 84 Candidate for the Master Degree of Sciences with Orientation in Sustainable Processes
Study area: Sustainable Processes

Purpose and method of the study: About 50% of the dyes used for textile dyeing end up in the wastewater. It represents an ecotoxic hazard and introduces the potential danger of bioaccumulation, and can significantly damage the flora, fauna and the human health. Redox mediators have been applied on anaerobic digestion treatment to increase biotransformation of azo dyes, but its continuous addition causes a high cost for the treatment. For this reason, different immobilization techniques have been tried to obtain a solid-phase redox mediator. Nevertheless, to achieve high immobilization stability and to avoid detachment of redox mediator, different expensive and complicated methods of immobilization have been tested. In this work, two easy ways of covalently immobilization for redox mediator onto activated carbon were investigated, and their application for enhance decolorization of congo red dye at typical concentration of dye textile wastewater (150 - 200 mg/L).

Contribution and Conclusions: Lawsone immobilization on granular activated carbon (GAC) was successfully achieved with high capacity (~1.56 mmol/g), and desorption studies shows chemical stability; immobilization conditions may suggest esterification mechanism between lawsone and activated carbon. Furthermore, an easy way for chlorination of activated carbon, duplicate adsorption capacity of AQDS onto chlorinated carbon, compared with adsorption on unmodified GAC, then nucleophilic substitution may covalently bonded sulfonate groups of AQDS onto activated carbon; both immobilization (physical and chemical) shows good stability. Kinetics with solid-phase redox mediator and their controls (GAC, GAC-Cl and anaerobic sludge) were proved for decolorization of congo red dye. Lawsone and AQDS immobilized on GAC reached ~85% and 87% of decolorization, compared to 28% reached by anaerobic sludge at 48 h. Gas chromatography show that methane production was slightly inhibited when redox mediator is applied. Moreover, selective hydrogen production was observed with GAC-Cl as redox mediator, duplicates and maintains the cumulative production compared to anaerobic sludge, attributed to a methanogenic inhibition and acid intermediates production. Finally, HPLC study shows a slow rate for benzidine production compared to decolorization; dye reduction mechanism is presented. Both synthetized solid-phase redox mediators are good candidates for application in continuous bioreactors.

Table of Contents

INDEX OF TABLES

INDEX OF FIGURES

1. General Introduction

Because of industrial development (chemicals and petrochemicals), huge amounts of toxic and recalcitrant pollutants are discharged into wastewater [1]. These pollutants can significantly damage the flora, fauna and human health. Such pollutants as aromatic compounds, poly-halogenated compounds, azo dyes and metalloids, all electron acceptor contaminants (EAC), are commonly discharged into the environment by those industries. Advance oxidation, aerobic and anaerobic processes have been employed to degrade EAC, but poor efficiency, high cost and major toxicity to microorganism have been cause that its application become unavailable. Nevertheless, research to overcome disadvantages of wastewater treatment technologies has taking force the last years. Anaerobic treatment presented high efficiency for biodegradation and biotransformation of electron acceptor compounds. However, the main disadvantage of these systems is the low rate reaction due to poor electron transfer or toxicity of certain pollutants, which eventually results in a low yield or collapse of the biological reactor [2]. Nowadays, significant advances have been reported on the use of model quinones and humic substances as redox mediators (RM) to enhance the electron transfer capacity between the microorganisms and the electron acceptors pollutants. Therefore it's important to explore the potential of RM to be applied in the treatment of wastewater contaminated with electron acceptor compounds [3]. However, continuous addition of these substances can cause a high cost for the treatment. For this reason, immobilization of RM will allow its application up-scale systems for anaerobic wastewater treatment.

1.1 Recalcitrant pollutants

Chemical and petrochemical industries has changed the ecosystem of the earth due the synthesis and purification of a wide range of chemicals to be used as solvents, herbicide, dyes, refrigerants and many other applications. More than 100,000 chemicals are manufactured on a daily basis [4]. Most of these substances that are produced are recalcitrant, they resist to biodegradation under aerobic and anaerobic conditions, and may result in severe destruction of the natural ecological balance and human health due the discharge and accumulation in air, water and soil. The removal and degradation of these compounds become focal in many research studies of air, water and soil treatment, and recovery of the compounds to reuse in some process.

1.1.1 Aromatic compounds

Next to glucosyl residues, the benzene ring is the unit of chemical structure most widely spread in nature. Moreover, the thermodynamic stability of the benzene ring increases its persistence in the environment; therefore many aromatic compounds are major environmental pollutants [5]. On the other hand, polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) are compounds whose physical/chemical properties led to their widespread commercial use. Although their production has been banned or severely limited in most countries since the 1970s, the persistence and stability of these compounds have resulted in a worldwide distribution, especially of PCBs [6].

1.1.2 Azo dyes

Azo dye represent the largest class of organic colorants listed in the Color Index and their relative share among reactive, acid and direct dyes is even higher, it can be expected that they make up the vast majority of the dyes discharged by textile-processing industries. Characterized by azo (N=N) bridges linking substituted aromatic structures [7]. It is estimated that 10 to 40% of the dyes used for textiles dyeing end up in the wastewater. This fraction has increased over the last decades because of the increasing use of reactive dyes, a class of water-soluble dyes, with a relatively low degree of fixation.

Textile-processing wastewaters, typically with a dye content in the range 10-200 mg/L, are therefore usually highly colored and discharge in open waters presents an aesthetic problem. As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments. The release of dyes may therefore present an ecotoxic hazard and introduces the potential danger of bioaccumulation that may eventually affect man by transport through the food chain [8]. Table 1 shows typical parameters of a textile wastewater contaminated with azo dyes.

Table 1. Textile industry wastewater typical parameters.

Parameters	Range
pH	7.0 – 9.0
Biochemical oxygen demand (mg/L)	80 – 6,000
Chemical oxygen demand (mg/L)	150 – 12,000
Total suspended solids (mg/L)	15 – 8,000
Total dissolved solids (mg/L)	2,900 – 3,100
Chloride (mg/L)	1000 – 1,600
Total Kjeldahl Nitrogen (mg/L)	70 – 80

Generally, the process of bacterial azo dye biodegradation consists of two stages. The first stage involves reductive cleavage of the dyes azo bond, resulting in the formation of aromatic amines, which is generally colorless but potentially hazardous. The second stage involves degradation of the aromatic amines under aerobic conditions.

Anaerobic bio-reduction of azo dye is a nonspecific and presumably extracellular process and comprises of three different mechanisms by researchers (Figure 1), including the direct enzymatic reduction, indirect/mediated reduction and chemical reduction. A direct enzymatic reaction or a mediated/indirect reaction is catalyzed by biologically regenerated enzyme cofactors or other electron carriers [9].

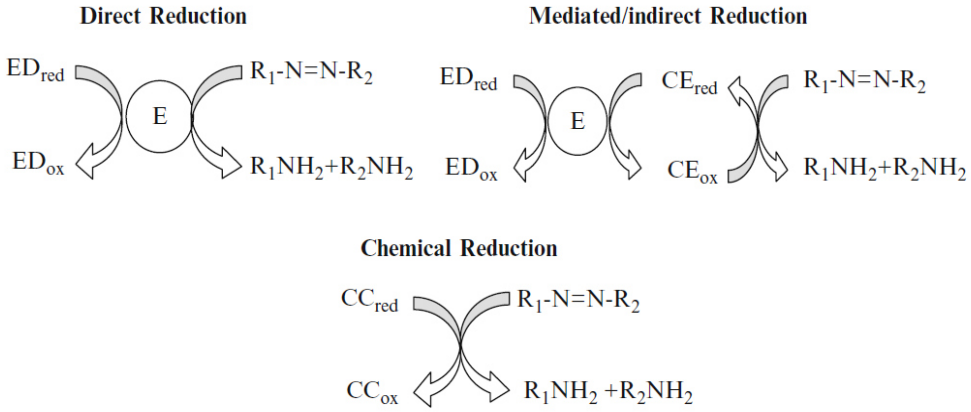


Figure 1. Different bio-reduction mechanism.

Under aerobic conditions, aerobes has so far been only found in studies capable of reducing azo compounds and produce aromatic amines by specific oxygen-catalyzed enzymes called azo reductases. These aerobic bacteria could grow with mostly simple azo compounds as solo source of carbon and energy and under strict aerobic conditions by using a metabolism

that started with reductive cleave of the azo linkage. Nevertheless, those dyes are generally persistent due high stability of azo linkage [10, 11].

Under anaerobic or anoxic conditions, the azo bond (-N=N-) cleavage is conducted by specific enzymes (catalyzing only the reduction of azo dyes) or non-specific enzymes (catalyzes the reduction of a wide range of compounds, including azo dyes). However, there is no clear evidence for the specific azoreductases in anaerobically grown bacteria. Further research shows that one of responsible enzymes it was flavoprotein capable of catalyzing the reduction of azo dyes as well as nitroaromatics [2]. The azo bond (-N=N-) cleavage proceeds through two stages, involving a transfer of four electrons (reducing equivalents). In each stage two electrons are transferred to the azo dye, which acts as a final electron acceptor (Figure 2). The rate of decolorization is dependent on the added organic carbon source, as well as the dye structure. But there is no correlation between decolorization rate and molecular weight, indicating that decolorization is not a specific process and cell permeability is not important for decolorization. Nonetheless, aerobic digestion or advance oxidation is required after the decolourisation to achieve mineralization of the by-products of dye reduction [12, 13].

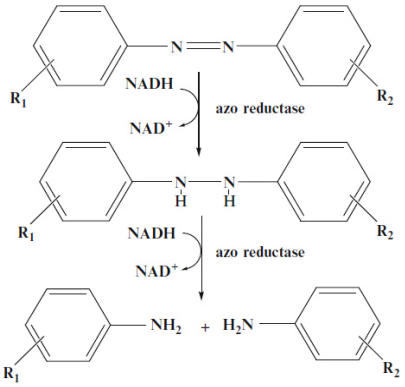


Figure 2. Anaerobic azo dye reduction mechanism.

1.2 Wastewater treatment

A number of wastewater treatments have been studied to achieve mineralization, degradation or transformation of recalcitrant pollutants. However, these treatments lead to an expensive process and unviability for application in continuous process. Nonetheless, research focused on find new ways for wastewater treatment and their possible application onto elimination of recalcitrant pollutants.

1.2.1 Advanced oxidation

Advanced oxidation process (AOPs) refers to a set of chemical treatment procedures designed to remove organic materials in wastewater by oxidation trough reaction with hydroxyl radicals (·OH) [14]. Table 2 shows the comparison of electrochemical potential for some species.

Table 2. Comparison of various electrochemical potential.

Oxidizing Agent	Oxidation Potential (V)
Hydroxyl radical	2.80
Oxygen (atomic)	2.42
Ozone	2.08
Hydrogen peroxide	1.78
Hypochlorite	1.49
Chlorine	1.36
Chlorine dioxide	1.27
Oxygen (molecular)	1.23

These reactive species (·OH) are the strongest oxidants that can be applied in water and can virtually oxidize any compound present in the water matrix, often at a diffusion controlled reaction speed. Consequently, ·OH reacts unselectively once formed and contaminants will be quickly and efficiently fragmented and converted into small organic molecules. Hydroxyl

radicals are produced with the help of one or more primary oxidants (e.g. hydrogen peroxide, ozone) and/or energy source (e.g. ultraviolet light, Iron (III)) or catalyst (e.g. titanium dioxide)[15]. A list of the different possibilities offered by advance oxidation process is given in the Figure 3.

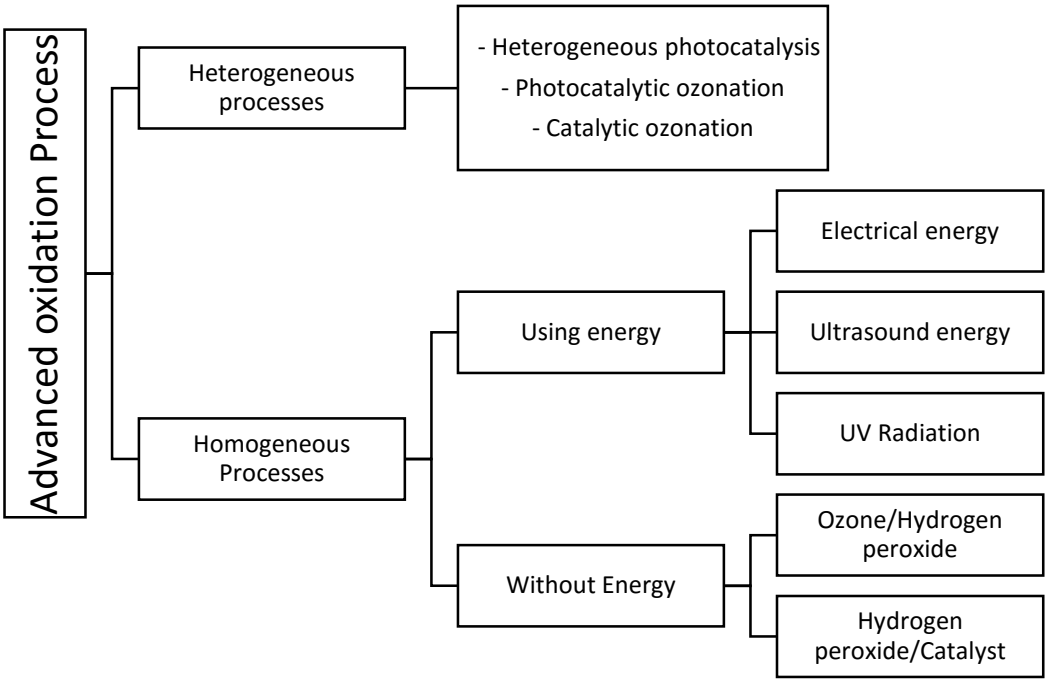


Figure 3. Advance oxidation technologies for wastewater treatment.

AOPs seems to be an excellent technology, nonetheless it present some disadvantages (Table 3). These have been employed for decolourisation with good results. Among AOPs, heterogeneous photocatalysis using TiO_2 as photo-catalyst appears as the most emerging destructive technology. The great part of the studies on the photocatalytic degradation of azo dyes relies only on the monitoring of solution decolorization, TOC or COD and inorganic ions. Monitoring the disappearance rate of the target dyes is not the most appropriate parameter to classify the efficiency of this process. Only few studies reported thorough

mechanisms with detailed reaction steps of the different pathways leading to several photoproducts [16-19].

Table 3. The advantage and disadvantages of advance oxidation processes.

Advantages	Disadvantages
-Rapid reaction rates -Small foot print -Potential to reduce toxicity and possibly complete mineralization of organics treated -Does not concentrate waste for further treatment with methods such as membranes -Does not produce materials that require further treatment such as "spent carbon" from activated carbon adsorption -Does not create sludge as with physical chemical process or biological processes	-Capital intensive -Complex chemistry must be tailored to specific application -For some application quenching of excess peroxide is require

1.2.2 Aerobic digestion

Aerobic digestion is the biochemical oxidative stabilization of wastewater sludge in open or closed tanks that are separate from the liquid process system. This method of digestion is capable of handling waste activated, trickling filter, or primary sludge as well as mixtures of the same. The aerobic digester operates on the same principles as the activated sludge process. As food, is depleted, the microbes enter the endogenous phase and the cell tissue is aerobically oxidized to CO_2, H_2O, NH_4^+, NO_2^- and NO_3^- [20].

Aerobic digestion of municipal wastewater sludge is based on the principle that, when there is inadequate external substrate available, microorganisms metabolize their own cellular mass. In actual operation, aerobic digestion involves the direct oxidation of any biodegradable matter and the oxidation of microbial cellular material by organisms. These two steps are illustrated by the following reactions [21].

Synthesis and oxidation

$$COHNS + O_2 + nutrients \xrightarrow{bacteria} CO_2 + NH_3 + C_5H_7NO_2 + other\ products$$

Endogenous respiration

$$C_5H_7NO_2 + 5O_2 \xrightarrow{bacteria} 5CO_2 + 2H_2O + NH_3 + energy$$

Some advantages have been claimed for aerobic digestion over other stabilization techniques particularly anaerobic digestion [22]. Based on all current knowledge, the following advantages and disadvantages can be cited for properly design and operated aerobic digestion processes [23 – 27]. As we mention, enzymes generated like azo-reductases on aerobic metabolism can enhance degradation of azo dye; several studies focal on isolated yeast strain from activated sludge for azo dye degradation with high efficiencies in a couple of hours [28-32]. Nevertheless extraction of yeast from activated sludge could cause a lot of operational problems on a full-scale application.

1.2.1 Anaerobic digestion

Anaerobic digestion refers to the anaerobic decomposition of organic matter, resulting in partial gasification, liquefaction, and mineralization. Anaerobic processes are used to treat strong organic wastewaters (Biochemical oxygen demand > 500 mg/L) and for further treatment of primary and secondary sludge from conventional wastewater treatment. Due the wastewater treatment and the potential for generating methane, anaerobic processes

become of interest for sustainability and energy conscious society. Research in this area has been suitable for the application of a renewable energy due a biodegradation of pollutants. Anaerobic treatment of wastewaters has been receiving added attention in recent years. In addition to methane production, some advantages and disadvantages for the anaerobic treatment process are summarized in Table 4.

Table 4. The advantage and disadvantages of anaerobic treatment.

Advantages	Disadvantages
-Low operational costs	-Generally require heating
-Low sludge production	-Long retention time required (>24h)
-Reactor sealed giving no odour or aerosols	-Corrosive and malodorous compounds
-Sludge is highly stabilized	produced during anaerobiosis
-Biogas are produced as end product	-Not as effective as aerobic stabilization for
-Low nutrient requirements due to lower	pathogen destruction
growth rate of anaerobes	-Hydrogen sulphide also produced
-Rapid start-up possible after acclimation	-Reactor may require additional alkalinity
	-Only used as pretreatment for liquid wastes

Anaerobic degradation occurs in the absence of oxygen with methanogens adversely affected by even trace levels of oxygen. Anaerobic biomass can form a granular sludge under certain circumstances and when this occurs the methanogens are protected from the effect of oxygen inside the granules. The basic difference between aerobic and anaerobic oxidation is that in the aerobic system, oxygen is the ultimate hydrogen acceptor with a large release of energy, but in anaerobic system the ultimate hydrogen acceptor may be nitrate, sulphate or an organic compound with a much lower release of energy.

The process is generally considered to be a two-stage biological process involving waste conversion and stabilization. However, three general stages involve the decomposition of contaminants and are listed as follows:

• Hydrolysis: Particulate matter is converted into soluble compounds which can be subsequently hydrolyzed to simple monomers.

• Fermentation (acidogenesis) amino acids, sugars and fatty acids are some volatile degraded; organic substrate serves as the electron donor and electron acceptor. The major products of fermentation are acetate, hydrogen, carbon dioxide, and propionate and butyrate.

• Methanogenesis: It is carried out by methanogenic organisms. Two groups of organisms are related to the production of methane. The first acetoclastic methanogens divides acetate to methane and carbon dioxide. The second group uses hydrogen as electron donor and carbon as an electron acceptor to produce methane. Bacteria in the anaerobic process are capable of using carbon dioxide to oxidize the hydrogen and form acetic acid.

A limited number of substrates are used by methanogenic organisms and the reactions that occur in the process are:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 2H_2O + 3CO_2$$

$$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$$

$$4CH_3OH \rightarrow 3CH_4 + 2H_2O + CO_2$$

$$4(CH_3)_3N + H_2O \rightarrow 9CH_4 + 6H_2O + 3CO_2 + 4NH_3$$

$$CH_3COOH \rightarrow CH_4 + CO_2$$

A COD balance can be used to determine changes in concentration during fermentation; loss of COD in the anaerobic reactor is accounted for by the production of methane. The

COD of methane is the amount of oxygen necessary to oxidize all the methane to carbon dioxide and water.

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$$

1.3 Redox mediators

Extensive research has been conducted to explore the catalytic effects of different electron shuttles on redox (bio) transformation processes. Electron shuttles, also referred to as redox mediators (RM), are organic molecules that can reversibly be oxidized and reduced, thereby conferring the capacity to serve as an electron carrier in multiple redox reactions. RM accelerates reactions by lowering the activation energy of the total reactions, also decrease toxicity of pollutants that inhibited anaerobic consortium. In some cases, the presence of RM may even need it for the reaction to take place [33]. RM is capable of transferring electrons in redox reactions between a wide variety of both inorganic and organic compounds. Reduction of RM can be promoted by chemical reaction with reductant commonly found in many anaerobic environments [34, 35] or by electron donors [36 – 38]. The most relevant RM that has been used in research and application are humic substances and their quinoid analogues. For a RM to be an effective electron shuttle, its standard redox potential (E_0') should ideally be in between those of the two eventual half reactions. Nevertheless, as the RM's efficacy will also depend on the activation energy of its reduction and oxidation, in comparison to that of the non-mediated total reaction, it may occur that even compounds with E_0' lower than the primary electron donor or higher than the terminal electron acceptor may also be effective RM.

Some physical-chemical properties of the shuttling compound may play an important role in determining its feasibility and strength as mediator for electron acceptor contaminants (EAC). These properties are list as follows:

- Redox potential
- Ability to cross cell membrane
- Solubility
- Temperature
- Electron donors
- Trophic groups and selective inhibitors

1.3.1 Humic substances

Humic substances constitute a very abundant class of organic compounds that are chemically heterogeneous and widely distributed in terrestrial and aquatic environments. These substances are generally considered recalcitrant because of their remarkable stability in the environment [39]. For instance, high molecular weight humic polymers have residence time longer than 500 years [40]. However, humus play different roles in the carbon and electron flown in anaerobic environments, evidences indicated that humic substances might be important electron acceptors for microbial respiration. Moreover, humic substances can also serve as electron donors for anaerobic respiration, it means as redox mediator [41-43]

1.3.2 Model quinones

Quinone moieties are the most likely candidate for the redox reactions observed in humic substances. Electron accepting capacity is correlated with quinone content in humus [44]. Therefore, quinone model compounds should be able to replace the function of humus as terminal electron acceptor. Also, quinones can enhance (bio) transformation of EAC as azo dyes, nitroaromatic compounds, polychlorinated and heavy metals, trough electron transport due electron external source (electron donor) and those contaminants. An schematic reaction mechanism is show in Figure 4.

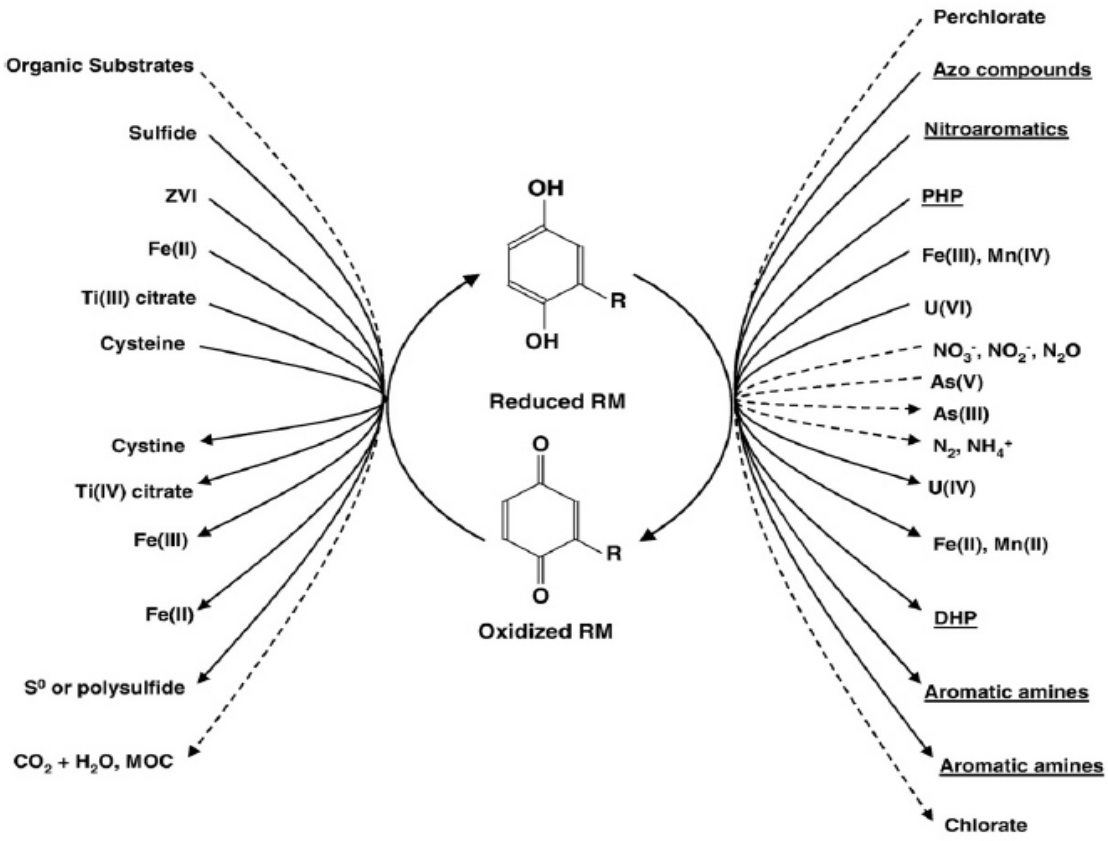


Figure 4. Abiotic and microbial reactions involved in the reduction and oxidation of electron shuttles.

1.4 Immobilization

The immobilization of enzymes is a process in which is confined or localized the enzyme in a defined region, to result in insoluble forms which retain their catalytic activity and which can be reused repeatedly [45]. This definition has been extended to that process by which are restricted, wholly or partially, the degrees of freedom of movement enzymes, organelles, cells, redox mediator, etc. Some advantage of employing immobilization list as follows:

I. Enhance stability
II. Possible reutilization
III. Possibility of ease management and control, enzymatic reactor design

From the other hand, some disadvantage has presented as follows:

I. Enzyme properties modification
II. Loss of catalytic activity
III. Expensive biocatalyst

There are some methods of immobilization that are divided in two great genders: physical retentions and binding support.

1.4.1 Physical retention

Entrapment

Involves the physical retention of the enzyme in the interior cavities of a solid porous matrix, generally constituted by polymers of polyacrylamide type, collagen, alginate or

polyurethane resins. The immobilization process is carried out by suspending the enzyme in a monomer solution. Next, polymerization is initiated by a temperature shift or the addition of a chemical reagent. Trapping can be gels or fibers, which are usually more resistant than gels. In the first case, the enzyme is entrapped within a gel, whereas in the second case the enzyme is entrapped within the microcavities of a synthetic fiber. Entrapment of great simplicity from the experimental point of view, little amount of enzyme required to obtain active derivatives. As a bonus, the enzyme does not undergo any change in its structure. Anyway, entrapment requires strict control of the polymerization conditions, as well as verification that the chemical nature of the process does not alter the reactive groups of the protein [46, 47].

Membrane inclusion

a. Microencapsulation

In this technique, the enzymes are surrounded by semipermeable membranes which allow passage of molecules of substrate and product, but no enzyme. These semipermeable membranes can be permanent (caused by interfacial polymerization) or permanent (generated by surfactants, also called "reverse micelles"). The microcapsules obtained are spherical in shape, with sizes ranging from 1 to 100 microns in diameter. By using this method may simultaneously encapsulate a variety of enzymes, cells or biomolecules, allowing it to carry out certain reactions that occur in multiple steps [48].

b. Membrane Reactors

The development of enzyme reactors or systems containing entrapped enzymes has aroused great interest in the industry. These reactors employ permeable membranes at the end, or not permeable to the initial and obviously impermeable substrate to the enzyme product. By a pump a liquid flow of substrate through the reactor is established.

In general, in this method, initially comes to adsorption of the enzyme to the membrane which forms the reactor. This adsorption can be performed in two ways:

1) By the passage of a buffered enzyme solution through the membrane;
2) By continuous contact of an enzyme solution with the membrane.

1.4.2 Binding support

The methods of immobilization by binding support are the most used and what is more information available. The choice of support and the type of binding are critical in the subsequent behavior of the biocatalyst. Care should be taken that immobilization increases the affinity for the substrate, decrease inhibition, expand the range of optimal pH and reduce possible contamination of reactor operation and easily separable from the liquid medium so that it can be reused. A wide variety of materials have been used as supports for the immobilization of many enzymes. These materials differ in size, density, porosity and shape, but generally we find them in the form of cylinder, sheets, fibers, and most commonly in the form of spheres. The supports can be classified into two groups:

1) Inorganic supports as metal oxides, silica, alumina, zeolite, etc.
2) Organic supports as chitin, chitosan, cellulose, starch, etc.

a. Adsorption

The biocatalyst is bound to a support via ionic interactions without functionalization, Van der Waals forces and hydrogen bonding. The main factors influencing the adsorption are:

1 pH: controls the number and nature of the charges presented on the surface of the protein and the solid;

2 Ionic strength: by increasing the ionic strength of the enzyme desorption occurs, since inorganic ions bind more tightly to the protein carrier;

3 Pore Diameter: should be about twice the size of the major axis of the enzyme;

4 The presence of ions that act as enzyme cofactors, since they can increase the charge of the derivative enzyme.

b. Covalently bond

Covalent binding of a biocatalyst to a carrier is perhaps the most interesting method of immobilization since the industrial viewpoint. The method of covalent attachment is based on chemical activation of groups of the support to react with nucleophiles of the biocatalyst. This immobilization technique presents some advantages:

1. Easy manipulation of immobilized derivatives
2. Immobilized specie is susceptible to ionization
3. Easy application on continuous processes

Also, some disadvantages have been observed:

1. Poor dispersion, which lead to inactive sites
2. Immobilization may take place on the active site

c. Cross-linking

The crosslinking method is to use bi-functional reagents which cause crosslinks between enzyme molecules. The results of the lattice are enzymes with irreversible intermolecular bonds can withstand extreme conditions of pH and temperature. The crosslinking strategy eliminates losses due to diffusional effects enzyme activity by crosslinking enzyme with a protein without enzymatic activity and rich in lysine residues (*e.g.* bovine albumin). A composite common immobilization procedure is to immobilize the enzyme by adsorption on an ion exchange resin or a polymeric support, and subsequently adding the bi-functional reagent. The increased stability is based on obtaining a crystal lattice, where the enzyme molecules are surrounded only by other protein molecules. In this way the enzyme itself acts as a support, and its tertiary structure is stabilized by intermolecular covalent linkages. In summary, Table 6 shows the main features that differentiate the various methods of immobilization and figure 5 illustrate the enzyme immobilization methods.

Table 6. Comparison between enzyme immobilization methods.

Method	Membrane inclusion	Entrapment	Cross-linking	Adsorption	Covalent bond
Preparation	Intermediate	Advanced	Intermediate	Simple	Advanced
Ionic strength	weak	medium	Medium weak	medium	Strong
Catalytic activity	Medium-high	Low	Low	Medium	High
Regeneration	Possible	Impossible	Impossible	Possible	Hard
Cost	Expensive	Medium	Medium	Cheap	Expensive
Stability	Medium	High	High	Low	High
Microbial resistance	Yes	Yes	Yes	No	No

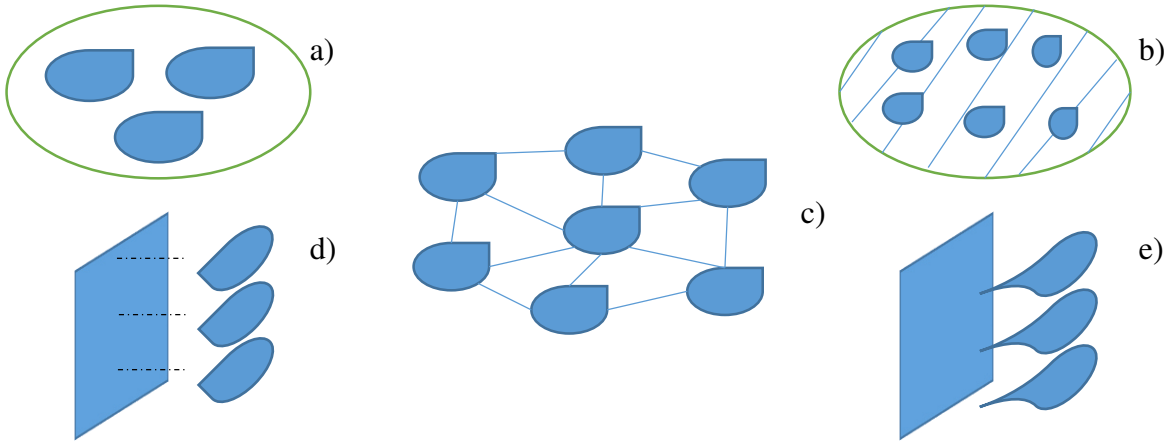


Figure 5. Immobilization methods: a) Membrane inclusion; b) Entrapment; c) Cross-linking; d) Adsorption; e) Covalent bond.

2. Background

2.1 Application of redox mediator for anaerobic treatment

For almost two decades the role of humic substances and model quinones has been studied for anaerobic biotransformation of EAC, by humus reducing bacteria. As we mentioned, redox mediator increase the rate of electron transfer from EAC by several orders of magnitude, decreasing the toxic effects due to an adsorption process and, in some cases, are required to be carried out the reaction [49]. Has been reported that different quinoid compounds serve as redox mediator; Lawsone (LQ) and anthraquinone-2,6-disulfonate (AQDS) are the most used model quinones as electron shuttles. Additionally, those redox mediators considerably increase the rate of the anaerobic reduction of azo dyes yielding high conversions (X ~ 0.90) during acclimation period. It was demonstrated that depending of the bacteria consortium, the redox mediator activity will be enhanced in different ways [50]. On the other hand, Riboflavin also has been employed as redox mediator for accelerating the reduction of azo compounds by anaerobic granular sludge. It displayed saturation kinetics at higher concentrations leads a rate of reaction 2-fold compared to the control without redox mediator [51], which has a poor redox activity unlike AQDS or LQ. Nonetheless, continuous addition of redox mediator could increase COD and toxicity of wastewater and finally lead to the inhibition of microorganism, which can cause the collapse of bioreactor.

2.2 Carbon materials as solid-phase redox mediator

Nevertheless, research of synthesis of a solid phase redox mediator has taken priority. Van der Zee was the first to apply granular activated carbon (GAC) as redox mediator for anaerobic reduction of azo dyes. But, the effect of quinone groups present on GAC surface on dye reduction was inefficient compared with the adsorption process the first 4 weeks [52]. Pereira *et al.* (2010), selectively modified GAC by chemical oxidation with HNO3 and O2, and thermal treatments under H2 and N2 flow, being the thermal treatment under H2 the fastest rate of decolorization, due reduced Surface sites of activated carbon. Batch experiments with low amount of GAC (0.1 g/L) demonstrated an increase of the first order rate of reduction, up to 9-fold, as compared with the control experiments without activated carbon. Also, reaction rate was independent of the amount of activated carbon concentration in the tested range (0.1 – 0.6 g/L), this could be attributed to high porosity and mass transfer limitations in the adsorption step of azo dyes [53]. Moreover, Amezquita *et al.* (2013) used activated carbon fibers (ACFs) as redox mediators in the anaerobic reduction of nitroaromatic compounds. The results indicate that ACFs are necessary to reduce nitroaromatics. Furthermore, the modification of ACFs highly improved its redox properties, increasing the concentration of quinone groups (1.68 times), and therefore the redox capacity (up to 1.38 times) [54]. Nevertheless, Rios-Del Toro et al (2013) observed that chemical oxidation of ACFs, increase adsorption capacity over redox mediating capacity, being adsorption process that plays major role on decolorization of methyl red [55]. Also, the application of carbon nanotubes as redox mediator was studied by Pereira *et al.* (2014), observing an increasing rate of decolorization, but the high cost and the mass

transfer limitations in continuous processes become its application unavailable [56]. Recently, graphene oxide (GO) has took interest in different research areas for it physical-chemical properties; Colunga *et al.* (2015) reported for the first time GO as an electron shuttle to increase redox activity of azo dye (reactive red 2) and nitroaromatic (3-chlronitrobenzene) reduction increasing 10-fold and 7.6-fold respectively. Characterization of GO shows that it has proper size distribution and redox potential to promote reduction of EAC [56].

2.3 Immobilization of redox mediator

On the other hand, the search of a support material biological inert for RM immobilization has taken interest the last ten years. Entrapment immobilization of AQDS on calcium alginate was conducted successfully by increasing the reaction rate of the denitrification process 2 fold compared to the control lacking RM. Repeated-batch operations show that AQDS immobilization beads appeared to exhibit a good reuse. However, some disadvantage like mass transfer limitations due RM is embedded within the polymeric material making its accessibility dependent on diffusion; gradual loss of redox activity due to long-term wash-out from bioreactors and disruption of calcium alginate owing to weak mechanical stability [57]. Furthermore, functionalized polypyrrole composites were prepared by incorporation of AQDS, as doping anion during the electropolymerization of pyrrole monomer on active carbon felt electrode. The resulting composite, ACF/PPy/AQDS, was employed on biotransformation of nitroaromatic compounds, increasing rate of reduction of 5 time higher than control without RM. Additionally, bio-reduction of azo dye reach an increment

between 1.3-2 fold on the rate of decolorization in relation to the control without composite. Nonetheless, the difficult of preparation make hard it production for industrial application [58]. On the other hand, adsorption immobilization through anion exchange resin has demonstrated easy preparation, reaching 1.87 mmol AQDS/g of adsorption capacity, maintaining its catalytic activity in batch studies. Compared to the treatment without RM, an increase on the rate of reduction of 8.8-fold was observed for methyl orange decolorization. Nevertheless, the immobilization capacity is highly dependent on the physical properties of resin. In addition, desorption of quinone molecules may occur when catalyst are exposed to high concentration of anions, due adsorption competition with AQDS, and operation temperature above 25°C [59]. Also, loss of redox activity of immobilized RM could be occurring for some reasons:

1. There is no interaction between RM active site and EAC
2. Blocked quinone groups
3. Detachment or deactivation of RM, due operational conditions

In contrast, increment or decrement of activity could be attributed to internal or external diffusional resistance, electrostatic forces and steric hindrance. Therefore is important to achieve strong immobilization and to consider its reactivation when activity becomes insufficient for the treatment.

Therefore, Alvarez *et al.* immobilized humic substances and AQDS on metal oxide nanoparticles by physical adsorption with high stability for anaerobic biotransformation of polyhalogenated pollutants and aromatic compounds. Nevertheless, inhibition of

microorganism due metal toxicity carries on a poor efficiency of anaerobic reduction process [60]. Since the last year some studies of covalent immobilization of redox mediators on a biologically inert support, showed an improvement activity and stronger immobilization for later use in continuous processes. AQS chlorinated reagent was employed for chemical adsorption on polyurethane foam and polyethylene terephthalate, making covalent bond due a nucleophilic substitution mechanism [61, 62]. Additionally, Amezquita *et al.* modified activated carbon fibers with thyonil chloride, for chlorination of phenolic groups and, consecutively, anchorage of AQS molecules reaching about 8% (w/w) of immobilization [63]. Nevertheless, the high cost of reagents, complicated and expensive modification of adsorbents make scale up immobilization unavailable.

2.4 Mechanism of anaerobic reduction with redox mediators

In addition, just a few researchers have reported complete anaerobic azo dye reduction. As discussed earlier, azo biological decolorization are mainly reduced in a direct reduction or mediated/indirect reduction with nonspecial azo reductase or reduce enzyme cofactors. According to the direct enzymatic reduction mechanism, nonspecial azo reductase can catalyze the transfer of reducing equivalents originating from the oxidation of original electron donor in the azo dyes. The acceleration mechanism of RM is presumed by van der Zee [2]. RM as reductase or coenzymes catalyzes reaction by lowering the activation energy of the total reaction. Artificial RM such as AQDS, can accelerate both direct enzymatic reduction and mediated/indirect biological azo dye reduction (Figure 6). In the direct

reduction, the accelerating effect of RM will be due to RM enzymatic reduction in addition to enzymatic reduction of the azo dye. In the mediated reduction, the accelerating effect of RM will either be due to an electron shuttle between the reduced enzyme cofactor and RM or be due RM enzymatic reduction in addition to enzymatic reduction of the coenzymes. In the latter case, the addition of RM simply increases the pool of electron carriers [64].

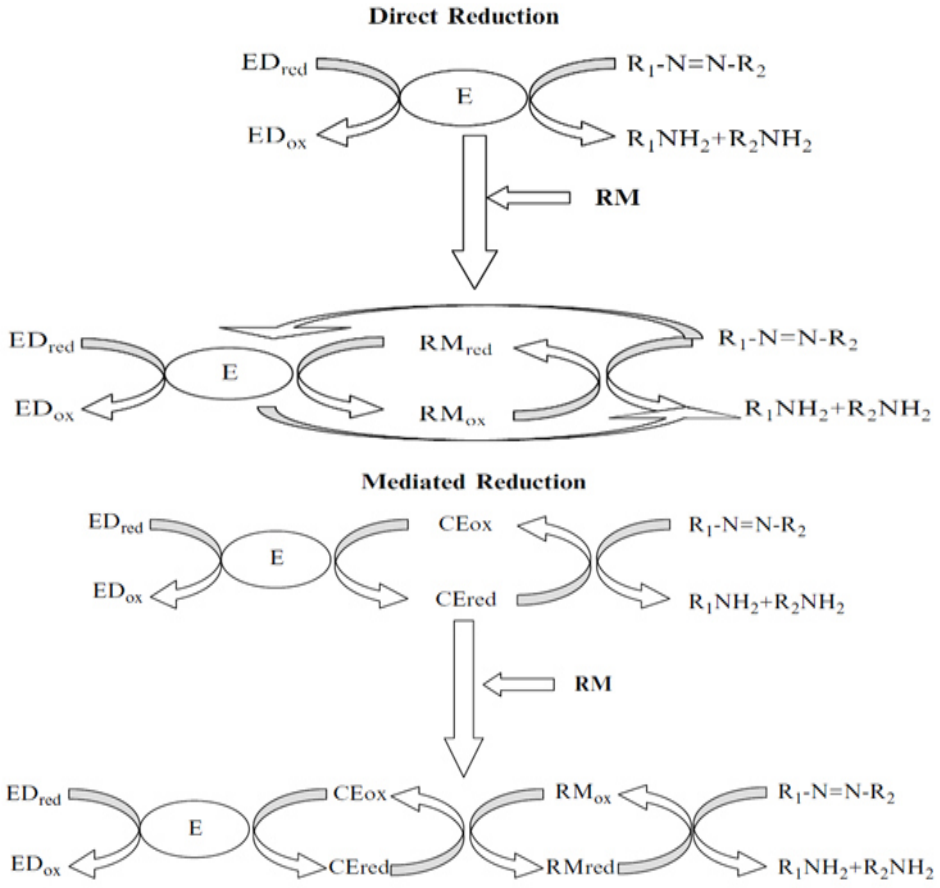


Figure 6. The presuming accelerating mechanism of redox mediators.

Additionally, Martinez *et al.* (2013) have showed the reduction mechanism of mono-azo dye (reactive red 2) mediated by AQDS supported on anion resins, leading to a production

of aniline [59]. Also, Amezquita *et al.* (2014) presented the reduction of the same dye mediated by AQS bound on ACFs, identifying aniline as one of by-products by HPLC analysis of the anaerobic catalytic reduction [53]. It is important to know the biotransformation pathway of azo dyes to choose the best treatment, economically and sustainability, for the complete degradation of those pollutants.

Carbon materials seem to be a suitable option for its application as redox mediator or support material for immobilization. Also, exploration of other quinone molecules like LQ has not been studied for it immobilization. In the best of our knowledge, there is no evidence of catalytic reduction mechanism of poly-azo dye mediated by RM, considering the rate of decolorization and rate of production of aromatic amines to attribute the controlling stage of the reaction.

The aims of the research are:

1. To propose an easy way to achieve covalent immobilization of redox mediator,
2. To compare the catalytic potential of immobilized RM and its control without RM on the decolorization of congo red dye at typical concentration of dye textile wastewater, and
3. To elucidate the catalytic mechanism of complete anaerobic azo dye reduction.

Methodology

3.1 Materials and reagents

Granular activated carbon (GAC) for support of redox mediator was obtained from mineral source (CLARIMEX), particle diameter was in the range 0.5 – 1 mm. Congo red dye, benzidine, LQ and AQDS were obtained from Sigma Aldrich (85% of purity).

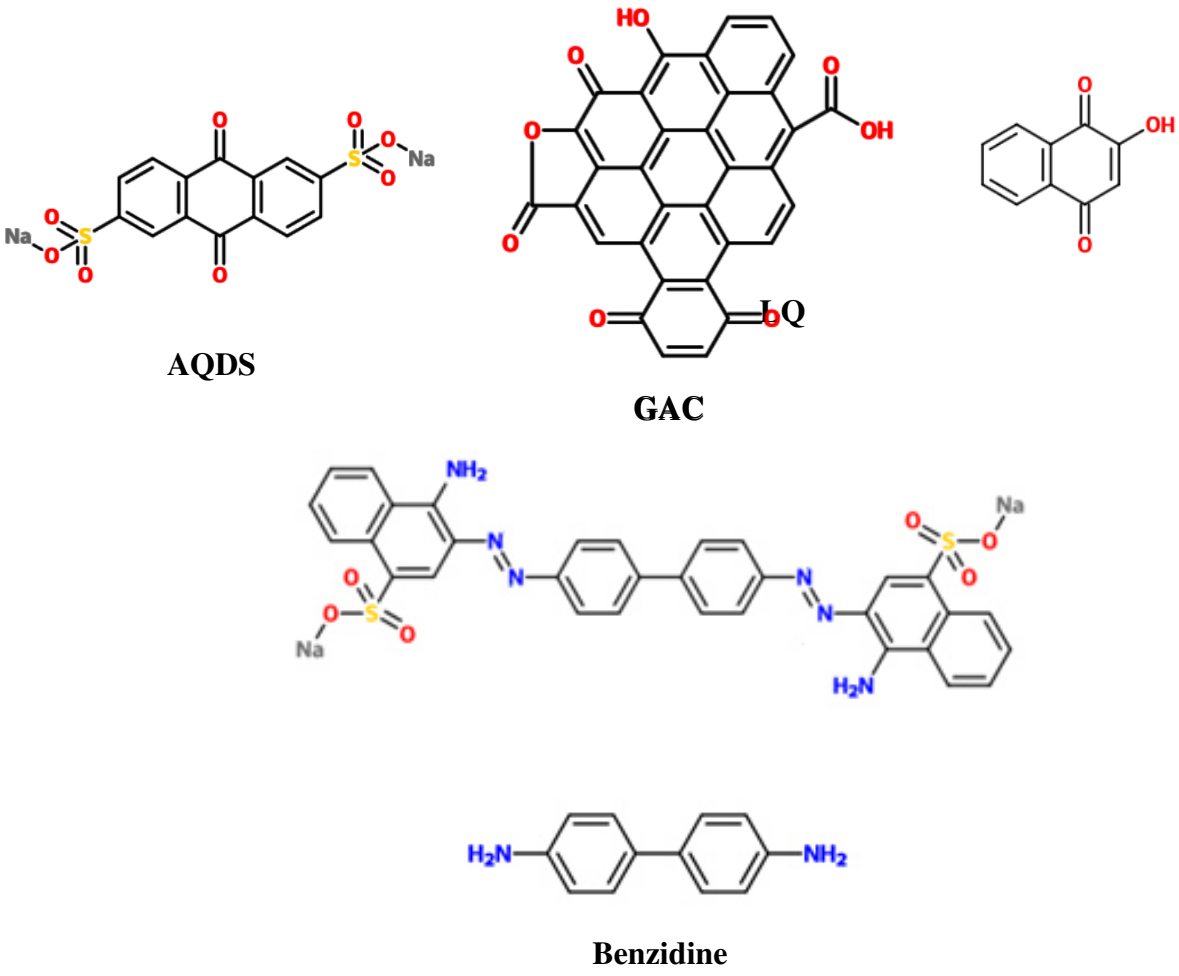


Figure 7. Chemical structure of AQDS, GAC, LQ, Congo red and Benzidine.

3.2 Inoculum and basal medium

Anaerobic granular sludge for batch and continuous experiments was obtained from an UASB anaerobic digester from a brewery industry (Cd. Obregón, Sonora, México). Granular sludge was activated before batch and continuous experiments with 1 g/L of glucose as energy source. The basal medium for the activation of anaerobic sludge was prepared as following composition (g/L): $NaHCO_3$ (1.68); NH_4Cl (0.3); KH_2PO_4 (0.2); $MgCl_2$•6H2O (0.03); $CaCl_2$ (0.1); and 1 mL/L of trace elements solution. The trace elements solution contained (mg/L): $FeCl_2$•$4H_2O$, (2000); H_3BO_3, (50); $ZnCl_2$, (50); $CuCl_2$•$2H_2O$,(38); $MnCl_2$•$4H_2O$ (500); (NH4)6Mo_7O_{24}•$4H_2O$, (50); $AlCl_3$•$6H_2O$, (90); $CoCl_2$•$6H_2O$, (2000); $NiCl_2$•6H2O, (92); Na_2SeO•$5H_2O$, (162); EDTA, (1000); and 1 mL/L of HCl (36%). The pH was buffered with the amount of bicarbonate added and head-space composed of N_2.

3.3 Effects of congo red and quinoid redox mediators on anaerobic sludge

Batch incubations were conducted in glass serum bottles of 120 mL. A first set of assay was conducted to evaluate the inhibitory effect of congo red on anaerobic sludge by evaluating the biogas production and decolorization. The microbial consortium was exposed to different concentrations (0.1, 0.5, 1.0, and 2.0 mM) of congo red. The second set of assays consisted in evaluating the adverse effect of LQ and AQDS on biogas production by the anaerobic sludge. The tested concentrations of LQ and AQDS were 4.0, 8.0, and 12 mM. In these two assays the working volume was 80% of the total volume of serum bottle and glucose was provided as energy source at 2 g/L. Finally, a third set of assays was conducted to assess the catalytic effect of LQ and AQDS (0.01, 0.05, 0.1, 0.5 and 1 mM), acting as RM,

on microbial decolorization of congo red. After to add congo red, the bottles were pre-incubated during 24 h to promote an initial reduction of quinoid compounds, then ~150 mg/L of the azo dye was dispensed in each bottle. Moreover, the cultures received a pulse of glucose of 1 g/L in both pre-incubation and incubation period. In all cases, anaerobic conditions were established by flushing the headspace of bottles with nitrogen (N_2), after to seal them with rubber stoppers and aluminum crimps. The bottles were placed in a shaker at 30 °C and 150 rpm. Decolorization of congo red was spectrophotometrically measured at their maximum wavelength of visible absorbance at 485. Samples (0.5 mL) were centrifuged and diluted up to an absorbance of less than 1 in a phosphate buffer (10.86 g/L $NaH_2PO_4 \bullet 2H_2O$; 5.38 g/L $Na_2HPO_4 \bullet H_2O$). The biogas volume was determined by liquid displacement using a 2% NaOH solution to solubilize the CO_2 produced. The VSS content of anaerobic sludge was determined according to Standard Methods [66].

3.4 Surface Characterization of GAC

Boehm titration has been used as a chemical method to identify surface groups on carbon materials [67]. The Boehm titration works on the principle that oxygen sites surface have different acidities and can be neutralized by bases of different strengths. Sodium hydroxide (NaOH) is the strongest base generally used, and is assumed to neutralize all Bronsted acids (phenolic, lactonic and carboxylic groups), while sodium carbonate (Na_2CO_3) neutralizes carboxylic and lactonic groups and sodium bicarbonate ($NaHCO_3$) neutralizes carboxylic groups, whereas sodium ethoxide ($NaOC_2H_5$) will react with all oxygen species, even extremely weak acids [68].

Boehm test were conducted in 50 mL falcon tube using 30 mL NaOH, $NaOC_2H_5$, Na_2CO_3, $NaHCO_3$ and HCl 0.1 N solution with 300 mg of activated carbon respectively during 3 days at 150 rev/min and 30°C. Next, the solution was filtrated and 10 mL aliquot was potentiometric titrated with corresponding solution for neutralization (HCl or NaOH 0.1 N) of residual solution to calculate acidic and basic sites of activated carbon.

3.5 Adsorption equilibrium of azo dyes

GAC congo red dye (Figure 6) adsorption capacity (Qe) was determined at different pH values in triplicate and the average values are presented. Samples of 30 mg of GAC, LQ-GAC and AQDS-GAC were added separately to 30 mL of congo red concentrations of 50 to 200 mg/L. These experiments were continuously stirred at 150 at 30 °C until equilibrium was achieved. Aliquots of 3 mL were taken to measure the initial and final congo red concentration by UV-vis spectroscopy at 485 nm.

3.6 Immobilization of Redox Mediator

LQ and AQDS were immobilized using the adsorption equilibrium technique in order to determine the adsorption capacity of GAC. Sorption capacity (Qe) were determined at different pH (3, 4, 5 and 7) in triplicate and the average values are presented. Samples of 30 mg of GAC were added to 30 mL of lawsone and AQDS concentrations of 50 to 200 mg/L. These experiments were continuously stirred at 150 rpm at 30 °C for 24 hours. The solution pH was initially adjusted by adding 1.0 M HCl. Aliquots of 3 mL were taken to measure the initial and final lawsone and AQDS concentration by UV-vis spectroscopy at 450 nm and 325

nm respectively in order to determine the adsorption capacity by mass balance relationship using Eq. (1):

$$Q_e = \frac{V(C_0 - C_e)}{W} \ldots Eq.1$$

Where Q_e is the adsorption capacity (mg/g); V is the volume of LQ or AQDS solution (L); C_0 and C_e are the initial and equilibrium concentration of LQ and AQDS (mg/L) respectively; and W the weight (g) of granular activated carbon. Additionally to determine LQ and AQDS chemically stable adsorption, from resulting GAC LQ and AQDS adsorption (LQ/GAC and AQDS/GAC), the materials were filtered and heated at 100°C for 24 hours to promote stronger interaction. Next, 30 mg of LQ/GAC and AQDS/GAC materials were added to 30 mL of basal medium solutions. Stability experiments were continuously stirred at 150 rpm at 30°C for 24 hours. Finally, 3 mL aliquots were taken to measure the desorption concentration and it was determined LQ and AQDS immobilized concentration.

Immobilization of LQ on GAC

LQ was immobilized covalently on granular activated carbon (LQ-GAC) based on maximum adsorption capacity from previous experiments. First, 1 L of 250 mg/L LQ solution was prepared with deionized water, adding a few drops of sodium bicarbonate 1.0 M to solubilize LQ; next, pH solution was adjusted to 3.0. Then, LQ solution and 1 g of GAC were added to an Erlenmeyer flask and it was continuously stirred 150 rpm at 30°C for 24 hours. Final solution was decanted and LQ-GAC material was washed until no LQ was observed in solution. Concentration of LQ was measured by UV-vis spectrophotometric technique at 450 nm.

Immobilization of AQDS on GAC

Activated carbon chlorination for AQDS chemical immobilization were conducted based on Lucas reagent test. Tertiary alcohols can be modified with HCl and zinc chloride as catalyst [68], for surface halogenation of GAC, consequently nucleophilic substitution (SN1) takes place with sulfonate groups of redox mediator and chloride groups of activated carbon and finally obtained a covalent immobilization of AQDS.

For Lucas reagent (LR) preparation, 64 g of $ZnCl_2$ was dissolved on 250 mL of concentrated HCl (37%), this conduct to an exothermic reaction, so LR preparation takes place on cold water (0 - 5°C). Then, 10 g of GAC and LR solution were added to an Erlenmeyer flask at 25°C for 24 hours. Chlorinated GAC (GAC-Cl) obtained was washed with deionized water three times. Next, 1 L of 250 mg/L AQDS solution and 1 g of GAC were added to an Erlenmeyer flask and it was continuously stirred 150 rpm at 30°C for 24 hours. Final solution was decanted and AQDS immobilized on GAC obtained was washed until no AQDS was observed. Concentration of AQDS was measured by UV-vis spectrophotometric technique at 325 nm.

3.7 Electron transferring capacity of GAC and RM immobilized on GAC

The electron transfer capacity (ETC) of all solid-phase redox mediators were determined by biological methods using 1 g/L of each material. Incubations were prepared with 0.1 g VSS/L at different temperatures (30 and 37 °C) with 2 g/L of glucose as electron donor. All ETC measurements were conducted by the ferrozine technique following the protocol described

by Lovely *et al.* [40]. Briefly, Fe (III) citrate was mixed with samples to obtain a final concentration of 20 mM, and allowed to react for 30 min. Then, equal volume of 0.5 M HCl was added to the sample, and an aliquot was mixed with ferrozine solution for spectrophotometric (562 nm) determination of Fe (II). The Fe (II) concentration was used to calculate the ETC from each material under all experimental conditions. All ETC measurements were corrected for intrinsic inoculum transfer capacity.

3.8 Catalytic potential of solid-phase RM on anaerobic bio-reduction assays

Kinetics of decolorization was conducted in 100 mL serum bottles using basal medium previously described. Portions of basal medium (47 mL) were placed in serum battles, which were immediately sealed with Teflon stoppers and aluminum caps. Anaerobic conditions were established by flushing the head-space (50 mL) of the bottles with N_2 for 5 minutes. Inoculation takes place by adding 0.1 g SSV/L of a stabilized anaerobic sludge described above. The anaerobic granular sludge was previously disintegrated by pressing the sludge suspension through sieve mesh #40. Glucose was provided as an electron donor (1 g/L·d). Congo red dye was added at initial concentration of 200 mg/L from a stock solution previously prepared. Sterile control provided with nutrients and GAC, in the absence of glucose and microbial consortium (dye adsorption on GAC). Endogenous control include sterilized microbial consortium, provided with nutrients, without redox mediator and source of energy (dye adsorption on sludge). Another control, provided with nutrients, glucose and microbial consortium without redox mediator. Soluble quinone control, provided with nutrients, glucose, microbial consortium and, 0.05 and 1mM of LQ or AQDS

respectively. Finally, complete experiment, provided with nutrients, glucose, active microbial consortium and 1 g/L of solid-phase redox mediator (GAC, GAC-Cl, LQ-GAC and AQDS-GAC). All experimental treatments were carried out by triplicate and incubated at 30°C at 150 rpm. Spectrophotometric screening was conducted during the incubation period in order to verify detachment of LQ and AQDS from activated carbon granules. The concentration of congo red dye was also measured by spectrophotometry UV-vis at 485 nm during the incubation period. Furthermore, due wavelength overlap on UV screening, benzidine (one of by-products of dye reduction) was measured by high performance liquid chromatography (HPLC). The HPLC was equipped with two reverse-phase columns, Synerg 4u Hydro-RP80A (250mm, 4.60 mm, 4µm) from phenomenex. The carrier liquid, composed of 100% of acetonitrile, was pumped at a flow rate of 1 mL/min. Benzidine was detected using a diode array at 280 nm. The content of methane and hydrogen was analyzed by gas chromatography (GC) equipped with a thermal conductivity detector and a column Hayesep D with following dimensions: 3.048 m X 3.18 mm X 2.16 mm. Nitrogen was used as carrier gas with a flow rate of 12 mL/min. Temperatures of the injection port, oven and the detector were 250, 60, and 250 °C, respectively.

3.9 Waste management

All waste (solid and water) was disposal according to stablished normativity by environmental, health and safety department of school of chemical science.

Results and Discussion

4.1 Effects of congo red and quinoid redox mediators on anaerobic sludge

The capacity of anaerobic sludge to decolorize was evaluated under different concentration of Congo red dye and during a period of about 170 h. The decolorization process followed a first-order kinetic and the rate constants were calculated at the maximum slope observed as follows (Eq. 2):

$$C = C_0 e^{-kdt} \quad \text{... Eq. 2}$$

where C is the concentration of the azo dye in a given time; C_0 is the initial concentration of azo dye; k_d is the first-order rate constant of decolorization (h^{-1}); and t is the elapsed time (h). Figure 8 shows the typical decolorization profiles (left) and biogas production (right) in absence of RM by anaerobic sludge under different concentrations of the dyes tested.

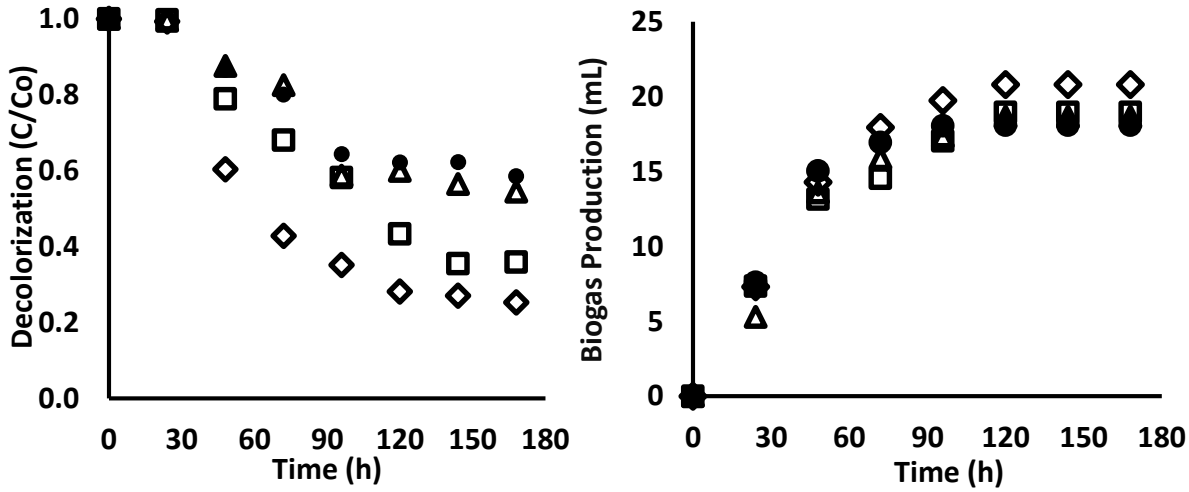


Figure 8. Decolorization profiles (left) and cumulative biogas production (right) by anaerobic sludge. Symbols represent azo dye concentration (mM): (◆) 0.1, (■) 0.5, (▲) 1.0, and (●) 2.0. In all cases standard deviation was ≤10%.

In general, a better decolorization process occurred at the lowest concentration of dye tested. For instance, after the incubation period (~170 h), the decolorization efficiency for congo red at 0.1 mM was 76% and decreased to 58% when 2.0 mM of dye was provided, respectively. Table 7 presents the rates of decolorization achieved under all conditions.

In all cases, lower rates of decolorization were observed by increasing the concentration of dye.

Table 7. First-order rate constants (k_d) of decolorization by anaerobic sludge using different concentration of azo dyes. Data represents k_d values (h^{-1}) ± standard deviation.

Concentration (mM)	k_d (h^{-1})
0.1	$0.0183 \pm 1.5x10^{-3}$
0.5	$0.0078 \pm 6.6x10^{-4}$
1.0	$0.0072 \pm 1.1x10^{-4}$
2.0	$0.0061 \pm 2.5x10^{-4}$

The production of biogas (Figure 7) was observed under all conditions tested; indicating that the anaerobic sludge remained physiologically active even in the presence of any concentration of dye. The biogas production was effected according to the concentration of azo dye and showed a similar behavior than the decolorization. Indeed, the assays with the lower concentration of azo dye produced the higher volume of biogas. This result indicates that methanogenic microorganisms present in the consortium are initially not affected by the presence of azo dyes. On the other hand, there is not a direct relationship between the affectation of decolorization process (rates of decolorization observed) and the biogas produced by the anaerobic consortium. In a previous study, Lee & Pavlostathis

(2004) found similar results during the decolorization of Reactive Blue 19 (RB19) [69]. Certainly, the extent of decolorization of RB19 for initial concentrations of 50–300 mg/L was ~90%, with a severe inhibition of the methane production, achieving maximum ~8% of the control lacking the dye. Moreover, this behavior was not observed for incubations with Reactive Blue 4. These authors suggested that methanogenic microorganisms were inhibited by the presence of RB19, which was evidenced by the accumulation of volatile fatty acids. Then, the high extent of reduction of dyes could be attributable to the reduced conditions created by the consortium. The inhibitory effects of AQDS and LQ on anaerobic consortium during biogas production were assessed in the presence of 4.0, 8.0, and 12 mM of these compounds. Figure 9 shows the cumulative biogas production after ~170 h of incubation, which indicates that the control lacking quinoid compounds, achieved the highest production of biogas with 19.8 mL. Considering all concentrations evaluated of quinoid compounds, LQ amended assays were more affected than AQDS assays, as indicated by the biogas production. The incubations reflected a decrement in biogas production up to 37, 53, and 58% at 4.0, 8.0, and 12 mM of LQ, in relation to the biogas produced by the control lacking this quinoid compound. Under the same conditions, AQDS affected the biogas production up to 5.1, 19, and 24% at 4, 8, and 12 mM of the quinoid compound, in relation to the control.

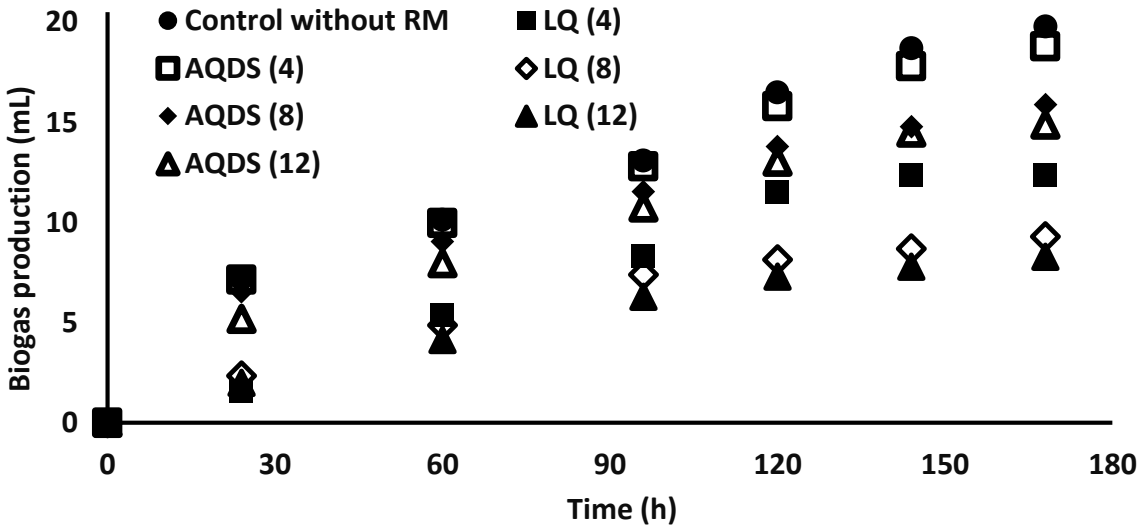


Figure 9. Cumulative biogas production by anaerobic sludge in the presence of different concentrations of AQDS and LQ.

The inhibitory effects of quinoid compounds can be explained by the redox potential of the culture solution. Previously, Cervantes *et al.* (2000) showed that a solution of 20 mM of AQDS increased the redox potential to +130 mV, but this value decreased gradually to about -175 to -250 mV according to the extent of reduction AQDS in cultures. The decrement of redox potential promoted the recovery of the capacity of the anaerobic consortium to produce methane. Certainly, the initial redox potential affected a biochemical process of methanogenesis because the methane production is only possible if redox potential in the medium is lower than -200 to -400 mV [70].

The catalytic effect of RM during the decolorization of congo red was evaluated at typical dye concentration of textile wastewater (150 mg/L). AQDS and LQ were added at different concentration (0.01, 0.05, 0.1, 0.5 and 1 mM), which are significantly lower than those concentrations applied in the incubations to assess the effect of these compounds during

biogas production. The endogenous and sterile controls showed negligible effect on decolorization efficiency, ranging between 8.7 to 13%, for both AQDS and LQ incubations. Figure 10 shows the performance of LQ acting as RM for the decolorization of congo red as compared to AQDS after 24 h of incubation. The catalytic activity of AQDS shows a remarkable rate of decolorization when a concentration >0.05 mM was employed, compared with sterile control. From the other hand LQ shows an inverse effect, this could be attributed that at high concentration, LQ wavelength (450 nm) overlap congo red wavelength (485 nm), being the steady state of assays the actual LQ concentration.

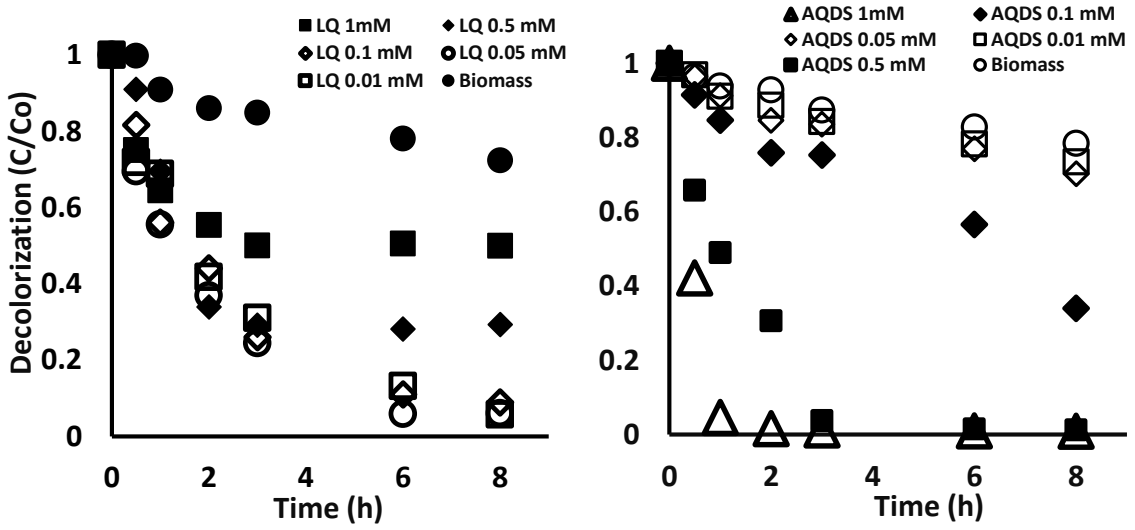


Figure 10. Decolorization profiles of congo red by anaerobic sludge using different concentration of LQ (left) and AQDS (right).

A mediator's potential should not be much lower than that of the biological reducing system, typically -320 mV for NADH otherwise it will not be reduced sufficiently [33]. On the other hand, the molar ratio RM/Azo here stablished is 3.4, which is similar than those reported during decolorization assays by anaerobic sludge [48, 49, 51]. In the study

developed by Van der Zee *et al.* 2001 it was also used a high concentration of AQDS (1.18 mM) with a molar ratio of RM/Azo of 3.7, achieving an increment up to 16-fold higher than the control without RM, but using anaerobic consortium at 2 g VSS/L (20 time higher than the applied in this study).

4.2 Surface Characterization of GAC

Activated carbon have been employed as a model adsorbent because have functional groups that can be interacting in different ways with organic compounds. These groups can be dissociated or protonated depending on the solution pH, which affects the surface charge and the degree of ionization and speciation of adsorbate [71]. Boehm method was studied to quantify functional groups on carbon surface. Boehm titration of two different samples of activated carbon was demonstrated that activated carbon surface has majority acid groups (~2.50 mEq/g) compared to basic groups (0.05 mEq/g). Table 8 shows that carboxylic groups are 4.0-6.8 fold lactonic and phenolic groups, respectively.

Table 8. Total acid and basic sites per gram of activated carbon (GAC1).

Mass (g)	Carboxylic (mEq/g)	Lactonic (mEq/g)	Phenolic (mEq/g)	Total acid sites (mEq/g)	Total basic sites (mEq/g)
0.10	1.70	0.25	0.42	2.43	0.01
0.20	1.80	0.28	0.56	2.62	0.03
0.30	1.75	0.22	0.52	2.45	0.05
μ ± σ	1.75 ± 0.05	0.25 ± 0.03	0.50 ± 0.07	2.50 ± 0.10	0.03 ± 0.02

μ: mean σ: standard deviation

This suggests that chemical immobilization process will have better affinity at acid pH, because acid groups are not dissociated, and there is a less electrostatic repulsion between redox mediator and activated carbon functional groups.

Several months later, carbon surface suffer a modification because it was constantly oxidized due ambient conditions, so phenolic and lactonic groups of activated carbon were increase. This suggests that carbon material has high heterogeneity, so changes in the surface are expected without chemical stimulation. Menéndez *et al.* (1996) confirmed that after a high temperature modification in inert conditions, carbon surface become highly reactive, capable of readsorbing oxygen and hence reacidifying in room-temperature air [72]. Table 9 shows acid and basic sites of oxidized activated carbon surface.

Table 9. Total acid and basic sites per gram of activated carbon (GAC2).

Mass (g)	Carboxylic (mEq/g)	Lactonic (mEq/g)	Phenolic (mEq/g)	Total acid sites (mEq/g)	Total basic sites (mEq/g)
0.20	0.02	1.35	1.31	2.68	0.02
0.30	0.03	1.63	0.97	2.63	0.03
0.40	0.03	1.55	1.15	2.73	0.01
μ ± σ	0.03 ± 0.01	1.51 ± 0.14	1.14 ± 0.17	2.68 ± 0.06	0.02 ± 0.01

μ: mean σ: standard deviation

4.1 Immobilization of Redox Mediator

Adsorption isotherms of LQ on GAC1 were obtained at different pH values (3, 4, 5 and 7) at 30°C (Figure 11). At acidic conditions, LQ adsorption capacity increase considerably compared to neutral conditions, reaching the maximum adsorption capacity (~ 299 mg/g)

at pH 3. According to speciation diagram (Figure 12), below pH 4 LQ has no dissociated groups, so esterification reaction between tertiary alcohol of LQ and carboxylic groups of GAC are expected. Spyroudis (2000) observed that hydroxyquinone molecules present high reactivity for synthesis of quinoid dyes, due hydroxyl group can be reacting in different manners with other species [73].

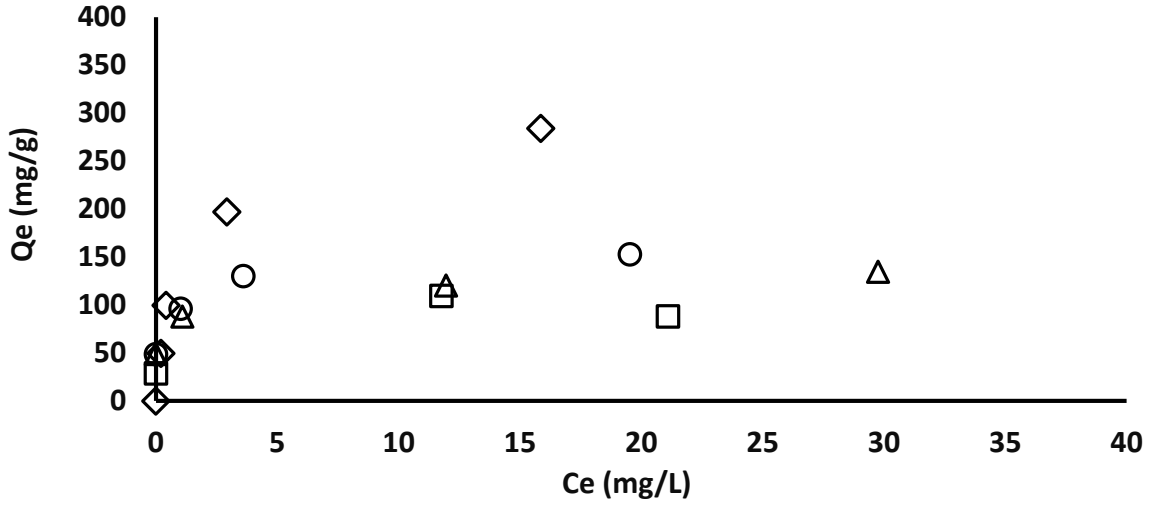


Figure 11. Adsorption isotherms of lawsone on activated carbon at 30°C, 150 rev/min, 30 mg GAC and 30 mL of solution; ◆ pH 3; ● pH 4; ▲ pH 5; ■ pH 7.

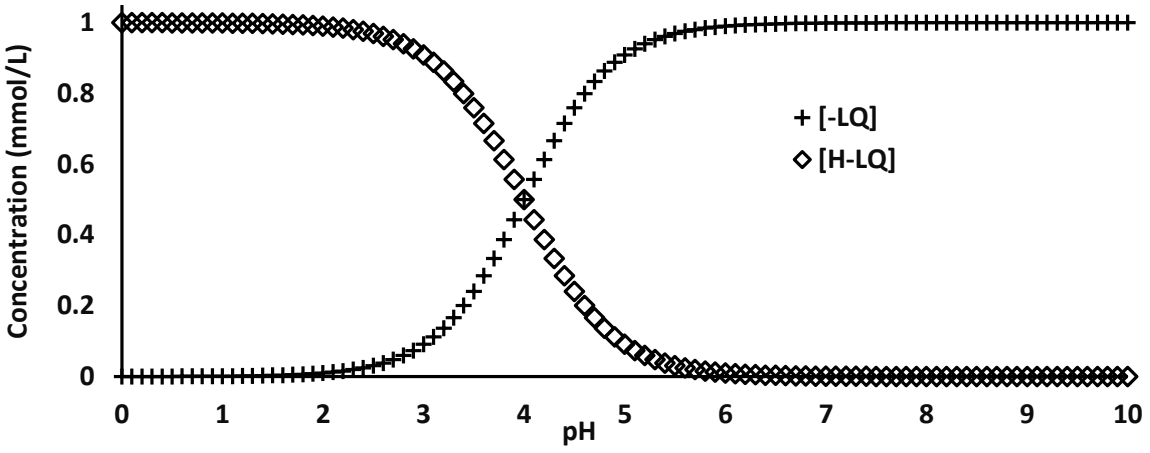


Figure 12. Speciation diagram of LQ.

Moreover, Gilles *et al.* (2015) showed high conversion of esterification of juglone (lawsone analogue) with long chain fatty acids at acidic conditions [74]. On the other hand, activated carbon has been shown being a suitable support of catalyst, because the variety and density of acid and basic groups that can be interacting with other species [75]. Also, previous studies suggest a Fischer esterification of phenolic groups on carbon materials [76].

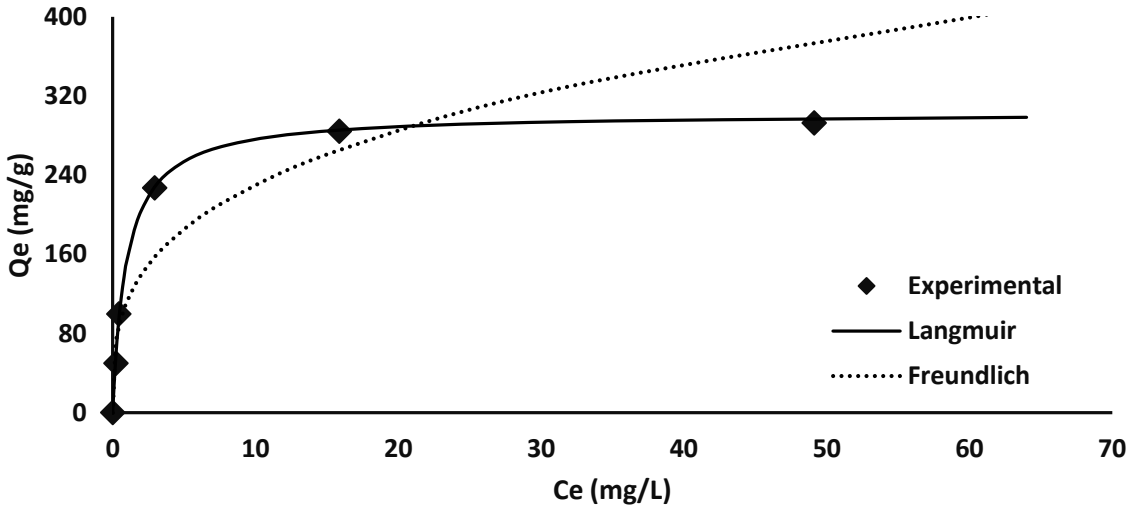


Figure 13. Adsorption isotherm of LQ on GAC1, with Langmuir and Freundlich prediction model.

As well, the equilibrium data of LQ adsorption on GAC1 at pH 3 (Figure 13) was predicted with the Langmuir isotherm model (Eq. 3) and Freundlich isotherm model (Eq. 4). Langmuir isotherm predicted better than Freundlich. Therefore, monolayer adsorption on specific sites is other evidence for chemical immobilization.

$$Q_e = \frac{Q_{max} b C_e}{1 + C_e} \dots Eq. 3 \qquad Q_e = k_f C_e^{1/n} \dots Eq. 4$$

In order to verify the strength of immobilization, desorption studies of adsorbed LQ on GAC were conducted with basal medium. Ionic interaction with activated carbon functional groups are expected, due high concentration of minerals. However, LQ has shown low desorption <2% (Figure 14), then high immobilization stability was attributed to covalent bonding. Okumura *et al.* (2011) studied esterification bond stability of long chain hydrocarbons, having a long-term stability over a pH range 5-7 at room temperature [77], suitable for anaerobic processes. In addition, maximum adsorption capacity estimated (1.71 mmol/g) may be limited to carboxylic group's concentration (1.75 mmol/g) of activated carbon surface, as well another evidence of esterification reaction. The proposed mechanism for chemical immobilization of LQ onto GAC is elucidated (Figure 15).

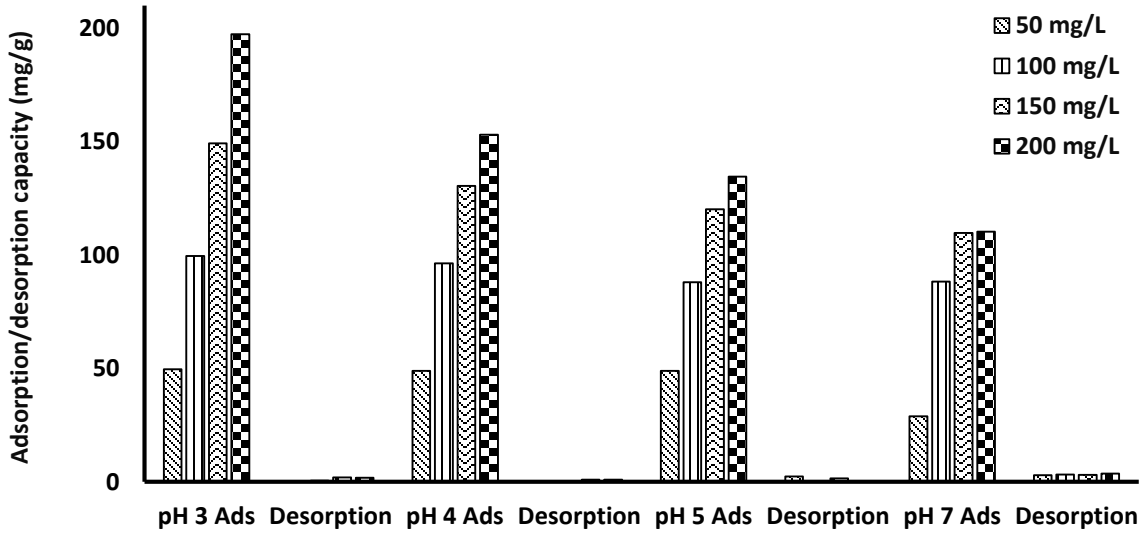


Figure 14. Adsorption capacity of LQ on activated carbon and desorption with basal medium at different pH and initial concentration values.

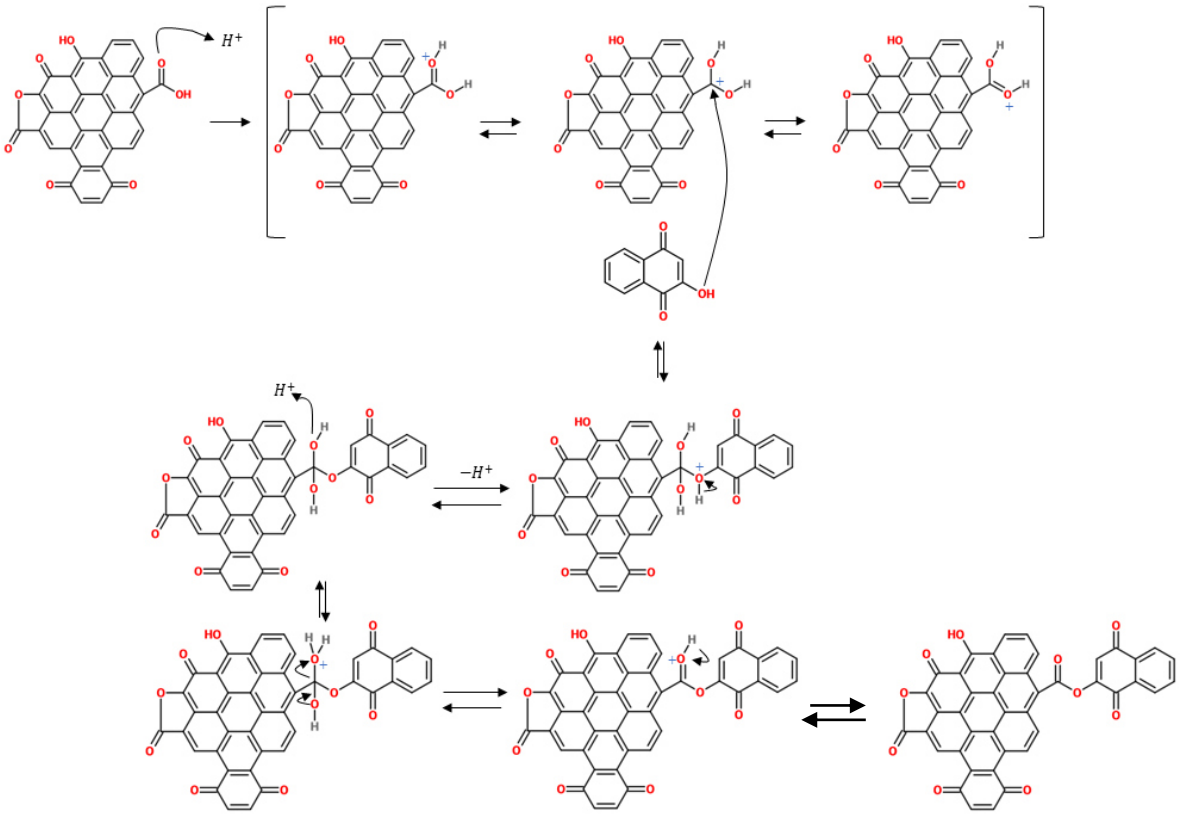


Figure 15. Fischer esterification mechanism for binding of lawsone onto activated carbon.

In the best of our knowledge there is no evidence of immobilization of LQ on carbon materials. Alternatively, henna leaves have been applied as co-substrate and electron shuttle on anaerobic processes. Rau *et al.* (2002) was the first author applying henna leaves, which contains 1% of LQ glucosides, as redox mediator with good catalytic potential on the decolorization of amaranth, reaching about 75% of decolorization compared with the soluble control (95%) at same LQ concentration (0.25 mmol/L) . Additionally, Liu *et al.* (2010) observed that LQ is an excellent redox mediator for anaerobic chromate reduction. Direct bacterial reduction of Cr(VI) can require days or even weeks for complete reduction of less than 50 mg/L, LQ-mediated reduction of 100 mg/L of Cr(VI) was completed in hours

[78]. Immobilization of LQ seems to be promising for it application on anaerobic bio-reduction. Furthermore, the high amount of immobilization obtained (1.56 mmol/g) can enhance the rate of decolorization of azo dyes.

AQDS adsorption isotherms were obtained at same values of pH than LQ-GAC1 adsorption experiments. Isotherms behavior of AQDS adsorption (Figure 16) has no regularity at different pH values. According to speciation diagram, AQDS was dissociated at entire pH range (Figure 17). Then, electrostatic repulsion between GAC and AQDS is expected due sulfonated quinone and acidic sites of GAC (Table 8).

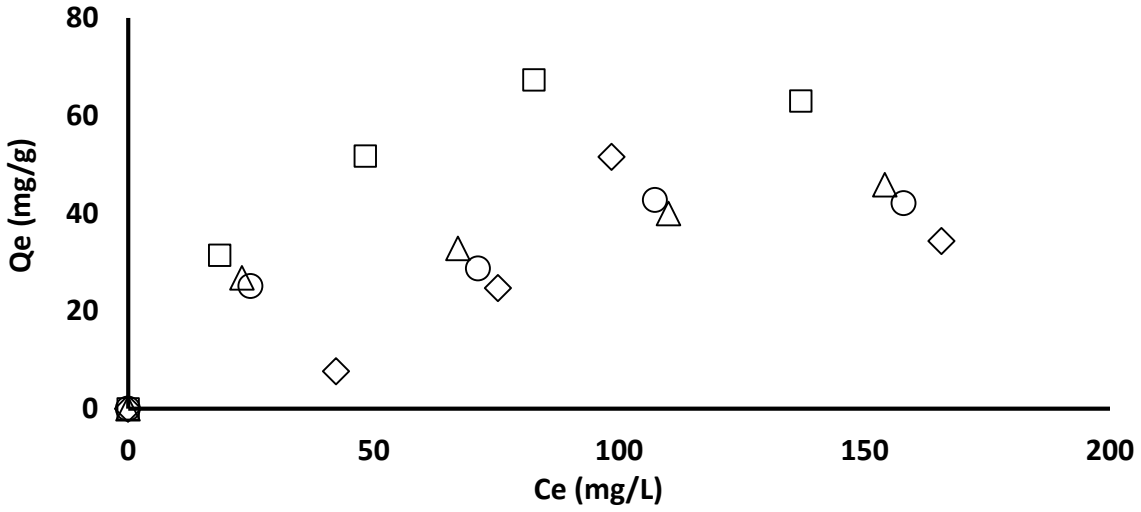


Figure 16. Adsorption isotherms of AQDS on activated carbon at 30°C, 150 rev/min, 30 mg GAC and 30 mL of solution; ■ pH 3; ▲ pH 4; ● pH 5; ◆ pH 7.

Immobilization process of AQDS on GAC by physical interaction between GAC and AQDS may be attributed to π dispersion. It is well known that aromatic molecules are adsorbed in flat position on the graphene layer, and in this situation the adsorption driving force would

be due π-π dispersion interactions between the aromatic ring of AQDS and the aromatic structure of the GAC layers [79]. The maximum adsorption capacity is 67 mg/g, which is much lower than reported by Cervantes *et al.* (2010) on anion exchange resins (~ 760 mg/g). Nevertheless, it suggest that one day adsorption on GAC will fix AQDS on the outer surface, which can interact more easily with the electron flow from bacteria and to EAC, decreasing intraparticle diffusion and other mass transfer resistances.

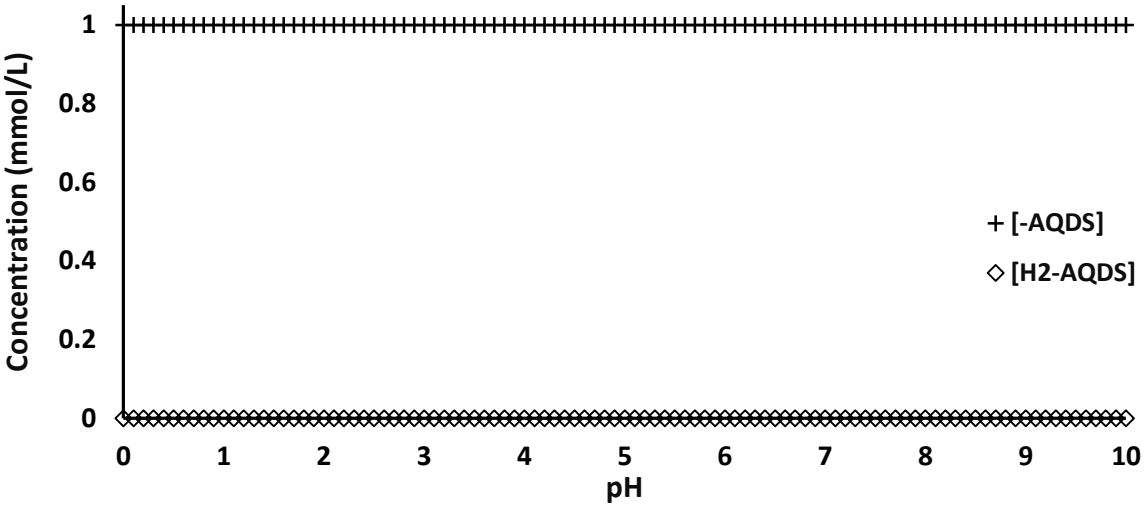


Figure 17. Speciation diagram of AQDS.

As well, desorption experiments of AQDS were conducted with basal medium. Unusually, high adsorption stability was observed (Figure 18). Mattson *et al.* (1969) described that acidic surface oxygen groups located at the edge of the basal planes of carbon surface, remove electrons from the π electron system, creating positive holes in the conducting π-band of the graphitic planes [80]. Then, donor-acceptor complex mechanism, between carbonyl or hydroxyl groups of activated carbon and aromatic ring or quinone group of AQDS may take place. Nonetheless, strength interaction will be maintained until redox

activity of carbonyl groups decay. This suggests that AQDS immobilized on GAC is going be detach on anaerobic reduction process, so its application become unavailable.

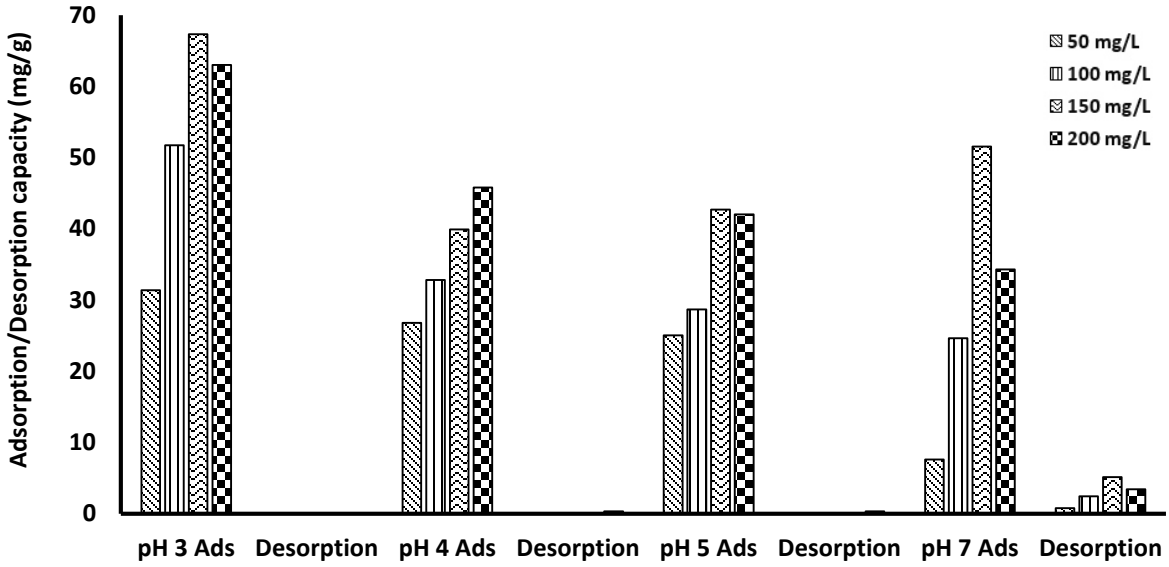


Figure 18. Adsorption capacity of AQDS on activated carbon and desorption with basal medium at different pH and initial concentration values.

To overcome physical immobilization disadvantages, modification of carbon surface takes places by chlorination of phenolic groups of GAC2 (GAC-Cl). In previous studies, Amezquita *et al.* (2014) showed chlorination of activated carbon fibers (ACF) with thionyl chloride and therefore anthraquinone-2-sulfonate (AQS) covalent immobilization on chlorinated ACFs. But, in despite to propose another chemical modification cheap and harmless, Lucas reagent technique was employed for chlorination of GAC. Adsorption isotherms of AQDS on GAC2 and GAC-Cl at neutral pH were obtained.

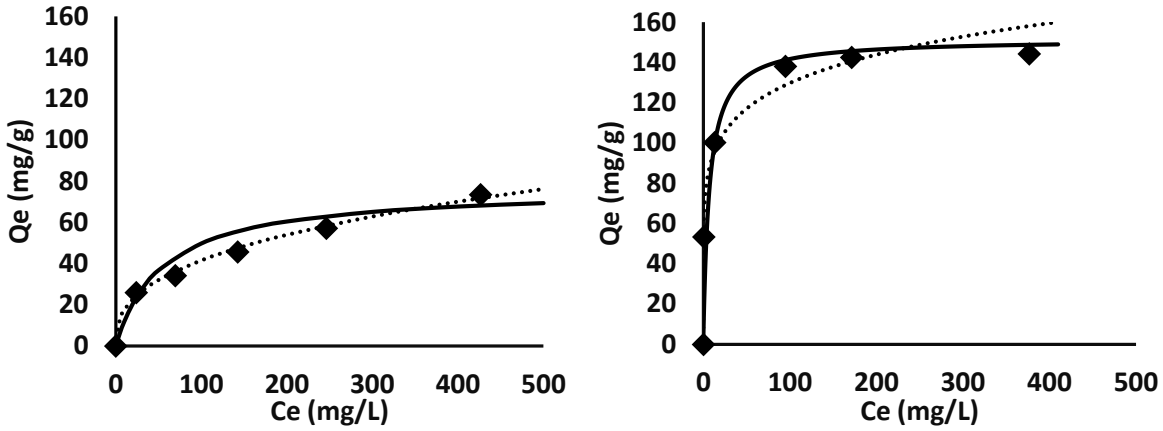


Figure 19. Adsorption of AQDS on GAC2 (left) and GAC-Cl (right), experimental data (◆), with Langmuir (—) and Freundlich (⋯) isotherm models.

Figure 19 shows the equilibrium data of AQDS adsorption onto GAC2 and a good prediction with Freundlich isotherm model (Eq. 4), then heterogeneous surfaces and multilayer adsorption are expected, indicating π stacking adsorption mechanism. On the other hand, AQDS adsorption on GAC-Cl was predicted with Langmuir isotherm model better than Freundlich (Figure 19), this can be attributed to monolayer specific sites adsorption; therefore, binding of sulfonate groups of AQDS on chlorinated GAC via nucleophilic substitution can take place. Furthermore, maximum adsorption capacity of AQDS/GAC-Cl increased twice (145 mg/g) in comparison with AQDS/GAC2 (71 mg/g). The proposed mechanism for chlorination of GAC and for chemical immobilization of AQDS onto GAC-Cl is show at Figure 20.

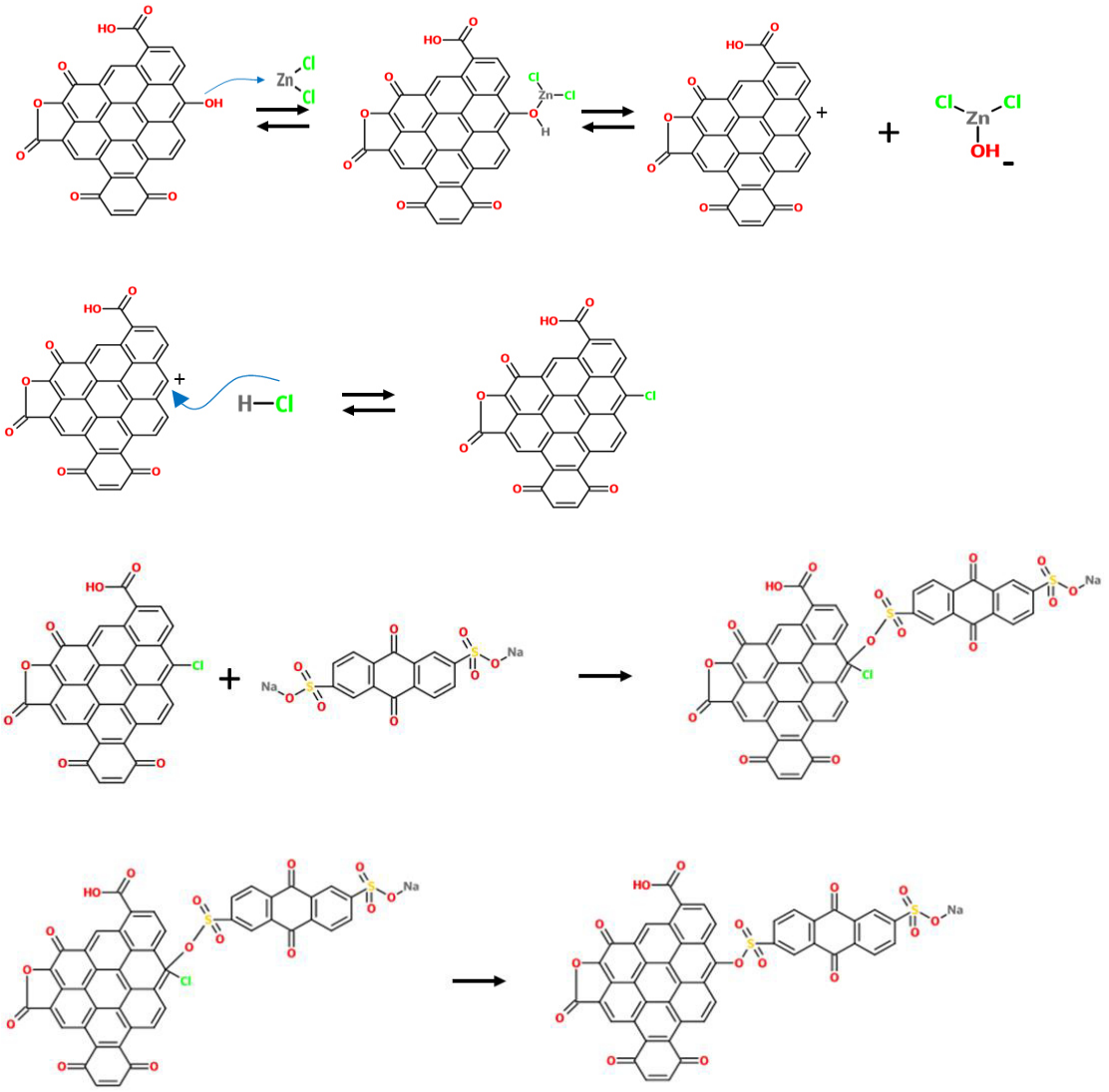


Figure 20. Proposed mechanism for chlorination of activated carbon, and AQDS immobilization on chlorinated activated carbon by nucleophilic substitution.

4.1 Adsorption equilibrium of azo dyes

The equilibrium isotherms of congo red at pH 7 and temperature of 30°C are presented in Figure 21. The maximum experimental adsorption capacity of congo red onto GAC was 0.013 mmol/g, decreasing when GAC has immobilized RM to 0.010 and 0.004 for AQDS-GAC and LQ-GAC, respectively.

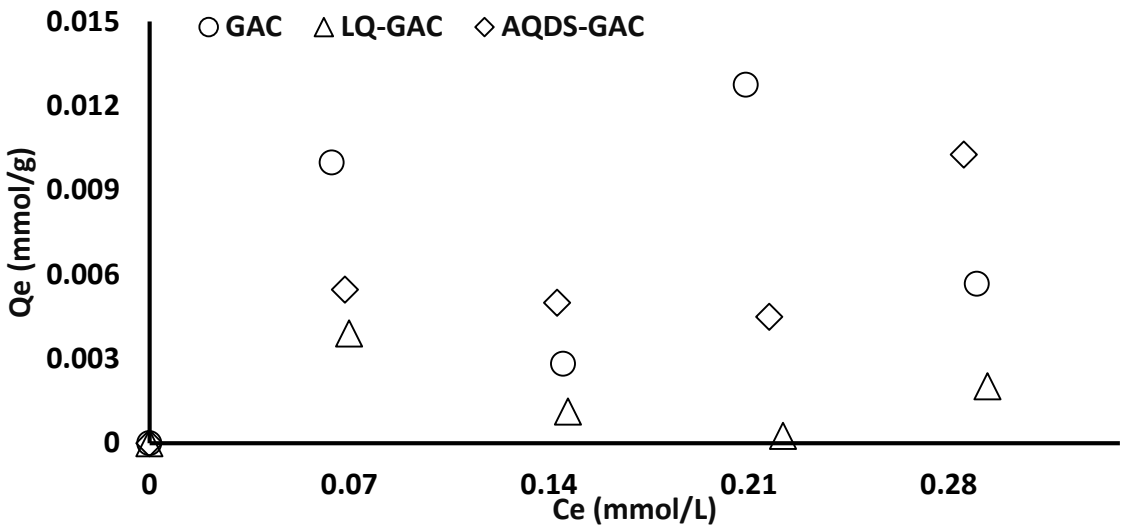


Figure 21. Adsorption isotherms of congo red dye onto GAC, LQ-GAC and AQDS-GAC.

Sulfonated azo dyes commonly have high negative charge, leading to poor interaction with acid groups of carbon surface due electrostatic repulsion mechanism. In previous studies, has been observed that with the increasing pH values, the adsorption of congo red on different adsorbents tends to decrease, which can be explained by the increasing electrostatic repulsion between the anionic dye adsorbate species and negatively charged adsorbent surfaces [81]. This mechanism is amplified when GAC has immobilized naphthaquinone (LQ) and anthraquinone (AQDS) molecules, blocking basic sites of carbon surface, decreasing interaction with sulfonated azo dye and, therefore, a low adsorption capacity. Moreover, owing high molecular size of congo red, intraparticle pore diffusion on activated carbon would be restricted and, hence, interaction of azo dye with carbon sites only will be accomplished on the outer carbon surface. Various adsorbents have been tried on congo red adsorption, but mostly achieved a low adsorption capacity. Table 10 shows adsorption capacity of congo red on different adsorbents.

Table 10. Maximum adsorption capacity of congo red on different adsorbents.

Adsorbent	Adsorption capacity (mg/g)	Reference
Activated coir pitch carbon	6.72	[82]
Activated carbon (LR)	1.88	[83]
Bagasse fly ash	11.88	[84]
Tamarind fruit shell	3.48	[85]
Kaolin	5.44	[86]
Na-Bentonite	5.70	[86]
Raw pine cone	19.18	[81]
GAC	9.05	This study
AQDS-GAC	6.96	This study
LQ-GAC	2.78	This study

Consequently, adsorption process will not contributed significantly (< 2% decolorization) on anaerobic bio-reduction assays, which favored its application as solid-phase RM on anaerobic processes.

4.2 Electron transferring capacity

Determinations of electron transfer capacity of microorganisms consortia suggest that species present on anaerobic sludge are highly capable of transfer electron to EAC. Moreover, at 30°C has major capacity than 37°C, which can be attributed to a competition between methanogenic and respiration activity of anaerobes. In previous studies, a competition between methanogenic and quinone respiration activity has been observed, predominating the last one when RM are present in anaerobic cultures [87]. On the other hand, quinone respiration pathway has been studied at mesophilic (~30°C) and thermophilic (55°C) conditions, showing a better electron transfer at high temperature [88]. Nonetheless, there is no evidence about this effect at methanogenic conditions (37°C). Table 11 shows the ETC of anaerobic cultures with solid-phase RM and the control lacking

RM. ETC of assays with GAC as RM are slightly better than anaerobic sludge, this can be attributed that carbon surface have acid and basic sites that can be interacting in the redox process. Several studies have been applied carbon materials as solid-phase redox mediator with good rates of reduction [52-56]. Pereira *et al.* (2010) observed that with different surface modification of activated carbon, redox activity could change positively or negatively. Oxidation of activated carbon with nitric acid and oxygen increase significantly increase oxygen-containing groups which can increase the rate of reduction, but high density of acid sites also promotes a higher repulsion between azo dyes and carbon surface. Alternatively, modification with N_2 and H_2 promotes an increasing on activated carbon basic sites, which increase considerably the rate of reduction. This can be attributed that electron withdrawing groups (-OH, -NH2) decrease the electron density around the azo bond, minimizing the steric hindrance of the molecule, and facilitate its reduction [52]. Therefore, ETC of GAC is attributed mainly to oxygen species and high acid group's concentration and a slightly contribution of basic sites (Table 8 and 9). Also, LQ-GAC has a higher ETC than unmodified GAC, due high concentration of LQ immobilized (1.56 mmol/g). Instead, AQDS-GAC has the highest ETC, even if has a less quinone groups than LQ-GAC. This behavior can be attributed to the high ETC of GAC-Cl, which is owing to adsorbed Zinc particles from Lucas reagent catalyst, acting as Lewis acid (electron acceptor), increasing the reactivity of azo dye (minimizing the steric hindrance). Therefore, increasing quinone groups of GAC-Cl with immobilization of AQDS rise the ETC.

Table 11. Electron transfer capacity of anaerobic sludge, GAC and RM-GAC.

System	Electron transfer capacity (mEq/g)	
	30 °C	**37.5 °C**
Anaerobic sludge	1.537	1.423
GAC1	1.636	1.512
GAC2	1.613	1.471
GAC2-Cl	1.812	1.745
LQ-GAC1	1.763	1.597
AQDS-GAC2-Cl	2.001	1.848

4.3 Catalytic potential of solid-phase RM on anaerobic bio-reduction assays

Anaerobic reduction kinetics of congo red dye with LQ and AQDS (soluble and immobilized) as RM is presented in figure 22 left and right, respectively.

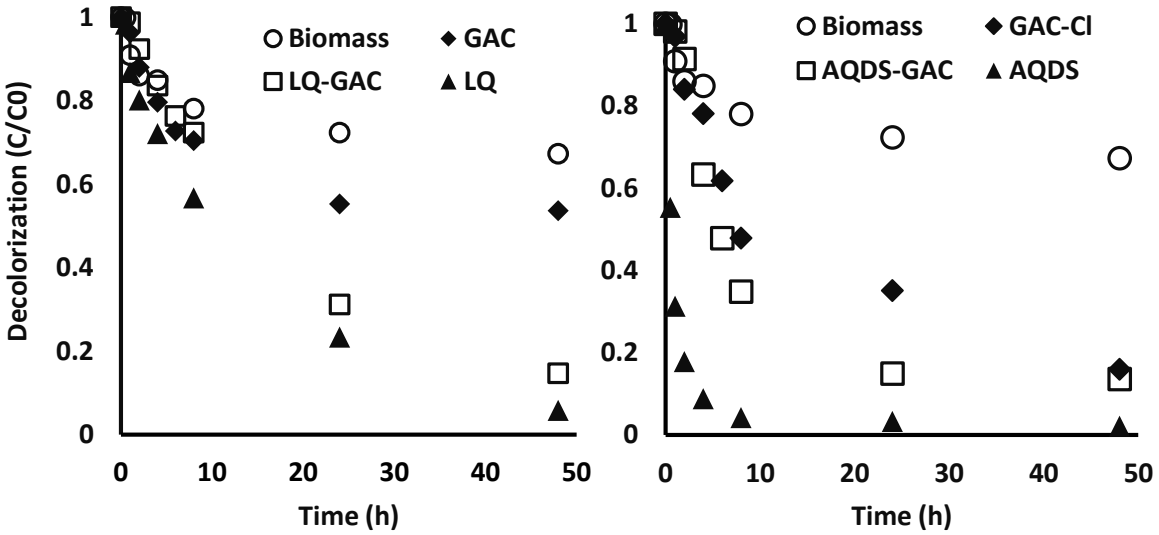


Figure 22. Decolorization profiles of congo red with and without RM; LQ (left) and AQDS (right).

Controls lacking RM were the same on both kinetic experiments achieving 28% of decolorization at 24h with low rate of reduction. Biotransformation of congo red dye lead

to the apparition of aromatic amines, which can inhibited the biological process. Sakkas *et al.* (2010) observed that transformation products of congo red dye are more toxic than dye [89]. Additionally, the rotational freedom of benzidine (by-product of complete congo red reduction) allows to orient itself on any desired conformation in the biological system leading to its toxic characteristics [90]. Therefore, inhibition of respiration activity may be the principal reason for the low performance of anaerobic bio-reduction. Unmodified GAC assay reached 46% of decolorization after two days, duplicating its rate of decolorization. In previous studies, carbon materials have been implemented as RM reaching high percentage of decolorization. Nevertheless, in some cases, adsorption process contributed significantly, even with modification of surface functional groups [53, 54]. On the other hand, LQ-GAC increase the rate of decolorization 2.3-fold, compared with unmodified GAC, reaching 85% of decolorization on 48 h. Comparable rates and percentage of decolorization were reaching by soluble and immobilized assay, but the concentration of immobilized is 31-fold larger than soluble control. It is well know that immobilization process decrease activity of catalyst. It's suggest that mass transfer limitations, highly steric hindrance and poor dispersion of quinone groups are the mainly reason for the poor redox activity. Nonetheless, the easy preparation of LQ-GAC makes its application highly available, and can be reused until its deactivation. Alternatively, GAC-Cl achieve a low rate of reduction in the first 8 hours, but high percentage of decolorization, being GAC-Cl a good option for anaerobic bio-reduction processes. As we mentioned, Zinc particles may acting as electron donor, minimizing the steric hindrance of congo red molecule, which increase the electron flow through carbon surface and azo-bond. Therefore, high rate of decolorization has been

achieved even without RM. Otherwise, soluble AQDS achieved the highest decolorization (98%) at 48h, followed by its immobilized counterpart (AQDS-GAC), reaching 87% of decolorization, with the highest rate of decolorization of all solid-phase RM synthetized. Table 12 shows the amount of quinone immobilized, rate (first order kinetic model), and percentage of decolorization of all anaerobic assays.

Table 12. Kinetic constants and decolorization efficiency of all experiments.

Assay	Quinone concentration (mM)	Rate of decolorization (h^{-1})	Decolorization at 48 h (%)
Biomass	0	0.049	24
GAC	0	0.0497	46
LQ-GAC	1.56	0.2373	85
LQ	0.05	0.4319	94
GAC-Cl	0	0.0893	86
AQDS-GAC	0.34	0.7028	87
AQDS	1	12.112	98

In table 13, its show the increasing rate of reduction of different solid-phase RM, compared with the control lacking RM. High rates have been achieving with carbon materials, but mostly adsorption mechanism are the mainly responsible for the process efficiency. On the other hand, immobilized quinone molecules (AQDS, NQS, AQS and fulvic acids) have increased more than twice the rate of reduction, compared with carbon materials, being fulvic acids on alumina particles the fastest of all studies. Nevertheless, physical immobilization, metal toxicity and fulvic acid extraction/purification limited its application. In this study, AQDS-GAC reach the highest rate of decolorization, employing immobilized RM, compared with previous studies. As well, LQ-GAC reached a good rate of decolorization being a better option for its application due it easily synthesis.

Table 13. Comparison of rate of reduction with previous reported studies.

Material/immobilization	Increase in rate of reduction (k)	Reference
Activated carbon (H_2)	2.0	[52]
Carbon nanotubes	1.5	[55]
Carbon xerogels	2.0	[55]
AQDS-Anionic resins	4.0	[91]
NQS-Anionic resins	8.8	[91]
Fulvic acid-Alumina	10.4	[60]
LQ-GAC	4.84	This study
GAC-Cl	1.71	This study
AQDS-GAC	14.34	This study

Figure 22 and 23 shows methane and hydrogen cumulative production on anaerobic decolorization assays. Typical methane and hydrogen production it's observed. Clearly, methane production decreased when redox mediator are presented, being the experiment with GAC-Cl as RM the lowest. Adsorbed zinc particles on GAC-Cl may be the responsible for methanogenic inhibition. It is well know that heavy metals are highly toxic to microorganism, even in low concentration [92]. Also, selective hydrogen production was observed, which could be explained by the accumulation of organic acid intermediates, as a result of methanogenic bacteria inhibition [93]. However, respiration activity is maintained over time, being a promising material for it application as electrode on microbial fuel cell system.

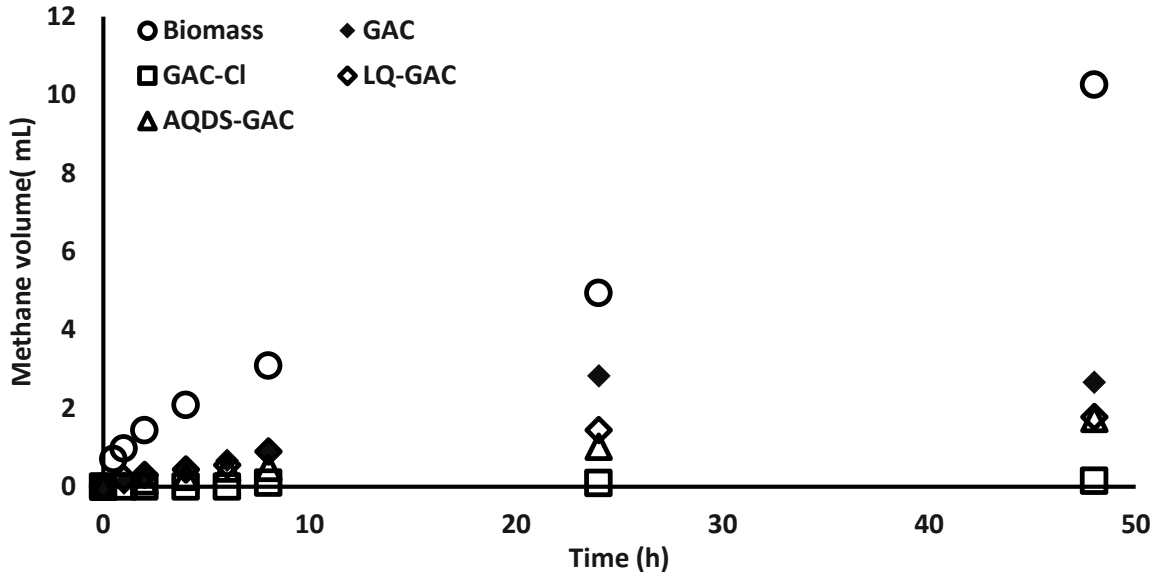


Figure 23. Cumulative methane production of different redox mediator on anaerobic decolorization of congo red dye.

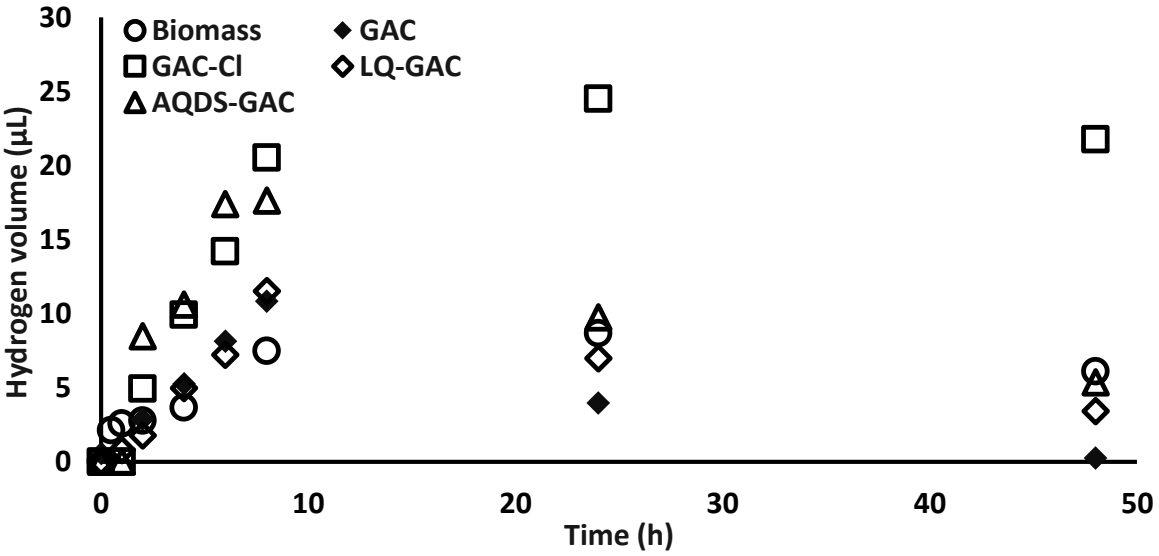


Figure 24. Cumulative hydrogen production of different redox mediator on anaerobic decolorization of congo red dye.

Finally, HPLC analysis shows a slow rate for benzidine production with LQ-GAC and AQDS-GAC as solid-phase RM compared to decolorization profiles (Figure 25). Nonetheless, reciprocal rate of decolorization and benzidine production is not observed.

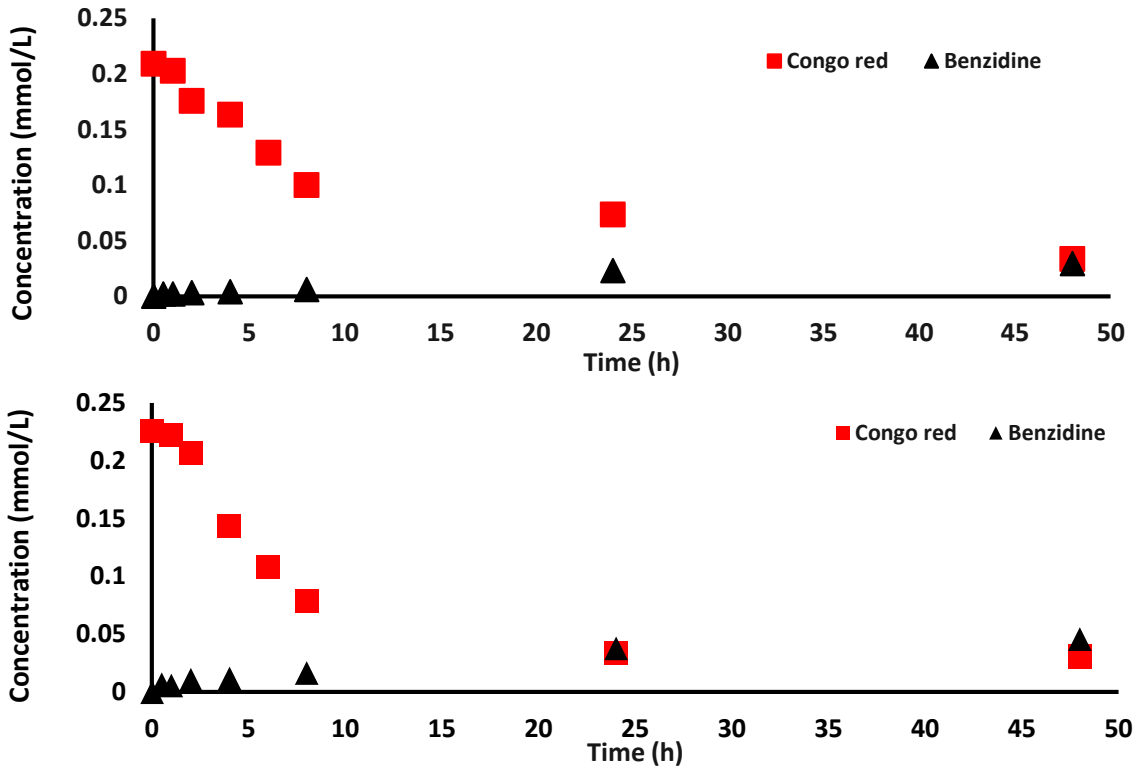


Figure 25. Decolorization profiles and benzidine production with LQ-GAC (above) and AQDS-GAC (below) as RM on anaerobic biotransformation of congo red dye.

Therefore, azo bridges of azo dye molecule are not broke at same time, and decolorization can be attributed to a breaking of one azo linkage. For this reason, complete anaerobic reduction requires a longer time, even with the application of RM. Amino-napthalene benzidine complex is formed as intermediate of complete reduction, and it is still a recalcitrant molecule for biological degradation. This suggests that molecules with poli-azo linkage may be loss electron affinity, attributed to an increasing steric hindrance; therefore,

congo red transformation could need a lower energy for its reduction than the amino-naphtalene complex produced. As a result, it is proposed that the reduction mechanism is carried out in two general steps. Because the molecule is symmetric, breaking of the first azo bond is indistinct, followed by the breaking of the second azo bond which is the limiting step of the reaction (Table 14). To the best of our knowledge, this is the first study that proposes the reaction mechanism of complete anaerobic reduction employing solid-phase RM, showing the limiting step of the reaction (Figure 26).

Table 14. Rates of decolorization and benzidine production.

Catalyst	Rate of decolorization	Rate of benzidine production	Decolorization at 48 h (%)
LQ-GAC	0.2373	0.0053	85
AQDS-GAC	0.7028	0.0055	87

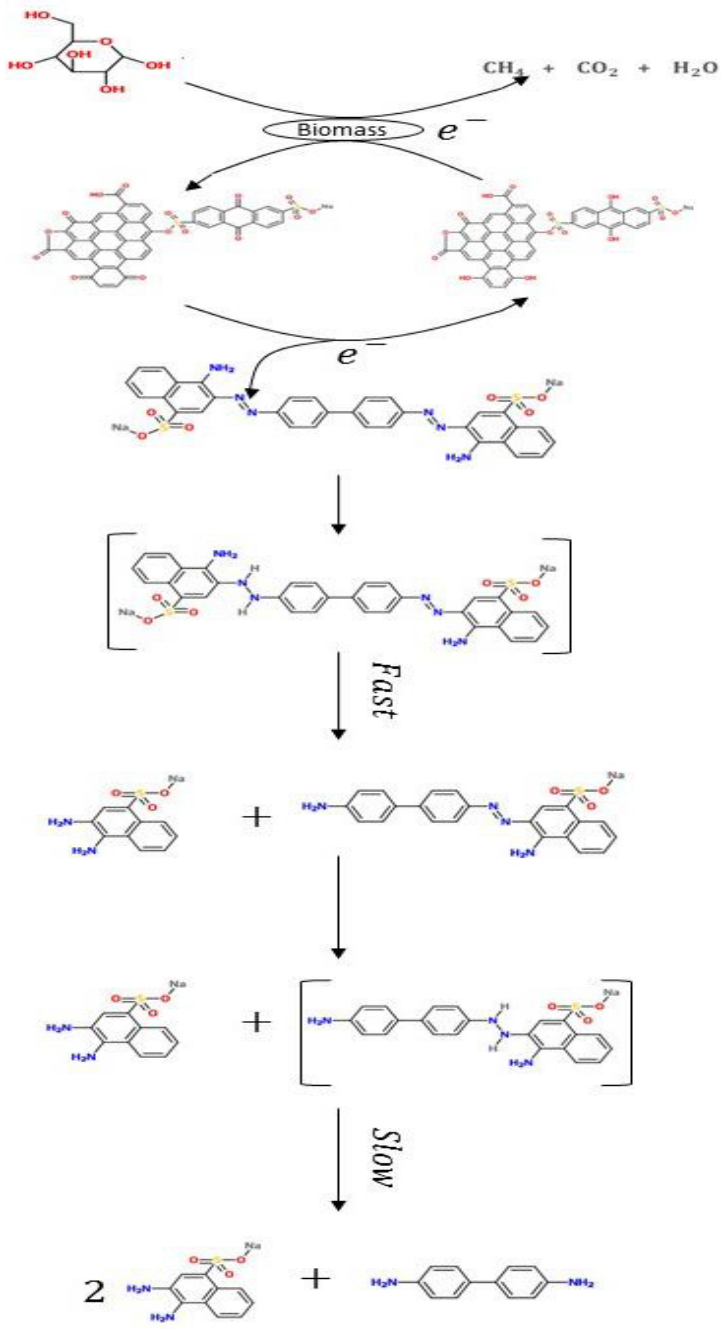


Figure 26. Proposed mechanism for anaerobic biotransformation of congo red dye, with dextrose as energy source.

Conclusions

Congo red dye does not have an inhibitory effect over methanogenic activity. On the other hand, quinone molecules show inhibition of methanogenesis, being LQ the most toxic. Binding of LQ is carried out by Fischer esterification mechanism between hydroxyl groups of LQ and carboxylic groups of GAC (1.56 mmol of LQ/gram of GAC), showing high rate of reduction (4.6-fold anaerobic sludge) reaching 85% of decolorization at 48 h. LQ seems to be the best option for its application at full scale process for economical, easily preparation and scale up viability. Moreover, an easy way for chlorination of GAC, based on Lucas reagent test, was presented and changed the adsorption mechanism of AQDS on carbon surface before and after its modification. Covalent immobilization was achieved by nucleophilic substitution and compared to unmodified activated carbon, chlorination enhanced AQDS adsorption capacity by twice following Langmuir adsorption model, which is immobilized on monolayer specific site (0.34 mmol of AQDS/gram of GAC-Cl). AQDS-GAC reached 87% of decolorization with a high rate of decolorization (0.7023 h^{-1}), being the highest reported on literature. Both LQ-GAC and AQDS-GAC are good candidates for its application on continuous bioreactors. Additionally, GAC-Cl material seems to have future application on redox mediating systems. Selective hydrogen and cumulative production was maintained over time compared to anaerobic sludge. Zinc may be playing a major role on electron flow, acting as an electron donor, minimizing steric hindrance of azo dye molecule and inhibiting methanogenic species, leading to acidic conditions, which can promote hydrogen production. Finally, mechanism of anaerobic bio-reduction of congo red is carried out in two steps, being the benzidine production the limiting step of the entire reaction.

Two ways for immobilization of quinone molecules, AQDS and LQ, was presented with high efficiency and promising application on continuous anaerobic reactors and limiting step of the entire reduction process was elucidated, being an essential information for better reactor design.

References

[1] Razo-Flores E, Macarie H, Morier F. Application of biological treatment systems for chemical and petrochemical wastewaters. In: Cervantes FJ, Pavlostathis SG, Van Haandel AC, editors. Advanced biological treatment processes for industrial wastewaters: principles & applications. London, UK: IWA Publishing; 2006, 267-297.

[2] Van der Zee FP, Bouwman RHM, Strik DPBTB, Lettinga G, Field JA. Application of redox mediator to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. Biotechnology Bioengineering 2003, 691-701.

[3] Lovley DR. Coates JD, Blunt-Harris EL, Phillips EJP, Woodward JC. Humic substances as electron acceptors for microbial respiration. Nature 1996, 445-448.

[4] Hong, Y., & Gu, J. D. Bacterial anaerobic respiration and electron transfer relevant to the biotransformation of pollutants. *International Biodeterioration & Biodegradation*, 2009, 973-980.

[5] Dagley, S. Biochemistry of aromatic hydrocarbon degradation in pseudomonas. *THE BACTERIA: A TREATISE ON STRUCTURE AND FUNCTION. 1986.*

[6] Wackett, L. P., Sadowsky, M. J., Newman, L. M., Hur, H. G., & Li, S. Metabolism of polyhalogenated compounds by a genetically engineered bacterium. *Nature*, 1994, *368*(6472), 627-629.

[7] Cariell, C. M., Barclay, S. J., Naidoo, N., Buckley, C. A., Mulholland, D. A., & Senior, E. Microbial decolourisation of a reactive azo dye under anaerobic conditions. *Water Sa*, 1995 *21*(1), 61-69.

[8] O'Neill, C., Hawkes, F. R., Hawkes, D. L., Lourenco, N. D., Pinheiro, H. M., & Delee, W. Colour in textile effluents–sources, measurement, discharge consents and simulation: a review. *Journal of Chemical Technology and Biotechnology*, 1999, *74*(11), 1009-1018.

[9] Guo, J., Kang, L., Wang, X., & Yang, J. Decolorization and degradation of azo dyes by redox mediator system with bacteria. In *Biodegradation of Azo Dyes.* Springer Berlin Heidelberg, 2010, 85-100

[10] Pagga, U., & Brown, D. The degradation of dyestuffs: Part II Behaviour of dyestuffs in aerobic biodegradation tests. *Chemosphere*, 1986, *15*(4), 479-491.

[11] Shaul, G. M., Holdsworth, T. J., Dempsey, C. R., & Dostal, K. A. Fate of water soluble azo dyes in the activated sludge process. *Chemosphere*, 1991, *22*(1), 107-119.

[12] Brown, D., & Laboureur, P. The aerobic biodegradability of primary aromatic amines. *Chemosphere*, 1983, *12*(3), 405-414.

[13] Munter, Rein. "Advanced Oxidation Processes–Current Status and Prospects.". *Proceedings of the Estonian Academy of Sciences. Chemistry, 2001,* 50 (2), 59–80.

[14] Glaze, William; Kang, Joon-Wun; Chapin, Douglas H. "The Chemistry of water treatment processes involving ozone, hydrogen peroxide and ultraviolet radiation. Ozone: Science & Engineering: The journal of the international ozone association, 1987, 9 (4), 335-352.

[15] Andreozzi, R., Caprio, V., Insola, A., & Marotta, R. Advanced oxidation processes (AOP) for water purification and recovery. *Catalysis today*, 1999, *53*(1), 51-59.

[16] Papić, S., Peternel, I., Krevzelj, Ž., Kušić, H., & Koprivanac, N. Advanced oxidation of an azo dye and its synthesis intermediates in aqueous solution: effect of Fenton treatment on mineralization, biodegradability and toxicity. *Environmental Engineering and Management Journal*, 2012, *1582*, 9596.

[17] Zhou, M., & He, J. Degradation of azo dye by three clean advanced oxidation processes: wet oxidation, electrochemical oxidation and wet electrochemical oxidation—a comparative study. *Electrochimica Acta*, 2007, *53*(4), 1902-1910.

[18] Nair, P. G., Vijayakumar, S., Lisluke, T., Mathew, M. S. P., & Aravindakumar, C. T. Degradation of Dyestuff Pollutant Sudan I Using Advanced Oxidation Process. *Journal of Water Resource and Protection*, 2014, *6*(14), 1276.

[19] Zuorro, A., & Lavecchia, R. Evaluation of UV/H2O2 advanced oxidation process (AOP) for the degradation of diazo dye Reactive Green 19 in aqueous solution. *Desalination and water treatment*, 2014, *52*(7-9), 1571-1577.

[20] J. A. Oleszkiewicz and D. S. Mavinic, Wastewater Biosolids: An Overview of Processing, Treatment and Management, *Can. J. Civil Eng*., Special Supplemental S1, 2001 28(3), 102–114.

[21] C. E. Randall, W. S. Young, and P. H. King, Aerobic digestion of trickling filter humus. *Proceedings 4th Environmental Engineering and Science Conference,* University of Louisville, Louisville, Kentucky, 1974.

[22] Air Products, *Aerobic Sludge Digestion,* Air Products and Chemicals Inc., Web Site:

www.airproducts.com/Markets/EnvironmentalWasteWaterTreatment/Sludge/Aerobic_Sludge_Digestion.html (2003).

[24] US EPA, *Construction Costs for Municipal Wastewater Treatment Plants: 1973-1977*, Office of Water Program Operations, Washington, DC, EPA 430/9-77-13, MCD-37, January, 1978.

[25] W. J. Jewell and R. M. Kabrick, Autoheated aerobic thermophilic digestion with air aeration, Presented at the *51st Annual Water Pollution Control Conference,* Anaheim, CA, October, 1978.

[26] N. R. Ahlberg and B. I. Boyko, Evaluation and design of aerobic digesters*,J. Water Pollut. Control Fed.,1972,* 44, 634.

[27] L. E. Ritter, Design and operating experiences using diffused aeration for sludge digestion, *J.Water Pollut. Control Fed.*, 1970, 42, 1982.

[28] Ramalho, P. A., Scholze, H., Cardoso, M. H., Ramalho, M. T., & Oliveira-Campos, A. M. Improved conditions for the aerobic reductive decolourisation of azo dyes by Candida zeylanoides. *Enzyme and Microbial Technology*, 2002, *31*(6), 848-854.

[29] Khalid, A., Arshad, M., & Crowley, D. E. Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains. *Applied Microbiology and Biotechnology*, 2008, *78*(2), 361-369.

[30] Pandey, A., Singh, P., & Iyengar, L. Bacterial decolorization and degradation of azo dyes. *International Biodeterioration & Biodegradation*, 2007, *59*(2), 73-84.

[31] Saxe, J. P., Lubenow, B. L., Chiu, P. C., Huang, C. P., & Cha, D. K. Enhanced biodegradation of azo dyes using an integrated elemental iron-activated sludge system: I. Evaluation of system performance. *Water environment research*, 2006, 19-25.

[32] Jafari, N., Soudi, M. R., & Kasra-Kermanshahi, R. Biodecolorization of textile azo dyes by isolated yeast from activated sludge: Issatchenkia orientalis JKS6. *Annals of Microbiology*, 2014, *64*(2), 475-482.

[33] Van der Zee, F. P., & Cervantes, F. J. Impact and application of electron shuttles on the redox (bio) transformation of contaminants: a review. *Biotechnology advances*, 2009, *27*(3), 256-277.

[34] Schwarzenbach RP, Stierli R, Lanz K, Zeyer J. Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqueous solution. Environ Sci Technol 1990, 24, 1566–1574.

[35] Curtis GP, Reinhard M. Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthraquinone disulfonate and humic acid. Environ Sci Technol 1994, 28, 2393–2401

[36] Tratnyek PG, Scherer MM, Deng BL, Hu SD. Effects of natural organic matter, anthropogenic surfactants, and model quinones on the reduction of contaminants by zero-valent iron. Water Res 2001, 35, 4435–4443.

[37] Dror I, Schlautman MA. Cosolvent effect on the catalytic reductive dechlorination of PCE. Chemosphere 2004, 57, 1505–1514.

[38] Keum YS, Li QX. Reduction of nitroaromatic pesticides with zero-valent iron. Chemosphere 2004, 54, 255–263.

[39] Cervantes, F. J., van der Velde, S., Lettinga, G., & Field, J. A. Quinones as terminal electron acceptors for anaerobic microbial oxidation of phenolic compounds. *Biodegradation*, 2000, *11*(5), 313-321.

[40] Lovley, D. R., Fraga, J. L., Blunt-Harris, E. L., Hayes, L. A., Phillips, E. J. P., & Coates, J. D. Humic substances as a mediator for microbially catalyzed metal reduction. *Acta hydrochimica et hydrobiologica*, 1998, *26*(3), 152-157.

[41] Lovley, D. R. Minimum threshold for hydrogen metabolism in methanogenic bacteria. *Applied and Environmental Microbiology*, 1985, *49*(6), 1530-1531.

[42] Van Bodegom, P. M., & Stams, A. J. M. Effects of alternative electron acceptors and temperature on methanogenesis in rice paddy soils.*Chemosphere*, 1999, *39*(2), 167-182.

[43] Fredrickson, J. K., Kostandarithes, H. M., Li, S. W., Plymale, A. E., & Daly, M. J. Reduction of Fe (III), Cr (VI), U (VI), and Tc (VII) byDeinococcus radiodurans R1. *Applied and Environmental Microbiology*, 2000, *66*(5), 2006-2011.

[44] Scott DT, McKnight DM, Blunt Harris EL, Kolesar SE, Lovley DR. Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ Sci Technol 1998, 32, 2984–2989.

[45] Wingard, L.B. Enzyme Engineering, Interscience Publishers, New York, 1972.

[46] Taylor, R.F. Protein Immobilization: fundamentals and applications, Marcel Dekker, New York, 1991.

[47] Klei, H.E.; Sundstrom, D.W.; Shim, D. Immobilization of enzymes by microencapsulation en Immobilized cells and enzymes: a practical approach J. Woodward, ed. IRL Press, 1985, 49-54

[48] Santos, A. B. D., de Madrid, M. P., Stams, A. J., Van Lier, J. B., & Cervantes, F. J. Azo dye reduction by mesophilic and thermophilic anaerobic consortia. *Biotechnology progress*, 2005, *21*(4), 1140-1145.

[49] Rau, J., Knackmuss, H. J., & Stolz, A. Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria. *Environmental science & technology*, 2002, *36*(7), 1497-1504.

[50] J.A. Field; J. Brady. Riboflavin as a redox mediator accelerating the reduction of the azo dye Mordant Yellow 10 by anaerobic granular sludge. Water Science & Technology 2003, 48(6), 187–193.

[51] Van Der Zee, F. P., Bisschops, I. A., Lettinga, G., & Field, J. A. Activated carbon as an electron acceptor and redox mediator during the anaerobic biotransformation of azo dyes. *Environmental science & technology*, 2003, *37*(2), 402-408.

[52] Pereira, L., Pereira, R., Pereira, M. F. R., Van der Zee, F. P., Cervantes, F. J., & Alves, M. M. Thermal modification of activated carbon surface chemistry improves its capacity as redox mediator for azo dye reduction.*Journal of hazardous materials*, 2010, *183*(1), 931-939.

[53] Amezquita-Garcia, H. J., Razo-Flores, E., Cervantes, F. J., & Rangel-Mendez, J. R. Activated carbon fibers as redox mediators for the increased reduction of nitroaromatics. *Carbon*, 2013, *55*, 276-284.

[54] Rios-Del Toro, E. E., Celis, L. B., Cervantes, F. J., & Rangel-Mendez, J. R. Enhanced microbial decolorization of methyl red with oxidized carbon fiber as redox mediator. *Journal of hazardous materials*, 2013, *260*, 967-974.

[55] Pereira, R. A., Pereira, M. F. R., Alves, M. M., & Pereira, L. Carbon based materials as novel redox mediators for dye wastewater biodegradation. *Applied Catalysis B: Environmental*, 2014, *144*, 713-720.

[56] Colunga, A., Rangel-Mendez, J. R., Celis, L. B., & Cervantes, F. J. Graphene oxide as electron shuttle for increased redox conversion of contaminants under methanogenic and sulfate-reducing conditions. *Bioresource technology*, 2015, *175*, 309-314.

[57] Guo J, Zhou J, Wang D, Tian C, Wang P, Salah Uddina M, Yu H. Biocatalyst effects of immobilized anthraquinone on the anaerobic reduction of azo dyes by the salt-tolerant bacteria. Water Research, 2007, 41(2), 426–432.

[58] Li L, Wang J, Zhou J, Yang F, Jin C, Qu Y, Li A, Zhang L. Enhancement of nitroaromatic compounds anaerobic biotransformation using a novel immobilized redox mediator prepared by electro-polymerization. Bioresource Technology, 2008, 99, 6908-6916.

[59] Martínez CM, Celis LB, Cervantes FJ. (2013) Immobilized humic substances as redox mediator for the simultaneous removal of phenol and Reactive Red 2 in a UASB reactor. Applied Microbial Biotechnology, 2013, 13, 5190-5195.

[60] Alvarez LH, Jimenez-Bermudez L, Hernandez-Montoy V, Cervantes FJ. Enhanced dechlorination of carbon tetrachloride by immobilized fulvic acids on alumina particles. Water Air Soil Pollutant, 2012, 223, 1911–1920.

[61] Zhou, Y., Lu, H., Wang, J., Li, J., Zhou, J., & Jin, R. Catalytic performance of functionalized polyurethane foam on the reductive decolorization of Reactive Red K-2G in up-flow anaerobic reactor under saline conditions. *Bioprocess and biosystems engineering*, 2015, *38*(1), 137-147.

[62] Zhang, H., Lu, H., Zhang, S., Liu, G., Li, G., Zhou, J., & Wang, J. A novel modification of poly (ethylene terephthalate) fiber using anthraquinone-2-sulfonate for accelerating azo dyes and nitroaromatics removal. *Separation and Purification Technology*, 2015, *132*, 323-329.

[63] Amezquita-Garcia, H. J., Razo-Flores, E., Cervantes, F. J., & Rangel-Mendez, J. R. Anchorage of anthraquinone molecules onto activated carbon fibers to enhance the reduction of 4-nitrophenol. *Journal of Chemical Technology and Biotechnology*, 2014, 90(9), 1685–1691.

[64] Guo, J., Kang, L., Wang, X., & Yang, J. Decolorization and degradation of azo dyes by redox mediator system with bacteria. In *Biodegradation of Azo Dyes*. Springer Berlin Heidelberg, 2010, 85-100.

[65] Rand, M. C., Greenberg, A. E., & Taras, M. J. *Standard methods for the examination of water and wastewater*. Prepared and published jointly by American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1976.

[66] Boehm, H. P. Chemical identification of surface groups. *Advances in catalysis*, 1966, *16*, 179-274.

[67] Boehm, H. P., Diehl, E., Heck, W., & Sappok, R. Surface oxides of carbon. *Angewandte Chemie International Edition in English*, 1964, *3*(10), 669-677.

[68] Lucas Howard J. A new test for distinguishing the primary, secondary and tertiary saturated alcohols. *Journal of the American Chemical Society*, 1930, *52* (2), 802-804

[69] Lee, Y. H. & Pavlostathis, S. G. Decolorization and toxicity of reactive anthraquinone textile dyes under methanogenic conditions. Water research, 2004, 38, 1838–1852.

[70] Fetzer, S. & Conrad, R. Effect of redox potential on methanogenesis by Methanosarcina barkeri. Arch. Microbiology, 1993, 160, 108–113.

[71] Özcan, A. S., Erdem, B., & Özcan, A. Adsorption of Acid Blue 193 from aqueous solutions onto BTMA-bentonite. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2005, *266*(1), 73-81.

[72] Menéndez, J. A., Phillips, J., Xia, B., & Radovic, L. R. On the modification and characterization of chemical surface properties of activated carbon: in the search of carbons with stable basic properties. *Langmuir*, 1996, *12*(18), 4404-4410.

[73] Spyroudis, S. Hydroxyquinones: synthesis and reactivity. *Molecules*, 2000, *5*(12), 1291-1330.

[74] Gilles, V., Vieira, M. A., Lacerda Jr, V., Castro, E. V., Santos, R. B., Orestes, E., & Greco, S. J. A New, Simple and Efficient Method of Steglich Esterification of Juglone with Long-Chain Fatty Acids: Synthesis of a New Class of Non-Polymeric Wax Deposition Inhibitors for Crude Oil. *Journal of the Brazilian Chemical Society*, 2015, *26*(1), 74-83.

[75] Aksoylu, A. E., Madalena, M., Freitas, A., Pereira, M. F. R., & Figueiredo, J. L. The effects of different activated carbon supports and support modifications on the properties of Pt/AC catalysts. *Carbon*, 2001, *39*(2), 175-185

[76] Abuilaiwi, F. A., Laoui, T., Al-Harthi, M., & Atieh, M. A. Modification and functionalization of multiwalled carbon nanotube (MWCNT) via fischer esterification. *The Arabian Journal for Science and Engineering*, 2010, *35*(1C), 37-48.

[77] Okumura, H., Kitazawa, N., Wada, S., & Hotta, H. Stability of sucrose fatty acid esters under acidic and basic conditions. *Journal of oleo science*, 2011, *60*(6), 313-320.

[78] Liu, G., Yang, H., Wang, J., Jin, R., Zhou, J., & Lv, H. Enhanced chromate reduction by resting Escherichia coli cells in the presence of quinone redox mediators. *Bioresource technology*, 2010, *101*(21), 8127-8131.

[79] Moreno-Castilla, C. Adsorption of organic molecules from aqueous solutions on carbon materials. *Carbon*, 2004, *42*(1), 83-94.

[80] Mattson, J. A., Mark, H. B., Malbin, M. D., Weber, W. J., & Crittenden, J. C. Surface chemistry of active carbon: specific adsorption of phenols.*Journal of Colloid and Interface Science*, 1969, *31*(1), 116-130.

[81] Dawood, S., & Sen, T. K. Removal of anionic dye Congo red from aqueous solution by raw pine and acid-treated pine cone powder as adsorbent: equilibrium, thermodynamic, kinetics, mechanism and process design. *Water research*, 2012, *46*(6), 1933-1946.

[82] Namasivayam, C., & Kavitha, D. Removal of Congo Red from water by adsorption onto activated carbon prepared from coir pith, an agricultural solid waste. *Dyes and pigments*, 2002, *54*(1), 47-58.

[83] Aygün, A., Yenisoy-Karakaş, S., & Duman, I. Production of granular activated carbon from fruit stones and nutshells and evaluation of their physical, chemical and adsorption properties. *Microporous and Mesoporous Materials*, 2003, *66*(2), 189-195.

[84] Mall, I. D., Srivastava, V. C., Agarwal, N. K., & Mishra, I. M. Removal of congo red from aqueous solution by bagasse fly ash and activated carbon: kinetic study and equilibrium isotherm analyses. *Chemosphere*, 2005, *61*(4), 492-501.

[85] Reddy, M. S. Removal of direct dye from aqueous solutions with an adsorbent made from tamarind fruit shell, an agricultural solid waste. *Journal of Scientific and Industrial Research*, 2006, *65*(5), 443.

[86] Vimonses, V., Lei, S., Jin, B., Chow, C. W., & Saint, C. Kinetic study and equilibrium isotherm analysis of Congo Red adsorption by clay materials. *Chemical Engineering Journal*, 2009, *148*(2), 354-364.

[87] Cervantes, F. J., van der Velde, S., Lettinga, G., & Field, J. A. Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiology Ecology*, 2000, *34*(2), 161-171.

[88] dos Santos, A. B., Bisschops, I. A., Cervantes, F. J., & Van Lier, J. B. Effect of different redox mediators during thermophilic azo dye reduction by anaerobic granular sludge and comparative study between mesophilic (30 C) and thermophilic (55 C) treatments for decolourisation of textile wastewaters. *Chemosphere*, 2004, *55*(9), 1149-1157.

[89] Sakkas, V. A., Islam, M. A., Stalikas, C., & Albanis, T. A. Photocatalytic degradation using design of experiments: a review and example [of the Congo red degradation. *Journal of hazardous materials*, 2010, *175*(1), 33-44.

[90] Parthasarathi, R., Padmanabhan, J., Sarkar, U., Maiti, B., Subramanian, V., & Chattaraj, P. K. Toxicity analysis of benzidine through chemical reactivity and selectivity profiles: a DFT approach. *Internet Electronic Journal of Molecular Design*, 2003, *2*(12), 798-813.

[91] Cervantes, F. J., Garcia-Espinosa, A., Moreno-Reynosa, M. A., & Rangel-Mendez, J. R. Immobilized redox mediators on anion exchange resins and their role on the reductive decolorization of azo dyes. *Environmental science & technology*, 2010, *44*(5), 1747-1753.

[92] McCarty, P. L., & McKinney, R. E. Salt toxicity in anaerobic digestion. *Journal (Water Pollution Control Federation)*, 1961, 399-415.

[93] Abdel-Shafy, H. I., & Mansour, M. S. Biogas production as affected by heavy metals in the anaerobic digestion of sludge. *Egyptian Journal of Petroleum*, 2014, *23*(4), 409-417.