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BIOSSORÇÃO & BIODEGRADAÇÃO DE AZO CORANTE – DESENVOLVIMENTO DE PROCESSOS UTILIZANDO RESÍDUOS COMO FONTE DE MICRO-ORGANISMOS

AZO DYE BIOSORPTION & BIODEGRADATION - PROCESS DEVELOPMENT WITH RESIDUES AS SOURCE OF MICROORGANISMS

GRAZIELY CRISTINA DOS SANTOS

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas - Microbiologia Aplicada.

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TÍTULO: BIOSSORÇÃO & BIODEGRADAÇÃO DE AZO CORANTE - DESENVOLVIMENTO DE PROCESSOS UTILIZANDO RESÍDUOS COMO FONTE DE MICROORGANISMOS

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"Only the one who does not question is safe from making a mistake."

Albert Einstein

RESUMO

As indústrias têxteis estão entre aquelas que utilizam a maior parte da água em seus processos, além de diversos produtos químicos tais como corantes, detergentes, sais e mordentes. Assim, grandes volumes de efluentes são gerados. O tratamento de águas residuais, às vezes, não é satisfatória. Inúmeros estudos têm sido realizados para encontrar maneiras mais eficientes e de baixo custo para o tratamento de efluentes têxteis. Os tratamentos biológicos têm se mostrado eficazes, uma vez que alguns micro-organismos têm demonstrado sua capacidade de descoloração de efluentes. O presente estudo teve por objetivo avaliar o processo de descoloração do azo corante Direct Red 75 (DR75) 100 mg/L utilizando diferentes fontes de micro-organismos em diferentes sistemas de tratamento, a fim de encontrar uma alternativa eficaz e de baixo custo para o tratamento biológico de efluentes têxteis. Esta avaliação é apresentada em três capítulos brevemente descritos abaixo. O primeiro capítulo mostra a comparação entre dois micro-organismos. O fungo *Phanerochaete chrysosporium* e a levedura *Saccharomyces cerevisiae* (fermento biológico) foram os micro-organismos utilizados no experimento do 1° capítulo, enquanto um consórcio de micro-organismos obtido a partir do inóculo de um biodigestor de alimentos foi utilizado no segundo capítulo. Ambos os experimentos tiveram DR75 como única fonte de carbono e os resultados foram expressos em porcentagem de descoloração e absorbância relativa. A absorbância relativa, obtida pela razão entre a absorbância no comprimento de onda dos grupos cromóforo e azo, sugere a ocorrência de biodegradação pela variação das razões das amostras em comparação com o controle. Os resultados do capítulo 1 mostraram que o tratamento com *S. cerevisiae* foi mais eficiente que com *P. chrysosporium*, e a absorbância relativa sugere a ocorrência de biodegradação para ambos. Portanto, o DR75 foi mais facilmente biodegradado pela levedura do fermento biológico, que pode ser facilmente adquirida, do que pela estirpe única de fungo, que parece ser uma opção cara e trabalhosa para o tratamento de efluentes têxteis. A análise de biossorção e biodegradação de DR75 pelo inóculo do biodigestor de alimentos foram apresentados no segundo capítulo. O consórcio testado mostrou eficiência na remoção, mostrando ter potencial para a remoção do corante. O terceiro capítulo apresenta a avaliação da utilização de um consórcio microbiano a partir de cascas de arroz em ambos os sistemas investigados, além da análise de glucose e extrato de levedura como fonte adicional de carbono. A remoção de DQO também foi analisada. Os sistemas investigados foram um biorreator anaeróbio (Biorreator A) preenchido com meio suporte de polietileno do tipo MBBR (Reator Biológico com Leito Móvel - *Moving Bed Biofilm Reactor*) e um biorreator de

leito fixo (Biorreator B) composto por 3 biorreatores anaeróbios e 1 aeróbio (sistema anaeróbio/aeróbio sequenciado) preenchido com cascas de arroz como meio suporte. O Biorreator B alimentado com meio de cultura de extrato de levedura apresentou as melhores taxas de descoloramento atingindo mais de 90% de descoloração, e a melhor remoção de DQO quando comparado ao Biorreator A. A descoloração utilizando glicose como fonte de carbono não foi tão eficiente como o extracto de levedura. As aminas aromáticas foram avaliadas por LC/MS, GC/MS e HPLC; no entanto, a de identificação das aminas não foi satisfatória devido à elevada porcentagem de compostos desconhecidos na composição do corante. Portanto, as cascas de arroz podem ser uma boa alternativa como meio suporte para o tratamento de efluentes têxteis, além do processo anaeróbio/aeróbio ser melhor indicado para ocorrência de biodegradação. Sendo assim, vimos que é possível o uso de resíduos agroindustriais e alimentícios serem usados como material de baixo custo para o tratamento de efluentes.

Palavras-chave Direct Red 75. Cultura mista. Cascas de arroz. MBBR. Aminas.

ABSTRACT

The textile industries are among the ones which use the most water in their processes, in addition to many chemical products such as dyes, detergents, salts and mordant. Thus, great volumes of effluents are generated. Sometimes the wastewater treatment is not satisfactory. Numerous studies have been conducted to find more efficient and low-cost ways to treat textile wastewater. Biological treatments have been proven to be efficient, since some microorganisms have shown the ability to effluent decolorization. The present study aims to evaluate the decolorization process of the azo dye Direct Red 75 (DR75) 100 mg/L by different sources of microorganisms in different treatment systems in order to find an effective and low-cost alternative for the biological treatment of textile wastewater. The evaluation is presented in three chapters briefly described below. First chapter shows the comparison between two microorganisms. The fungus *Phanerochaete chrysosporium* and the fresh yeast *Saccharomyces cerevisiae* were the microorganisms used in the experiment of the first chapter, while a consortium of microorganisms obtained from an inoculum of food waste biodigester was used in the second chapter. Both experiments had DR75 as the only carbon source and results expressed as percentage of decolorization and absorbance ratio. Absorbance ratio, obtained by the ratio between the absorbance at a wavelength of the chromophore and azo groups, suggests the occurrence of biodegradation by varying the values of the samples compared to control. The results from chapter 1 showed that treatment with *S. cerevisiae* was more efficient, than P. *chrysosporium* and the absorbance ratio suggests the occurrence of biodegradation for both. Therefore, the DR75 was more easily biodegraded by fresh yeast that can be easily acquired, than by single strain of fungus, which seems to be an expensive and labored option for the treatment of textile wastewater. Analysis of biosorption and biodegradation of DR75 by the inoculum of food waste biodigester were presented in the second chapter. The tested consortium showed removal efficiency presenting its potential for dye removal. The third chapter presents the evaluation of the use of a microbial consortium from rice husks in both systems investigated besides the analysis of glucose and yeast extract tested as additional carbon source. COD removal was also analyzed. The systems investigated were an anaerobic bioreactor (Bioreactor A) filled with polyethylene carriers used to Moving Bed Biofilm Reactor (MBBR) process and a fixed bed bioreactor (Bioreactor B) composed by 3 anaerobic bioreactors and 1 aerobic bioreactor forming (sequenced anaerobic/aerobic system) filled with rice husks as carriers. Bioreactor B fed with yeast extract culture medium presented the best decolorization rates reaching more than 90 % of decolorization and the best

COD removal when compared with Bioreactor A. The decolorization using glucose as carbon source was not as efficient as the yeast extract. The aromatic amines were evaluated by LC/MS, GC/MS and HPLC; however the attempt did not succeed well due to the high percentage of unknown compounds in the dye composition. Therefore, the rice husks can be a good alternative as a support material to textile wastewater treatment and the anaerobic/aerobic process is better indicated to dye degradation. Thus, it can be noticed that the use of residues from agro-industry and food industry, used as low-cost material, for wastewater treatment is possible.

Keywords Direct Red 75.Mixed culture. Rice husks. MBBR. Amines.

Contents

1 INTRODUCTION

With the continuous increase in human population comes the increase in the use of water, both for human use and for the utilization in agriculture and in industrial processes. Industries consume a great amount of water in their processes. According to the United Nations Educational, Scientific and Cultural Organization (UNESCO) (2003), approximately 22% of the total use of water in the world is for industry and, in developing countries, 70% of the industrial residues are discharged in rivers without treatment.

The textile industries are among the ones which use the most water in their processes, in addition to many chemical products such as dyes, detergents, salts and mordant. As great water consumers, these industries are also great generators of effluent.

The fashion industry is each day more exigent. In order to supply new textiles and new colors, the textile industry has been making a great effort to develop new technologies. Since new textiles have been developed, new dyes have been produced as well, aiming to present suitable characteristics for better fixation to the new fabric. Thus, the textile wastewater is increasingly complex and hard to be treated.

Since a certain amount of dye does not fix at the fabric fiber, the wastewater tends to be strongly colorful. Hence, as the dyes are easily visible to the unaided eye, there is a pressure by the population and authorities to stop the discard the treated wastewater still colorful in the watercourses. For this reason, it is crucial to have some information about the wastewater composition, i.e., about the dyes and other compounds used in processes such as dyeing, bleaching and washing.

The azo dyes are widely used by the textile industries and their degradation is a great challenge for the researchers. New methods for the dye degradation have been investigated and developed in order to improve the textile wastewater treatment. The treatment can be physical, chemical and biological.

From the point of view of some industries, the cheapest treatment system is always the best one. However, the cheapest system can be sometimes the most polluting. Thus, researchers have struggled to find an eco-friendly and low-cost treatment system. The biological treatment has demonstrated to be the most suitable choice.

Therefore, the dye biological treatment should be investigated in order to understand the biodegradation process and optimize the treatment system. For this purpose, microorganisms from different sources were evaluated regarding the ability to biodegrade an azo dye. Moreover, the presence of a carbon source to improve the decolorization process and the use of a support material were also analyzed.

Chapter 1 analyzes the biodegradation of the azo dye DR75 by *Phanerochaete chrysosporium* and *Saccharomyces cerevisiae*, pure cultures, by means UV-VIS spectrophotometry.

Chapter 2 evaluates both biosorption and biodegradation of DR75 by a mixed culture obtained from a food waste biodigester using the same method as chapter 1.

Chapter 3 investigates the DR75 biodegradation process in an anaerobic bioreactor using plastic carriers of Moving Bed Biofilm Reactor (MBBR) technique and an anaerobic/aerobic fixed bed bioreactor using rice husks as carrier. Rinse water of rice husks was used as a source of microorganisms for the anaerobic bioreactor, while the rice husks was used as a source of microorganisms for the fixed bed bioreactor. The research reported in chapter 3 was performed at Linnaeus University, Växjö, Sweden financed by the Program of sandwich doctorate abroad (*Programa de doutorado sanduíche no exterior* - PDSE) from CAPES.

2 AIMS

2.1 General aim

It is to evaluate the decolorization process of the azo dye Direct Red 75 (DR75) using different sources of microorganisms in different treatment systems in order to find an effective and low-cost alternative for the biological treatment of textile wastewater.

2.2 Specific aims

- Identifying a low-cost alternative and an effective treatment system to the biodegradation of the DR75 azo dye;
- Investigating different sources of microorganisms for the DR75 treatment;
- Analyzing the biodegradation capacity of the fungus *Phanerochaete chrysosporium*, the fresh yeast *Saccharomyces cerevisiae* and the mixed culture of microorganisms obtained from a food waste biodigester and rice husks;
- Evaluating the dye biodegradation in systems with DR75 as only carbon source as well as investigating the role of glucose and yeast extract in the biological treatment.
- Evaluating different methods and support materials to the dye biodegradation in bioreactors;
- Checking the presence of aromatic amines as generated metabolites of the Direct Red 75 azo dye biodegradation process in bioreactors with different materials support.

3 BACKGROUND

3.1 Dyes

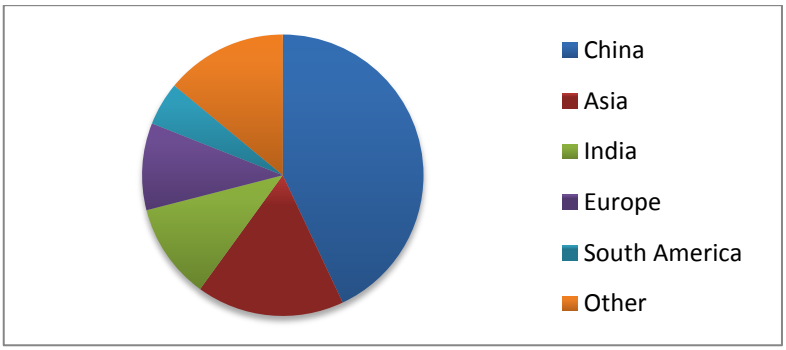
The clothes have become increasingly colorful. Not only the clothes, but also many other things such as pictures, plastic articles, posters, magazines, makeup, cars, household appliances, etc. are more colorful. This wide range of colors is possible due to the dyes and pigments. The difference between dyes and pigments is related to solubility. Dyes are soluble colored compounds, while pigments are insoluble compounds and it is incorporated by a dispersion process into products such as plastics (CHRISTIE, 2001).

The dyes are applied mainly to textile materials from solution in water. Before the existence of synthetic dyes, the textile materials were dyed with natural dyes from plants or animals until the mid-nineteenth century. Synthetic textile dyes are exclusively organic compounds (CHRISTIE, 2001, ERKURT, 2010).

William Henry Perkin discovered the first synthetic dye, a purple dye, in 1856. The original name was Aniline Purple, but later had become known as Mauveine (CHRISTIE, 2001, HUNGER, 2003).

Nowadays, countless synthetic dyes have been applied in the textile industry to supply the fashion world which dictates the types of colors used. The colors used for textiles have an effect on the total dyes consumption. Figure 1 presents the world consumption of synthetic dyes in 2013 and it can be seen that China has become the major consumer accounting for 40–45% and India accounts for about 10% of world consumption (IHS, 2014).

Figure 1 - World consumption of synthetic dyes in 2013



Source: Adapted from IHS (2014).

It is expected that the dye market increases at an average annual rate of about 3% (IHS, 2014).

Since there are numerous dyes produced in the world, there is the necessity to register and to classify every one properly. The responsibility for this task is held by The Society of Dyers and Colorists from England which manages a database of dyes known as Colour Index.

The nomenclature is related to the classification of the dyes. Dyes can be classified according to chemical structure such as azo dyes, anthraquinone dyes or by the application method such as direct dyes, reactive dyes, etc (HUNGER, 2003).

According to Hunger (2003), the chemical classification is more appropriate than the usage classification, since it is easier to identify a dye belonging to a group with specific characteristics, for instance, azo dyes are strong, good all round properties and cost effective.

Despite that, Colour Index has adopted the application method as the main system to the dye nomenclature. For this reason, the colorants have a C.I. generic name given in accordance with the application method, its hue and a serial number related to the chronological order in which the colorant has been registered in the Colour Index (CHRISTIE, 2001, ERKURT, 2010). For instance, Direct Red 75. Direct is the class of dye, red is the hue and 75 is the chronological number which this specific dye was registered.

Besides the differences between the classification systems and the dye nomenclature, there are also different nomenclatures for the groups that compose the dye. Dyes contain 2 special groups called chromophore and auxochrome groups. Common chromophores are –C=C–, –C=N–, –C=O, –N=N–, $–NO_2$, quinoid rings, while $–NH_3$, –COOH, $–SO_3H$, –OH are common auxochromes.

Therefore, chromophores are electron systems with conjugated double bonds and auxochromes are electron withdrawing or electron donating substituents that cause the color of the chromophore by altering the overall energy of the electron system; which means the excitation of the electrons in the molecule makes the light be transmitted, in certain wavelength, and then, the color can be seen (FORSS, 2013, GHALY *et al*., 2014).

One of most representative dye classes in the world is the azo dye, which has at least one azo chromophore group (–N=N–) in its structure. About 60 to 70% of the dyes used in the

industries belong to azo class. For this reason, they are present in a large amount of the textile wastewater, besides being the most researched class of dyes (HUNGER, 2003, GHALY *et al*., 2014).

3.1.1 Azo dyes and aromatic amines

In 1858, Peter Griess produced a diazonium salt, generated by the mixing of an aromatic amine with nitrous acid, that could be used to prepare colored compounds (CHRISTIE, 2001). From there, many other azo dyes were produced.

Currently, about three thousand azo dyes are known and are in use worldwide (VALIZADEH *et al*., 2015). Most of these compounds are monoazo, which contain one azo group, usually linking two aromatic rings, but it is also possible to contain two (diazo), three (triazo) or more azo groups (polyazo) (HUNGER, 2003).

The azo dyes are widely used by industries such as food and textile industries. One of the probable solutions to the wastewater treatment which contain azo dyes is the degradation, whether by physical-chemical treatment or biological treatment. However, azo dyes are able to split off some genotoxic and carcinogenic amines (SCHNEIDER *et al*., 2004).

For this reason, the azo dyes that generate aromatic amines proven to be carcinogenic were banned by the Textile sector since 2003, following the recommendation of Directive 2002/61/EC of the European Parliament as regards the limitation of the marketing and use of certain dangerous substances and preparations (BASTIAN, 2009).

Aromatic amines are formed when the azo dyes are reduced. Since the azo bond is broken and the amines are still present, the wastewater becomes colorless and toxic (ERKURT, 2010). Some textile industries are not concerned about the efficiency of the treatment which removes color and toxicity. They want just the color removal since this phase can be faster and cheaper than the whole process.

Researchers all over the world have struggled to find a way to eliminate the aromatic amines from the wastewater treated. Regarding the biological treatment, the aromatic amines degradation by microorganisms has been extensively investigated (HOSSEINI KOUPAIE *et al*., 2011, JONSTRUP *et al*., 2011, BRÜSCHWEILER *et al*., 2014, BAÊTA *et al*., 2015).

According to van der Zee and Villaverde (2005), the aromatic amines are generated by the reductive cleavage of the azo bond during the anaerobic treatment and it can be degraded during the aerobic treatment. Thus, the sequenced anaerobic-aerobic treatment seems to be suitable for this purpose, even though there is still much study to be done.

3.1.2 Direct dyes

Previously in history, it was necessary to fix the dye in the fibers using mordants. However the direct dyes were the first class that could be applied directly to the fibers, giving rise to the class of direct dyes (CHRISTIE, 2001).

Some azo dyes are direct dyes as well, since the dyes have two different classifications. Direct dyes are widely used by the textile industry due to some specific characteristics, for instance solubility and ease of application.

Direct dyes are mainly applied in the dyeing of cellulosic fibers, as cotton and viscose, in paper, though it can be applied in leather as well. They are anionic compounds, designed to be soluble in water due to the addition of some groups such as –OH, $–SO_3^-$, and they are commonly used because of the low cost and easy application, despite the fastness to satisfy the requirements moderately. Moreover, they are available in a wide range of hues (HUNGER, 2003, SEKAR, 2011, WAWRZKIEWICZ, 2011, SANTOS and BOAVENTURA, 2015).

The solubility of these dyes is provided by the sulphonic acid groups in the structure and H-bonds and Van der Waals force keeps the dye retained by the fiber (CHRISTIE, 2001, HUNGER, 2003, CHAKRABORTY, 2014).

Direct dyes are almost completely dissociated in water bath containing electrolytes and ionic salts. A disadvantage of these dyes is related to the bond strength. The bonds are not strong enough to allow the dye keep itself attached to the fiber (WAWRZKIEWICZ, 2011, GHALY *et al*., 2014). For this reason it is better to wash the fabric dyed with direct dyes separated from others.

3.1.3 Direct Red 75

The dye investigated in this research is the Direct Red 75 (DR75) (Figure 2), an azo dye. Its structure is composed by a diphenyl urea group, 4 sulphonic groups, 2 naphthol

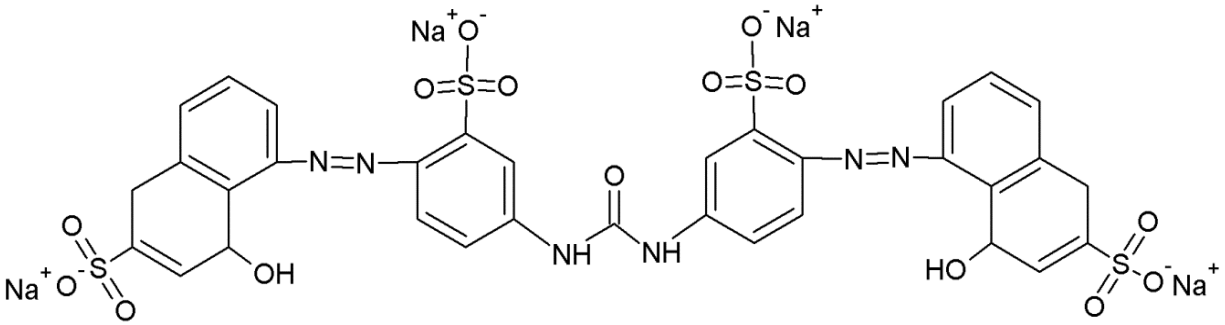
groups and 2 azo groups; therefore, a diazo dye (SEKAR, 2011). The structural formula is presented in Figure 3.

Direct Red 75 is also known as Chlorazol fast pink BK and Sirius Rose BB. The molecular weight is 990.79.

Figure 2 – Direct Red 75 in solution (100 mg/L)

Source: Made by the author.

Figure 3 – Structural formula of the azo dye Direct red 75



Source: Made by the author.

DR75 has 30% of dye content and, since this dye is patented, it was not possible to have detailed information about the composition of the 70% left. According to Semeraro *et al*. (2015), the direct dyes content is about 40-50% of dyestuff, that is more than DR75 has, and the remaining part (60-50%) consists of auxiliaries such as sodium sulfate besides other unknown substances. It is complicated to prove it without any information from the manufacturer.

This diazo dye has been applied not only in the textile industry, but in other industries as well. DR75 has been used as ink-jet ink, exhibiting high lightfastness, and as biological stain, for instance, to determine the amount of hepatic collagen of a rat liver (HUNGER, 2003, GHOSH *et al*., 2010).

3.2 Textile industry

Textile industry is one of the industries in the world that provides employment that does not require special skills. Due to this fact, it is one of the major employers in developing countries such as China, India, Bangladesh, Vietnam, Sri Lanka and Mauritius (GHALY *et al*., 2014).

The Brazilian textile market plays an important role in the worldwide market as well. It is the fifth largest textile industry in the world and the fourth largest clothing industry; the second largest denim producer and third in production of knitted fabric. Brazil is a world reference in beachwear, jeanswear and homewear (CONFEDERAÇÃO, 2012).

In spite of these numbers and of the importance of the textile industry to the country, the legislation on the use of dyes by the textile industries in Brazil and to protect the environment and the human health is not as developed as in the European Union.

According to the National Confederation of Industry (*Confederação Nacional da Indústria*) and the Brazilian Association of Textile and Clothing Industry (*Associação Brasileira da Indústria Têxtil e de Confecção*) (2012), there are six laws and federal resolutions that can be applied to textile industries and wastewater treatment in Brazil:

1. Conama Resolution nº 430/2011 – Conditions and effluent discharge standards

This resolution provides for the conditions, parameters, standards and guidelines for management of effluent discharge into receiving water bodies, partially changing and supplementing the Resolution nº 357 of 17 March 2005 from the National Environmental Council (*Conselho Nacional de Meio Ambiente* – CONAMA). The Resolution nº 357/2005 provides for the classification and environmental guidelines for the surface water bodies' framework as well as establishes the conditions and effluent discharge standards.

2. Law nº 6.938/1981 – National Environmental Policy, its purposes and mechanisms of formulation and application

The aim is the preservation, improvement and recovery of environmental quality to life, in order to ensure conditions in the country to the socio-economic development, the interests of national security and the protection of the dignity of human life.

This law defines the textile and clothing industries as potentially polluting activity of medium grade and the aim is to reduce the environmental impact by up to 10 years for investments in the sector with modern and clean technology.

3. Law nº 10.165/2000 – Implantation of rates linked to the National Environmental Policy

The textile and clothing industry is liable to tax with an average rate of potentially polluting activity.

4. Conama Resolution nº 313/2002 – Destination of industrial solid waste

This resolution regulates the recycling and presents very positive outlook for the correct destination of the sludge, there are even practical projects in that direction, as well as for textile waste from clothes manufacturing.

5. Law nº 9.984/2000 – National Water Resources Policy and Management System

This law represents, for the textile industry, the start of the National Agency of Water (*Agência Nacional de Águas* – ANA) activities and its relationship with the Ministry of Environment, resulting in direct consequences for the textile and clothing industry such as the search for water consumption indicators in the various links of industry production as well as signaling for constant reassessment of these indicators in order to reduce and reuse of water resources.

6. Conama Resolution nº 237/1997 – Environmental licensing incorporated to the environmental management tools

Among the activities or projects subjected to environmental licensing that directly or indirectly affect the textile sector are the chemical industry by the manufacture of resins and fibers besides artificial and synthetic yarns, and the textiles and clothing industries, footwear

and fabric artifacts by the processing of textile fibers, vegetable, animal and synthetic origin fibers; manufacturing and finishing of yarns and fabrics; dyeing and printing.

Many environmental problems can be generated if those laws and resolutions are not fulfilled.

Besides the application of many dyes and other chemicals in different processes, the textile industry also has to deal with an important issue, the excessive use of water, since they use a large volume of water to dyeing, washing and rinsing the fabrics. According to Ghaly *et al*. (2014), about 200 L of water are used to produce 1 kg of fabric. Though, the exact volume of water required differs from industry to industry depending on the dyeing process and the type of fabrics produced.

As mentioned before, the amount of chemicals applied in all process in a textile industry is huge and due to the variety of chemicals compounds, some of them are low or non-biodegradable compounds, besides salt and organic load in the effluent make it complicated to be accordingly treated. Moreover, when the wastewater is released into the watercourses, they are able of harming the environment and human health (BENZINA *et al*., 2012, GHALY *et al*., 2014, PUNZI *et al*., 2015). For instance, the presence of some chemicals in excess can cause the eutrophication of the rivers, causing the death of aquatic organisms. The impact of the dyeing process to the environment can be seen in Table 1.

Table 1 – Impact of the dyeing process with direct dyes to the environment

Input in the system	Output in the system
- Formaldehyde (dye fixation), direct dye, salts (sodium chloride, sodium sulfate and sodium carbonate, in some cases), surfactant, cationic resin (condensation of dicyandiamide salts / ammonia or formaldehyde used in the dye fixation), complexing agents (EDTA, DTPA, DTPMP) and equalizing agents (fatty amine ethoxylate), etc; - Electricity; - Steam; - Water (dyeing operations, washing the textile material and equipment); - Compressed air (compressed gas cylinders).	- Air Pollution: ↳ Air emissions (heat, through heat exchange). - Water Pollution: ↳ Generation of wastewater (residual bath dyeing and washing water from the washing of textile material and equipment). - Soil Pollution: ↳ Generation of waste (packaging).

Source: Adapted from Bastian (2009)

The generation of colorful wastewater is among the main environmental impacts identified in a textile industry. The effluent composition changes according to the productive process which makes the characterization of the wastewater difficult. Since each sector of the industry generates an effluent with specific characteristics and certain organic load, it is required an specific treatment to comply the environmental legislation (BASTIAN, 2009).

3.2.1 Textile wastewater treatment

Dyes are colorful compounds, highly visible. Thus, even minor releases into the environment may cause the appearance of color in watercourses, which attracts the critical attention of the public and local authorities, limits water uses, affects sunlight penetration and, consequently, affects the photosynthesis, due to the reduction of dissolved oxygen concentration, causing damage to the aquatic life (CHRISTIE, 2001, SARATALE *et al*., 2011, SANTOS and BOAVENTURA, 2015).

The government environmental agencies stipulated some rules about the textile effluent discharges that should be followed by the textile industries. The treated water must have lower concentration of nitrogen than the wastewater and must not be colorful. Nevertheless, the textile industries still have not a proper treatment to remove these compounds from the wastewater (BENZINA *et al*., 2012).

The wastewater is colorful due to the presence of non-fixed dyes, in other words, a small percentage of the dye is not fixed to the fibers, remaining in the effluent. The percentage of non-fixed direct dyes that could remain in the effluents is 5-30% (BASTIAN, 2009). Therefore, the dyes must be removed from the wastewater, as well as the organic load and other chemicals in general, to avoid environmental pollution.

However, it is a challenge to succeed in the end of the wastewater treatment. Many researchers accepted this challenge and they are working to find a solution. Generally, it is required that the effluent passes through several processes to obtain water quality (WAWRZKIEWICZ, 2011).

Every treatment method has advantages and drawbacks and the choice of the most appropriate methods will depend on the characteristics of each effluent (WAWRZKIEWICZ, 2011). The options are physical, chemical or biological treatment. Some physical and chemical parameters of the wastewater must be known to enable the choice of the most appropriated treatment. Some examples of the physical parameters are color, odor, pH, temperature, suspended and dissolved solids while chemical parameters are organic and inorganic compounds (SHULER and KARGI, 2002).

The physical treatment is performed by different techniques, mostly non-destructive since the dye molecules bonds are not broken. The dye can be removed from the wastewater by adsorption, filtration, coagulation/flocculation and sedimentation (FERNÁNDEZ *et al*., 2010).

Adsorption of dyes occurs when the dye molecules diluted in the wastewater are attached onto a solid surface (FORSS, 2013). This method has been employed for the dye removal from wastewater and several adsorbents have been evaluated such as activated carbon, sugarcane bagasse, rice husk ash (RHA) and microorganisms which the method is known as Biosorption and it is a biological method as well (CORSO and ALMEIDA, 2009,

KHALED *et al*., 2009, LAKSHMI *et al*., 2009, CORSO *et al*., 2012, MEZOHEGYI *et al*., 2012, MITTER *et al*., 2012, ALMEIDA and CORSO, 2014).

Filtration is a technique to separate particles from liquids and membranes have been commonly used to this purpose. There are membranes made of different materials, polymers and pores sizes. The three filtration methods widely used are ultrafiltration, nanofiltration and reverse osmosis (FORSS, 2013). As the filtration has been used for water reuse and chemical recovery, this technique has been probed by textile industries in order to use membranes that are able to separate the hydrolyzed dyestuffs and dyeing auxiliaries (BUSCIO *et al*., 2015, CHEN *et al*., 2015). Simultaneously, it is possible to reduce the color, BOD and COD of the wastewater (SARATALE *et al*., 2011).

Coagulation/flocculation are processes mostly used as pre-treatment to remove suspended particles and coloring materials by reducing their surface charge and gather them to form larger particles prior to other treatment (DRAGAN and DINU, 2008, AHMAD *et al*., 2015). Coagulants are added with intense mixing of the wastewater with the purpose to reduce or neutralize the surface charge of fine suspended particles while flocculants are added with gentle mixing and its purpose is to bring the fine particles close together to form larger particles (AHMAD *et al*., 2015). Coagulation and flocculation agents have been used to remove and even to degrade dyes in water solution. For instance, the green refined laterite soil investigated by Lau *et al*. (2014), was able to degrade the azo dye Acid Orange 7 and Dragan and Dinu (2008) evaluated the azo dye removal in aqueous solution by coagulation/flocculation using polycations which are synthetic flocculants.

Sedimentation is generally applied to remove the larger particles, formed during the flocculation, by using the gravity to deposit the suspended particles in the bottom of the wastewater treatment tank (GHALY *et al*., 2014, AHMAD *et al*., 2015).

The chemical treatment is usually destructive. The oxidation occurs by the application of oxidizing agents, such as ozone (O_3), hydrogen peroxide (H_2O_2) and permanganate (MnO_4), to modify the dye molecule by chemical reactions. Afterwards, the dye will be susceptible to the degradation (VON SONNTAG, 2008, FERNÁNDEZ *et al*., 2010, SARATALE *et al*., 2011).

In the advanced oxidation process (AOP), the radicals $OH^{\cdot}$ formed are more reactive than the others oxidizing agents already cited (VON SONNTAG, 2008). According to Fernández *et al.* (2010), the AOP are divided in two classes: non-irradiation process including Fenton process, ozonation, sonolysis, electrochemical oxidation, electrical discharges and wet air oxidation, and irradiation process including photolysis and photocatalysis. The application of these methods for the dye textile wastewater treatment has been evaluated by some researchers as Punzi *et al*. (2015), Yu *et al*. (2014) and Jóźwiak *et al*. (2007) with satisfactory results.

Though the physical and chemical methods have demonstrated certain efficiency in the textile wastewater treatment, these techniques are still expensive; furthermore they have some operational problems as high sludge formation and regeneration requirement (ERKURT, 2010). Thus, the biological treatment has become an interesting alternative to treat textile wastewater.

The biological treatment is the most common technique used to wastewater treatment, having been applied for over 150 years (HUNGER, 2003). According Ahmad *et al*. (2015), the principle of the biological treatment system is the conversion of biodegradable wastes into simpler and harmless species through biological processes by many microorganisms such as bacteria, fungi and algae.

There are two different processes in the biological treatment, aerobic and/or anaerobic process (SHULER and KARGI, 2002, HUNGER, 2003). Aerobic process takes place by aerobes microorganisms capable of growth at full oxygen tensions with carbon dioxide, water and biomass as final products. Anaerobic process takes places by microorganisms that cannot tolerate oxygen and the final products are carbon dioxide, methane and biomass (CHRISTIE, 2001, MADIGAN and MARTINKO, 2006, AHMAD *et al*., 2015). However, there are exceptions in both groups of microorganisms. Microorganisms that can tolerate lower level of oxygen in the air are called Microaerophiles. The facultatives microorganisms are able to growth with or without oxygen in the atmosphere, despite to growth better with oxygen. The anaerobes can be aerotolerants or strict anaerobes. Aerotolerants tolerates oxygen though they cannot use it to their metabolism while strict anaerobes can be inhibited or killed by oxygen (MADIGAN and MARTINKO, 2006).

Biological treatment has been constantly chosen by textile industry to degrade dyes and to treat the wastewater. Since the azo dyes are among the most applied dye in fabrics, the azo dye biodegradation process has become an interesting target of researches.

3.2.2 Biodegradation of azo dyes

Biodegradation process offers the attraction of the potential for the decolorization of the colorful wastewater with possible mineralization of the organic materials present (CHRISTIE, 2001).

Several techniques have been developed in order to improve the quality of the treatment. The selection of the most appropriate technique will depend on many factors such as the effluent conditions, type of dye and concentration, in this case the azo dye structure, operating conditions, treatment quality needed, costs, flexibility, environmental impact, carbon and nitrogen sources, salinity, pH, temperature, the presence or absence of oxygen, etc. *(SOLÍS et al., 2012, AHMAD et al*., 2015).

According to Santos and Boaventura (2015), the biodegradation process of azo dyes is based on the cleavage of chemical bonds that produces hazardous aromatic amines followed by biodegradation of these metabolites to release a colorless and not harmful effluent. Then the aromatic amines are generated by anaerobic reaction, since the azo dyes can be used as electron acceptors. Afterwards, the aromatic amines are biodegraded by aerobic reaction (BAÊTA *et al*., 2012).

The biodegradation mechanism of azo dyes by microorganisms involves enzymatic reactions responsible for the reduction or oxidation of compounds, leading to the bonds break. The efficiency of this process depends on the adaptability and the activity of the selected microorganisms (SARATALE *et al*., 2011).

Punzi *et al*. (2015) evaluated the microbial community of a continuous anaerobic biofilm reactor used to treat a synthetic textile wastewater and they observed that not only bacteria, but also fungi and algae may play a major role in the decolorization and biodegradation of that wastewater.

3.2.2.1 Bacteria

Bacteria are one of the most often employed microorganisms in the biological treatment of textile industry. Some of the reasons are the ease to cultivate and rapid growth under aerobic or anaerobic conditions. Some species are adapted to extreme conditions and they are able to express different types of enzymes (SOLÍS *et al*., 2012), that could be an advantage for the success of the treatment.

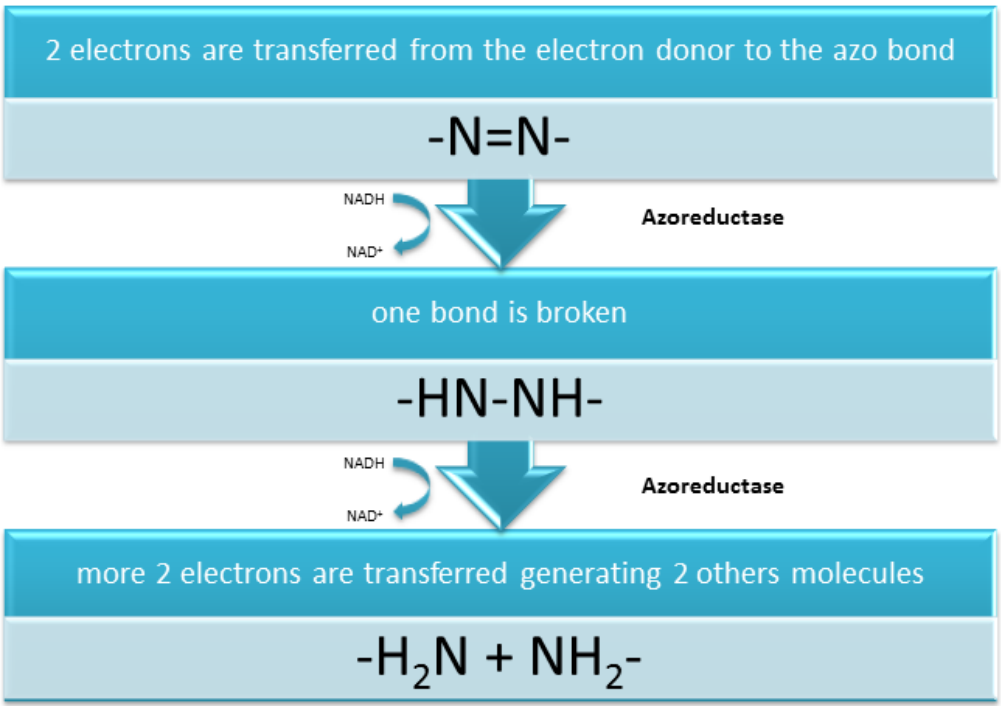
Some researchers have analyzed the microbial community associated to the dye biodegradation and they noticed that it is common to find more bacteria than other microorganisms. Punzi *et al.* (2015) evaluated the microbial diversity in anaerobic reactors and they verified, by means of DGGE fingerprints of bacterial and eukaryotic rDNA, that the bacterial diversity is higher than the eukaryotic diversity. The reason can be the high metabolic flexibility of the bacteria. Yang *et al*. (2012) also demonstrated that the bacteria are the predominant microorganism in the treatment system. They analyzed the microbial diversity in a full-scale printing and dyeing wastewater system under aerobic and anaerobic conditions. Forss (2013) investigated the microbial community in anaerobic and aerobic reactors filled with rice husks and reported the presence of bacteria and fungi. The bacteria were more abundant in the anaerobic and aerobic reactors than fungi, likewise the previously cited research.

The biodegradation of azo dye by bacteria takes place by means of enzymes, reductases and oxidases. Reductases start the degradation breaking the bonds while oxidases complete the degradation mineralizing the compounds (SARATALE *et al*., 2011).

Azoreductase, an specific enzyme, catalyzes the reductive cleavage of azo bonds, in other words, the enzyme helps to break the azo bonds and then aromatic amines are generated (MOUTAOUAKKIL *et al*., 2003). Azoreductases can use reduced Nicotinamide Adenine Dinucleotide (NADH), Nicotinamide Adenine Dinucleotide Phosphate (NADPH), reduced Flavin Adenine Dinucleotide (FADH) or Flavin Mononucleotide (FMN) as electron donors (SARATALE *et al*., 2011).

The azo bond cleavage takes place in two steps as showed in Figure 4.

Figure 4 – Steps of the azo bond cleavage



Source: Adapted from Guo *et al*. (2010).

In the first step, two electrons are transferred from NADH, for example, to the azo bond (-N=N-), breaking one bond (-HN-NH-). In the second one, more two electrons are transferred, generating two others molecules (-H_2N + NH_2-) (GUO *et al*., 2010). This reaction occurs in anaerobic environment and the azo dye acts as an electron acceptor. As the NADH is consumed by oxidative phosphorylation in aerobic conditions, it was suggested that the oxygen could inhibit the azoreductases interfering in the decolorization process (CHANG *et al*., 2001). On the other hand, some specialized aerobic bacteria are capable of reducing the azo group by special oxygen-tolerant azoreductases.

Moutaouakkil *et al*. (2003) investigated the azoreductase purified from *Enterobacter agglomerans* isolated from dye-contaminated sludge. This strain presented an ability to grow on culture medium containing an azo dye, under aerobic conditions, and it was able to initiating the azo dye degradation.

Chang *et al*. (2001) analyzed the activity of cell-free extract of *Pseudomonas luteola* under anaerobic and aerobic conditions and they reported that the presence of oxygen had no direct influence in the azoreductase activity since the specific activity under anaerobic

conditions was 1.98 nmol mg protein^{-1} min^{-1} and 2.08 nmol mg protein^{-1} min^{-1} under aerobic conditions.

The oxidases can be also applied to the azo dye biodegradation. The most known enzymes used for this purpose are tyrosinase, laccase, manganese-dependent peroxidase, and lignin peroxidase (ERKURT, 2010, SARATALE *et al*., 2011). Ligninolytic enzymes (laccase, manganese-dependent peroxidase, and lignin peroxidase) are commonly used by fungi to degrade azo dye. However, some researchers have reported the action of bacterial oxidases for this purpose (MOLINA-GUIJARRO *et al*., 2009, TELKE *et al*., 2009, PHUGARE *et al*., 2011).

Therefore, both reductases and oxidases play an important role in the dye biodegradation.

3.2.2.2 Fungi

Fungi are as capable of dye removal as bacteria and there are several studies showing the efficiency in the decolorization by fungi (AHMAD *et al*., 2015). For instance, Saroj *et al*. (2014) demonstrated that *Penicillium oxalicum* SAR-3 was successful in the biodegradation of azo dyes Acid Red 183, Direct Blue 15 and Direct Red 75 since they reached higher levels of degradation, between 95–100%. Niebisch *et al*. (2010) used *Lentinus crinitus* extracellular extract in order to degrade the dye Reactive blue 220. Santos and Corso (2014) evaluated the biodegradation of the azo dye Direct Blue 71 by pellets of *Phanerochaete chrysosporium* and *Aspergillus oryzae* they reported that both fungi were able to degrade the dye.

Fungi are widely used in biodegradation of azo dyes due to metabolic versatility and production of extracellular enzymes capable of acting in the breakage of the dye chemical bonds (SANTOS and CORSO, 2014). Thus, the application of fungi in the decolorization process is an attractive alternative due to the capacity of dye degradation and also because it is a low cost alternative (SARATALE *et al*., 2011).

Furthermore, filamentous fungi are ubiquitous in the environment. They can live in soil, plants, organic waste material and because of this, their metabolism can quickly adapt to several carbon and nitrogen sources (SARATALE *et al*., 2011, SOLÍS *et al*., 2012).

White-rot fungi (WRF), for instance, are producers of extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases. This enzymes group is

called ligninolytic system because of the powerful ability of extensively degrading lignin (ERKURT, 2010, MA *et al*., 2014). However, the enzymes from the ligninolytic system are relatively nonspecific, which allows them to act in the degradation of organic persistent compounds (SARATALE *et al*., 2011, MA *et al*., 2014).

Similar to what happens to bacteria, the laccases, for instance, are able to oxidize a wide range of other aromatic compounds when in the presence of suitable redox mediators (BOURBONNAIS and PAICE, 1990). Redox mediators are compounds that can accelerate the electron transfer from the electron donor to a final electron acceptor (DOS SANTOS *et al*., 2005). Riboflavin and Anthraquinone-2,6-disulfonate (AQDS) are examples of redox mediators.

Phanerochaete sp. is a WRF widely studied due to its good ability to biodegradation of organic compounds (GLENN and GOLD, 1983, MARTINS *et al*., 2001, FARACO *et al*., 2009, ENAYATIZAMIR *et al*., 2011, SANTOS and CORSO, 2014).

Very little research has been done to explore the dye biodegradation by yeast (MARTINS *et al*., 1999, YU and WEN, 2005, JADHAV and GOVINDWAR, 2006, JADHAV *et al*., 2007) when compared to filamentous fungi. Some studies are mainly related to the adsorption of dyes (DÖNMEZ, 2002, AKSU, 2003, KIM *et al*., 2015).

3.2.2.3 Mixed cultures

Despite the good performance in dye degradation shown by some strains of microorganism, sometimes, the use of pure cultures in the textile wastewater treatment cannot achieve satisfactory results, besides it seems not viable for industries. The cultivation of pure culture can demand a great effort, making the treatment a high cost process and time consuming.

According to Solís *et al*. (2012), the microorganisms work synergistically in a microbial consortium. Then, the degradation of organic persistent compounds such as dyes can be improved by the action of microorganisms from the consortium.

Some researchers have evaluated mixed cultures efficiency in the dye biodegradation (DAFALE *et al*., 2008, FORSS and WELANDER, 2009, YANG *et al*., 2009, PHUGARE *et al*., 2011, FORSS *et al*., 2013).

Among some advantages of the use of consortia mixed culture are the degradation of persistent compounds with the assistance of several enzymatic reactions, there are different strains that can act in the dye decolorization by different ways, they can use the metabolites produced by others strains and they use to be less sensitive to toxic compounds than pure cultures (SOLÍS *et al*., 2012, PUNZI *et al*., 2015).

On the other hand, there is a disadvantage as well, and it is the reproducibility of the system, since the results are not easily reproduced due to the complexity of the reactions and interaction among the different strains (SARATALE *et al*., 2011). Still, the effects of the application of a mixed culture seem to be more positives than negatives.

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4 CHAPTER 1

Comparison of decolorization capacity of the azo dye Direct Red 75 by *Phanerochaete chrysosporium* and *Saccharomyces cerevisiae*

4.1 Abstract

In order to assess the biodegradation of the azo dye Direct Red 75 (DR75) by different forms of fungi, this study aims to investigate the decolorization capacity of the fresh yeast, *Saccharomices cerevisiae,* and the pellets of filamentous fungi *Phanerochaete chrysosporium*. The results were expressed as percentage of decolorization and absorbance ratio values obtained by the ratio between the absorbance at a wavelength corresponding to the group's chromophore and azo (A_{522}/A_{320}) indicated the occurrence of biodegradation by varying the values of the samples compared to control. The percentage of decolorization showed that treatment with *S. cerevisiae* was more efficient, removing 77.14, 90.84 and 92.86% after 24, 96 and 192 hours, respectively, while treatment with *P. chrysosporium* removed 56.04, 61.84 and 81.91% after 24, 96 and 192 hours, respectively. The absorbance ratio suggests the occurrence of biodegradation, since the values of the treatments of 1.44, 1.02 and 0.91 for yeast and 1.54, 1.46 and 1.20 for *P. chrysosporium* showed a great variation in comparison to the absorbance ratio to the control 1.79 times evaluated. Therefore, DR75 was more easily biodegraded by fresh yeast, which can be easily acquired, than by single strain of fungus, which seems to be an expensive and labored option for the treatment of textile wastewater.

Keywords biodegradation, fungi, yeast, physical paramorphogenesis, pellets, *P. chrysosporium, S. cerevisiae*

4.2 Introduction

Synthetic azo dyes are extensively used in the textile industries. According to Martins *et al*. (2001), the azo dyes are considered persistent xenobiotic compounds due to the presence of a -N=N- bond and other groups that are not easily biodegraded, for instance, the sulphonic group (SO_3^-).

Several physical and chemical methods have been suggested for the treatment of dye contaminated wastewater but not widely used because of the high cost and secondary pollution that can be generated by excessive use of chemicals. Microbial degradation does not have a similar problem since they are a low cost treatment (JADHAV *et al*., 2007). Hence, establishing a biological wastewater treatment to the azo dye degradation seems to be a plausible option.

The use of microorganisms in the treatment of industrial effluents has been widely studied. Each species has metabolic versatility that helps in removing contaminants such as textile dyes. The fungi play an important role in the nature and in biodegradation of chemical compounds as well. Researchers have reported the ability of filamentous fungi and yeasts to biodegrade azo dyes (AKSU, 2003, YU and WEN, 2005, VITOR and CORSO, 2008, CORSO and ALMEIDA, 2009, ALMEIDA and CORSO, 2014, SANTOS and CORSO, 2014).

Yeast is one of the most ubiquitous microorganisms available for bioremediation of textile dyes, mainly at lower pH, besides being an inexpensive and easily available source of biomass (DÖNMEZ, 2002, AKSU, 2003). The yeast is a common waste from fermentation process in some industries such as brewery and it can be used for other purposes, for instance as substrate for the biodiesel production by another yeast and adsorption of dye (RYU *et al*., 2013, KIM *et al*., 2015).

According to Dönmez (2002), the yeast can remove the dye through the physical biosorption to the periphery of the cell, followed by specific accumulation in the wall and interior of the cell. However, yeasts are able to act in the biodegradation of dyes as well, including azo dyes. This is possible because of the production of enzymes by the yeast (JADHAV and GOVINDWAR, 2006).

The genus *Saccharomyces* has been used in the biodegradation of some dyes. The yeast cells of this genus are non-pathogenic, easily available, as already mentioned, and can grow on simple inexpensive medium (JADHAV and GOVINDWAR, 2006).

S. cerevisiae is one of the most important commercial yeasts since they are applied in the manufacture of bread and beer. Probably, the commercial yeasts are improved by selection and genetic manipulation by the industrial microbiologists (MADIGAN and MARTINKO, 2006). Since they are genetic manipulated to have a high fermentative power, it can be advantageous to use fresh yeast in the biodegradation of persistent compounds.

The use of *S.cerevisiae* cells for biodegradation of the dyes methyl red and malachite green was evaluated by Jadhav *et al*. (2007) and Jadhav and Govindwar (2006), respectively. *S. cerevisiae* MTCC 463 was able to degrade both dyes and enzymatic studies indicate the involvement of reductases as prominent enzymes in both researches.

The ability to dye degradation by filamentous fungi is also widely investigated. *Phanerochaete chrysosporium* is commonly studied (GLENN and GOLD, 1983, MARTINS *et al*., 2001, FARACO *et al*., 2009, ENAYATIZAMIR *et al*., 2011, SANTOS and CORSO, 2014). This filamentous fungus is known as white-rot fungi and they are capable of aerobic lignin depolymerization and mineralization because their capacity to produce one or more extracellular lignin (MARTINS *et al*., 1999, WESENBERG *et al*., 2003).

Ligninolytic enzymes are extracellular and they can degrade a wide variety of persistent compounds due to their lack of substrate specificity (WESENBERG *et al*., 2003, KAUSHIK and MALIK, 2009). Laccase, Manganese Peroxidase (MnP), Manganese Independent Peroxidase (MiP), Lignin Peroxidase (LiP) and Tyrosinase are some of the enzymes involved in dye decolorization (KAUSHIK and MALIK, 2009)

The biodegradation of dyes by *P. chrysosporium* has been evaluated by Enayatizamir *et al*. (2011), Santos and Corso (2014), Faraco *et al*. (2009), Martins *et al.* (2001), Knapp and Newby (1999) and others, and they reported the ability to dye removal and biodegradation.

A challenge of use filamentous fungus in the wastewater treatment is the growth of hyphae that can disturb the system. However, it is possible to transform hyphae into pellets, and then it is easier to have control of the biomass, moreover enable to quantify the pellets used to treat the wastewater.

Marcanti-Contato *et al*. (1997) developed a procedure, known as physical paramorphogenese, to transform hyphae of *Aspegillus* sp into pellets. Santos and Corso (2014) applied the physical paramorphogenese method in *Aspergillus oryzae* and *P. chrysosporium*. The fungi in pellets form were able to biodegrade the azo dye Direct Blue 71 in 240 hours of treatment, showing that the pellets also can be effective in wastewater treatment.

In order to assess the biodegradation of the azo dye Direct Red 75 by different forms of fungi, this study aims to investigate the decolorization capacity of the fresh yeast, *S. cerevisiae,* and the pellets of filamentous fungi *P. chrysosporium*.

4.3 Material and Methods

4.3.1 Dye

The azo dye Direct Red 75 (DR75) was acquired from Sigma-Aldrich Chemical Company, Inc. The maximum wavelength (λ_{max}) analyzed in this study was 522 nm.

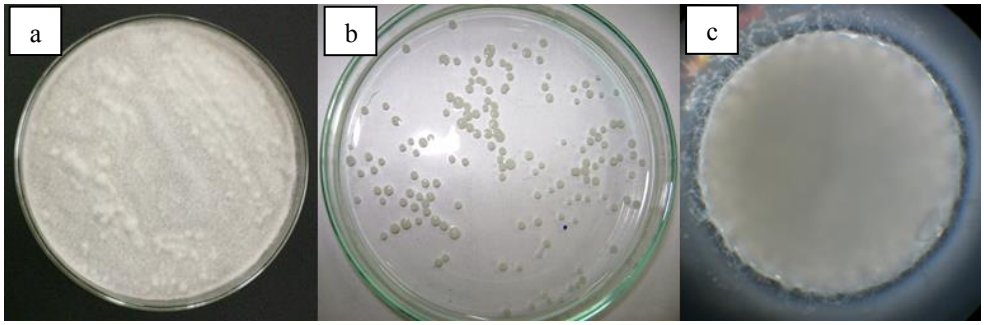
The stock solution of dye (1000 mg/L) was prepared from the dye dissolved in water. The stability of the dye in pH 2.5, 4.5 and 6.5 was analyzed by spectrophotometry. H_2SO_4 0.01 M ou NaOH 0.01 M were applied to correct the pH when needed.

4.3.2 Microorganisms

The fungi assessed in the present chapter were *P. chrysosporium* and the yeast *S. cerevisiae.*

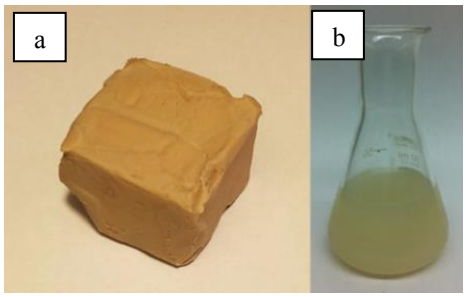
P. chrysosporium (CCB 478) (Figure 1) was obtained from the culture collection of the São Paulo Institute of Botany and it was kept in test tubes with 2% malt medium in room temperature. *S. cerevisiae* (Figure 2) was obtained from the fresh yeast (Fleischmann®) and the cellular viability was tested.

Figure 1 – *P. chrysosporium* in 2% Malt culture medium (a), in paramorphogenic form (b), pellet at 40x mag (c) in optical microscope



Source: Made by the author.

Figure 2 – *S. cerevisiae* as fresh yeast (a) and suspension diluted in distilled water (10^{-2}) (b)



Source: Made by the author.

4.3.2.1 S. cerevisiae washing

10 g of fresh yeast was dissolved in distilled water. Afterwards, the solution was centrifuged for 8 minutes at 6000 rpm and then the supernatant was discarded. The procedure was repeated until the supernatant was clear.

In order to get a 10% yeast suspension, the washed yeast was added in a graduated cylinder and the volume was completed to 100 mL with distilled water.

4.3.2.2 Cellular viability test

The cellular viability (GILLILAND, 1959) was tested using 1 mL of the 10% yeast suspension diluted in distilled water (10^{-2}) and 1 mL of erythrosine stain prepared by the dilution of 1ml of erythrosine solution 10 g/L in 50 mL of 0.1 M sodium phosphate buffer (pH 6.8 – 7.0). Afterwards, the cells were observed in optical microscope to verification and

counting the live and dead cells. The dead cells were stained while the live cells were not stained.

The percentage of viability was calculated as follows (Equation 1):

$$\%\ of\ viability = \left(\frac{number\ of\ living\ cells}{number\ of\ living\ cells + number\ of\ dead\ cells}\right) x\ 100 \qquad (1)$$

The yeast counting was performed with a Neubauer chamber under an optical microscope.

4.3.2.3 Culture medium for P. chrysosporium (modified from Lodder (1970)*):*

2% Malt medium:

- 2% Malt extract

- 2% Agar

- 4% glucose

- 1% peptone

- distilled water

4.3.2.4 Minimum Mineral Medium (MMM) (modified from Pontecorvo *et al*. (1953)*):*

- 6 g/L of $NaNO_3$

- 1 g/L of KH_2PO_4

- 0.5 g/L of KCl

- 0.5 g/L of $MgSO_4$. $7H_2O$

- 10 g/L of Glucose *

- 1 g/L of yeast extract

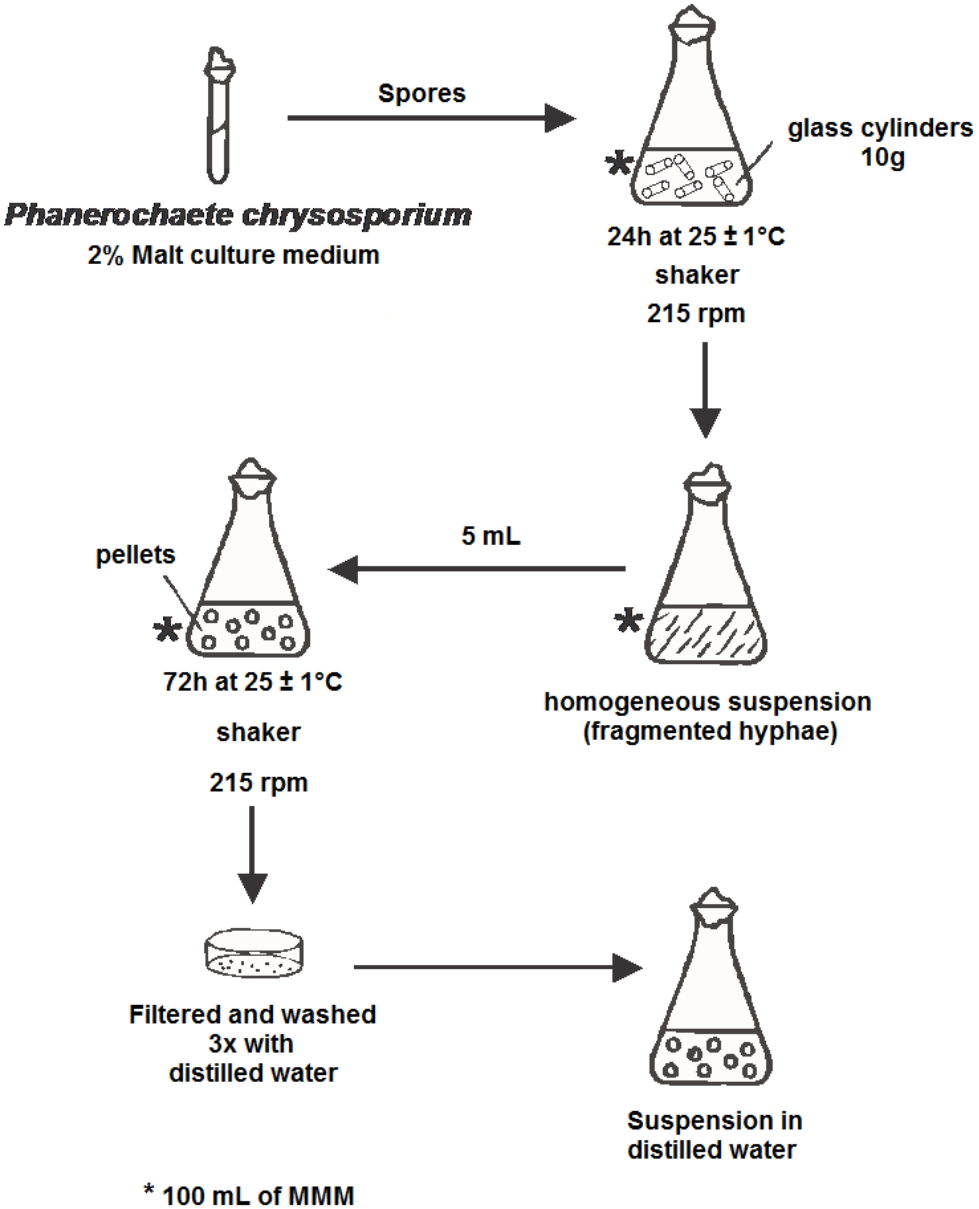
- distilled water q.s. 1L

* The glucose was sterilized separately to avoid carbonization in presence of salts.

P. chrysosporium was inoculated in test tube containing Malt culture medium. The culture was kept in 30°C for 4 days and removed afterwards, remaining at room temperature for 3 days before being inoculated in the MMM that was used for the growth of the mycelial pellets.

After 7 days of culturing, *P. chrysosporium* culture was used in the paramorphogenic process. The method was adapted from Marcanti-Contato *et al*. (1997) and it can be seen in Figure 3.

Figure 3 – Paramorphogenic process adapted from Marcanti-Contato *et al*. (1997)



Source: Made by the author.

The pre-inoculum and inoculum were prepared in flasks with 100 mL of sterilized MMM. Pre-inoculum consists of the addition of a spore suspension and 10 g of small glass cylinders (6mm) to divide the fungal mycelium. The pre-inoculum flasks were under constantly shaking for 24 hours in room temperature. Afterwards, 5 mL of pre-inoculum was aseptically collected and added to the MMM with the same conditions. However, the time needed for the pellets growth was 72 hours to *P. chrysosporium*. The pellets were washed with distilled water before storage in refrigerator at 4°C.

The dry weight of the pellets was measured so it was possible to quantify the biomass used in the treatment.

4.3.3 Decolorization test

Samples were prepared, in test tube, with 1 mL of stock solution of the dye to 1000 mg/L, 8 mL of distilled water at pH 2.5 and 1 mL of 4% yeast suspension for tests with *S. cerevisiae* and 1 mL of *P. chrysosporium* pellets, corresponding respectively to 11.3 mg/mL and 11.7 mg/mL. The control solutions were prepared with 9 mL of distilled water at pH 2.5 and 1 mL of stock solution of the dye. The acid pH was chosen to the decolorization test because, according to Boyle *et al*. (1992), it favors the induction of enzymatic activity and, consequently, it favors the azo dye reduction.

The samples were incubated at 30°C for 192 hours and centrifuged at 4000 rpm for 30 minutes to perform the scans on UV-VIS spectrophotometer after 24, 96 and 192 h. The results were expressed as percentage of decolorization and were obtained from the Equation 2 applied by (VITOR and CORSO, 2008):

$$\%\,decolorization = \frac{A_{522nm(\lambda\,\max)}\,initial - A_{522nm(\lambda\,\max)}\,final}{A_{522nm(\lambda\,\max)}\,initial}\;x\;100 \qquad (2)$$

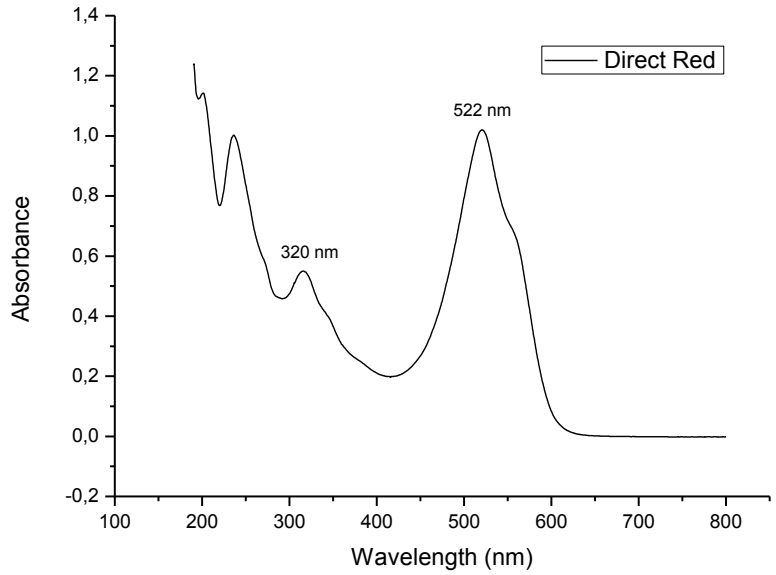
The biodegradation of the dye in solution was evaluated by absorbance ratio according to Glenn and Gold (1983). The absorbance ratio values can be obtained by the ratio between the absorbance at a wavelength corresponding to the chromophore and azo groups (A_{522nm}/A_{320nm}), respectively, indicating the occurrence of biodegradation by varying the values of the samples compared to control.

4.4 Results and Discussion

4.4.1 Dye

Figure 4 shows the dye absorption spectrum of DR75 100 mg/L scanned by UV-VIS spectrophotometer from 800 to 200 nm. The peak of chromophore group (522 nm) and azo group (320 nm), according to Silverstein *et al*. (1994), are indicated at the spectrum.

Figure 4 - DR75 (100 mg/L) absorption spectrum with chromophore group (522 nm) and azo group (320 nm) indicated



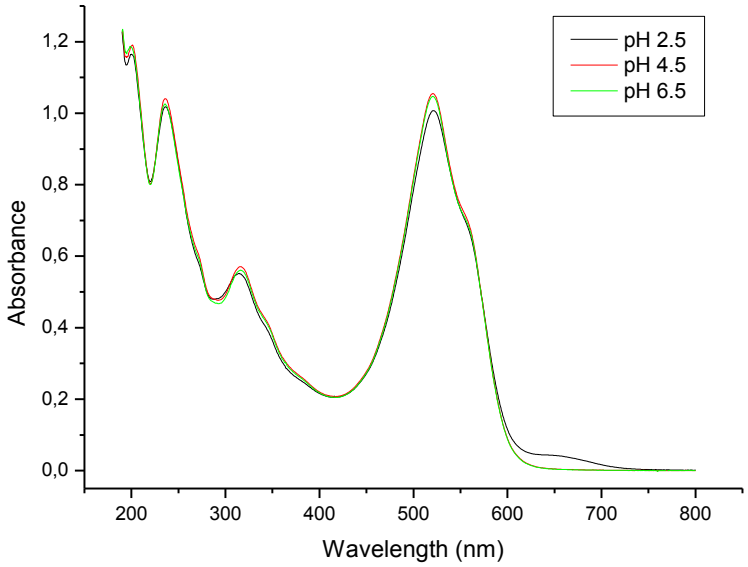
Source: Made by the author.

4.4.1.1 pH stability test

The pH stability test purpose is to check the dye behavior in acid and neutral environment, since the decolorization test was performed in pH 2.5. Santos (2011) evaluates the biosorption of the Direct Blue 71 dye in solution with pH 2.5, 4.5 and 6.5 by *P. chrysosporium* and *A. oryzae*, and it was reported that the samples with pH 2.5 achieved better rates of decolorization, showing that the acid pH could be applied to the dye decolorization. For this reason, pH 2.5 was chosen for this experiment.

The absorption spectrum of DR75 in pH 2.5, 4.5 and 6.5 is shown in Figure 5 and the visual evaluation is shown in Figure 6.

Figure 5 – pH stability test of DR75 solution in three different pH values



Source: Made by the author.

Figure 6 – Visual evaluation of DR75 solution in four different pH values, the control (c) with pH around 7.0 and the samples with pH 2.5, 4.5 and 6.5

Source: Made by the author.

It can be observed in Figure 4 that there is a small shift in the spectrum regarding to pH 2.5. Little is known about the effect of pH on the dye structure acidic or basic solutions; however, it is believed that the difference of the color intensity and the spectrum of DR75 is because of the sulphonate groups.

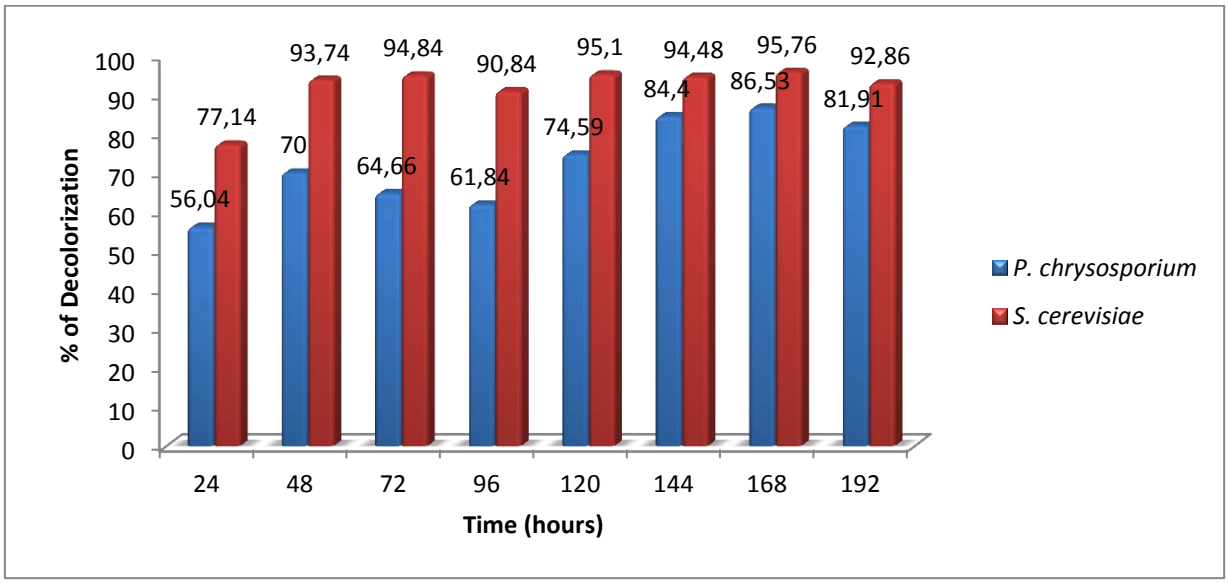
According to Mall *et al*. (2005), the initial pH of the dye solution affects the dye molecules and, consequently, it may affect the structural stability of the dye and the intensity

of the color. In fact, this shift probably is due to the four sulphonate groups in the dye molecule. The sulphonate group is dissociated in the dye solution and converted in anionic dye ions (-SO_3Na to $–SO_3^-$ + Na^+) (MALL *et al.*, 2006). Thus, since the dye solution in pH 2.5 has high concentration of H^+, the H^+ bonded to SO_3^- and it can be responsible for the shift in the spectrum.

4.4.2 Decolorization test

The percentage of decolorization showed that treatment with *S. cerevisiae* was more efficient, removing 77.14, 90.84 and 92.86% after 24, 96 and 192 hours, respectively, while treatment with *P. chrysosporium* removed 56.04, 61.84 and 81.91% of the dye as it can be seen in Figure 7.

Figure 7 - Percentages of decolorization of DR75 dye in solution pH 2.5 after 192 hours of treatment with *P. chrysosporium* and *S. cerevisiae*



Source: Made by the author.

Decolorization means to remove color of the wastewater, in other words, to remove dye molecules dissolved in the wastewater. It is possible to accomplish this by means of adsorption or degradation. Since microorganisms were used to dye removal, or the dye may have been removed by biosorption of the dye molecules by yeast cells and *P. chrysosporium* pellets or by biodegradation.

The solution treated with *S. cerevisiae* may have a certain percentage of decolorization due to biosorption, because their cells were free in solution and had a greater contact surface when compared with the pellets of *P. chrysosporium*. The pellets of *P. chrysosporium* were also free in solution, though the pellets are a tangle of hyphae and the center of this tangle does not have contact with the solution as the hyphae in the external part of the pellets.

According to Jadhav and Govindwar (2006), the biosorption occurs in the first 3 hours and, afterwards the decolorization takes place, mainly, by biodegradation. They analyzed the biodegradation of malachite green by *S. cerevisiae* by visual observation for 8 hours collecting yeast cells from the medium. After 3 hours, the cells were dark green; however, cells collected after 7 and 8 hours were almost white. Thus, the cells adsorbed the dye for the first 3 hours and then the biodegradation started, probably, after 5 hours. Therefore, the decolorization of malachite green occurred mainly due to microbial biotransformation and not to biosorption (JADHAV and GOVINDWAR, 2006).

Santos and Corso (2014) analyzed the biodegradation of Direct Blue 71 by *P. crhysosporium* from 24 to 240 hours and after 24 hours the biodegradation had already started. The biosorption test was performed by Santos (2011). Five different biomass concentrations (1.1-5.5 mg/mL) were tested in 3 different pH values (2.5, 4.5 and 6.5) for 2 hours. The higher the biomass concentration was, the higher was the percentage of decolorization which means the more biomass in the solution has, the greater is the absorption surface. Considering the size of the yeast cells and *P. chrysosporium* pellets and the time of treatment, it is clear that yeast cells could adsorb more than the pellets in the first hours.

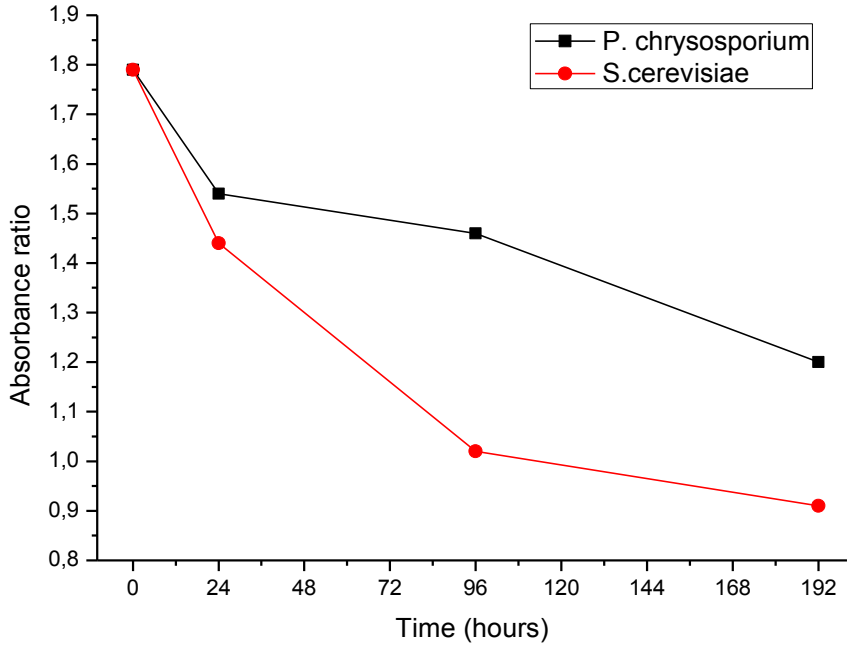
S. cerevisiae demonstrated has higher capacity to decolorize the dye solution in 192 hours of treatment than *P. chrysosporium*. Regarding the time analyzed and observing the absorbance ratio of the samples (Figure 8), it is possible to say that the biodegradation started already in the first 24 hours.

Regarding the time of treatment, after 144 hours the rates of decolorization for both microorganisms were quite similar, which means that 144 hours was enough time to decolorize the dye solution under the conditions tested.

The absorbance ratio indicates the occurrence of biodegradation, since the values of the treatments with yeast and *P. chrysosporium* showed a great variation when compared to the absorbance ratio of the control.

According to Glenn and Gold (1983), the absorbance ratio is an indicative of biodegradation because if the ratio between the absorbance of the chromophore group (522 nm to DR75) and the absorbance of the azo group (320 nm According to Silverstein *et al.* (1994) varies from the absorbance ratio of the control solution, i. e., the peaks will decrease in different proportions which means that the dye is being degraded, otherwise the peaks would decrease in the same proportions. This variation suggests that the molecular structure of the dye has changed.

Figure 8 - Absorbance ratio values of DR75 dye in solution after 24, 96 and 192 hours of treatment with *P. chrysosporium* and *S. cerevisiae*



Source: Made by the author.

The ability to decolorize dye by *S. cerevisiae* was also reported by Jadhav *et al.* (2007). They analyzed the decolorization of Methyl Red by *S. cerevisiae*. The Methyl Red dye was decolorized completely within 16 min when the concentration of the dye was 100 mg/L, while higher dye concentration (200 and 300 mg/L) requires more time for complete

decolorization. Thus it may be concluded that *S. cerevisiae* effectively decolorizes Methyl red due to biodegradation.

Thus, the fresh yeast *S. cerevisiae* showed its capacity in the biodegradation of DR75 proving that it may also be applied in the wastewater treatment, since is a cheaper material than a single strain microorganism; furthermore, it can be easily acquired.

P. chrysosporium was able to degrade DR75; however not with the same efficiency of the yeast. The white-rot fungi have been shown to be able to degrade several dyes by oxidative mechanisms involving their ligninolytic enzymatic system (MARTINS *et al*., 1999).

Yeasts, as white-rot fungi, use the enzymes in the biodegradation process. On the other hand, instead to use the oxidative enzymes to break the molecular bonds, the *S. cerevisiae* seems to increase the reductase activity and decreases the oxidative activity during the biodegradation process (JADHAV and GOVINDWAR, 2006). Probably the difference in the biodegradation capacity of both fungi is in the enzymes action. Enzymatic assays should be performed later to evaluate the enzymatic capacity of the microorganisms tested in the azo dye biodegradation.

4.5 Conclusion

It can be concluded that *S. cerevisiae* presented better capacity to biodegrade the azo dye Direct Red 75 than *P. chrysosporium*.

The dye removal in the first hours seems to have occurred mainly by biosorption and, afterwards the biodegradation process was responsible for the decolorization.

Even presenting good rates of decolorization, there are still few studies about the application of yeasts in the textile wastewater treatment compared to the white-rot fungi.

Regarding the cost of treatment, the paramorphogenic process of *P. chrysosporium* for wastewater treatment is slower, complex and expensive. Therefore, the use of fresh yeast is a cheaper and faster alternative, since it can be easily acquired, besides to be even more efficient than using pellets of white-rot fungi.

Therefore, *S. cerevisiae* proved to be effective and that it may also be applied in the wastewater treatment, since it has the ability to resist adverse environments as filamentous fungi.

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5 CHAPTER 2

Use of inoculum of food waste biodigester in the azo dye Direct Red 75 biodegradation and biosorption

5.1 Abstract

The textile industry has grown each year and, therefore, there is an increase in wastewater volume generated by such industries. However, treating these effluents is not always done in an effective way and thus it is discarded in rivers with high pollutant loads which can be toxic to aquatic organisms. Numerous studies have been conducted to find more efficient and low-cost ways of treating textile effluents. Biological treatments are most valued for their efficiency and have been proven in research to successfully remove dyes and other organic compounds. Some microorganisms have the ability to biodegrade and even remove the dye molecules. The source of microorganisms used in this research was found in a biodigester of food residues. The biodigester was fed with organic waste generated in the kitchen of a university restaurant and at the end of the digestion process. The organic waste was processed separately in two phases: the inoculum, which contained microorganisms, and Recycled Organic Nutrient. In order to find more efficient processes and low-cost treatment of textile wastewater, this study evaluated the potential for removal and/or biodegradation of the azo dye Direct Red 75 in solution by microorganisms in the inoculum biodigester. After the analysis of biosorption and biodegradation, the tested inoculum showed removal efficiency, presenting microorganisms with potential capacity for biodegradation of the dye molecules.

Keywords azo dye, biodegradation, biosorption, biodigester, food waste

5.2 Introduction

The increasing demand for different fabrics and colors has required an increased production in textile industries and, consequently, an increase in the generation of wastewater. Wastewater not properly treated is the reason for great environmental concern because they are discarded into rivers. Sometimes it is still colorful and can contain possibly toxic molecules to aquatic life. In order to avoid this problem, it is necessary to invest in a more efficient treatment system. It would be interesting for industries if the treatment could be not only efficient but also low-cost. Thus, studies have been conducted to find new alternatives for the wastewater treatment using organic and industrial waste as adsorbent or source of microorganisms (CHUAH *et al*., 2005, FORSS and WELANDER, 2009, MITTER *et al*., 2012, FORSS *et al*., 2013, KANAGARAJ *et al*., 2015).

One of the biggest problems of the textile industries is the removal of dye from its wastewater. It is estimated that 5-30% of textile dyes are discarded in the wastewater during the dyeing process because there is not 100% of fixation into the fabric fibers (BASTIAN, 2009).

Degradation of organic compounds by microorganisms has been studied; however, there is still more research to be done. In an attempt to achieve more efficient wastewater treatment and to reduce the environmental pollution, a large number of micro-organisms belonging to different taxonomic groups of fungi, yeasts and algae have been evaluated. Some of them have the ability to decolorize azo dyes (VITOR and CORSO, 2008, CORSO and ALMEIDA, 2009, FORSS and WELANDER, 2011, BAÊTA *et al*., 2012, CORSO *et al*., 2012, ALMEIDA and CORSO, 2014, SANTOS and CORSO, 2014, SAROJ *et al*., 2014).

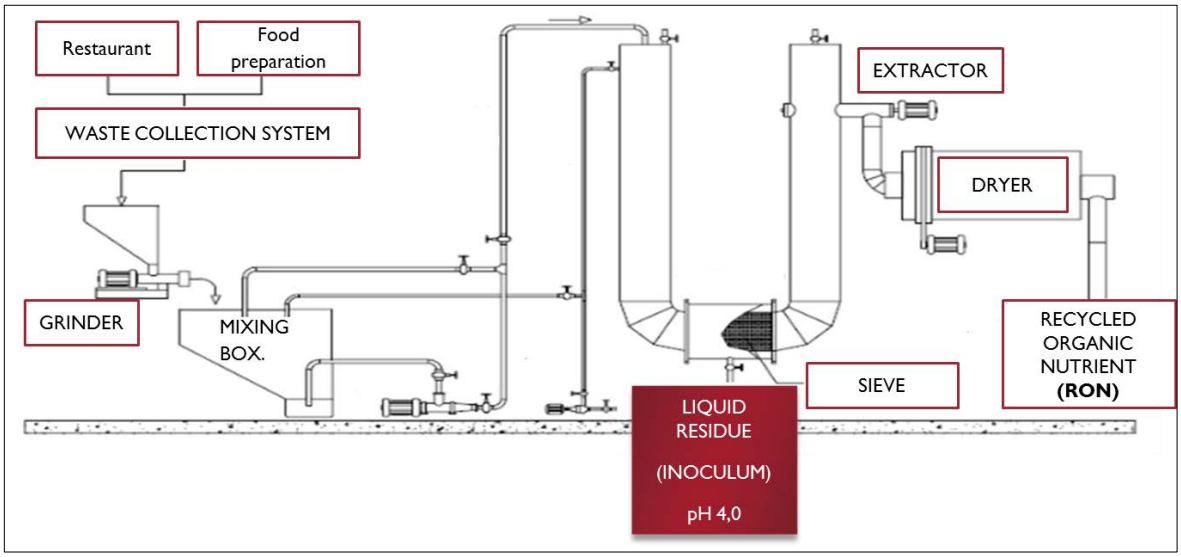
There are two mechanisms by which microorganisms can remove the dye from the wastewater: biodegradation and biosorption. The first one occurs by means of enzymes which act to break chemical bonds of the dyes. The second mechanism occurs by the adsorption of the dye molecules by the cellular membrane or cellular wall of microorganisms (MOU *et al*., 1991).

The digesters are artificial environments used for ex-situ treatment of organic waste, contaminated water or soil that can be transferred from its original location to the system (ALMEIDA, 2008).

The process that takes place inside the digesters is known as anaerobic digestion. According to Ruiz (1992), anaerobic digestion is the degradation of organic matter, by a mixed culture, in the absence of free oxygen. Methane (CH_4) and carbon dioxide (CO_2) are the final products of the digestion. The anaerobic digestion occurs naturally in several natural ecosystems such as swamps, rivers and lakes sediments, digestive system of superior animals and into the deeper layers of the soil.

The food waste digester used in the present research was continuously fed with food residues. The waste is initially crushed, mixed to the SOECCO activator (consortium of microorganisms) and sent to the biological reactor through a pump where the anaerobic digestion will take place. After this phase, the residual liquid, used as inoculum, is filtered by a static sieve, while the solid residue continues until the end of the process. Figure 1 shows the biodigester diagram and where the inoculum is collected.

Figure 1 – Diagram of food waste biodigester of SOECCO



Source: Personal files of Prof. Gilson Coutinho Jr.

According to Almeida (2008), the mixed culture can be able to adsorb and degrade several types of pollutants.

In order to evaluate new techniques for removal of azo dye Direct Red 75 of the solution, this study used the inoculum of the food waste digester as source of microorganisms to perform the biosorption and biodegradation.

5.3 Material and Methods

5.3.1 Dye

The azo dye Direct Red 75 (DR75) was acquired from Sigma-Aldrich Chemical Company, Inc. and the maximum wavelength (λmax) used to analyze the dye in this study was 521 nm.

The stock solution of dye (1000 mg/L) was prepared from the dye dissolved in water. The pH was the same of the distilled water, approximately 7.

5.3.2 Inoculum

The inoculum was obtained from the food waste biodigester Bioconvert 300 (Figure 2), of the SOECCO Environmental Solution Company, installed at Hermínio Ometto University Center (UNIARARAS), in Araras/SP, under supervision of Professor Gilson Coutinho Júnior.

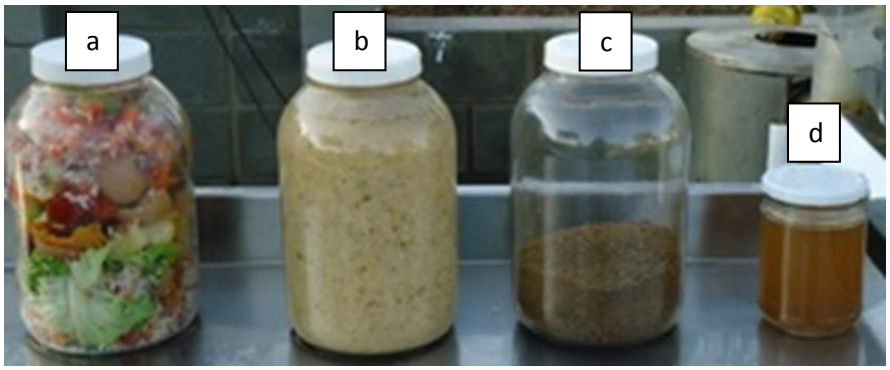
Figure 2 – Food waste biodigester of SOECCO

Source: Personal files of Prof. Gilson Coutinho Jr.

The biodigester was fed with organic waste from the university restaurant kitchen. At the end of the digestion process, the already processed organic waste was separated into two phases: liquid phase, i.e. the inoculum, pH 4.0, where are the microorganisms and other

dissolved compounds, and solid phase also called Recycled Organic nutrient (RON) (Figure 3).

Figure 3 – Samples of the organic waste from the university kitchen (a); processed organic waste in the biodigester (b); Recycled Organic Nutrient (RON), after drying (c) and liquid residue (Inoculum) (d)



Source: Personal files of Prof. Gilson Coutinho Jr.

5.3.3 Biosorption test

The test solutions were prepared in test tubes with 100, 150, 200, 250, and 300 µL of inoculum by adding 1 mL of stock solution of the dye DR75 and enough distilled water to prepare 10 mL. The control solution was prepared only with 9 mL of distilled water and 1 mL of dye. Afterwards, the test-solutions were mixed and incubated at 28°C±1 for 2 hours as proposed by Corso and Almeida (2009). After this period, the samples were centrifuged for 20 minutes at 5000 rpm and analyzed by UV-VIS spectrophotometry from 800 to190 nm in 5 mm quartz cuvette.

The spectra were observed for evaluation of the dye removal of the solution. The equation (1) was used to calculate the percentage of decolorization:

$$\%\,decolorization = \frac{A_{521nm(\lambda\,\max)}\,initial - A_{521nm(\lambda\,\max)}\,final}{A_{521nm(\lambda\,\max)}\,initial}\,x\;100 \qquad (1)$$

5.3.4 Biodegradation test

The test solutions for the degradation test were prepared in test tubes with 1 mL of inoculum by adding 1 mL of stock solution of the dye DR75 and 8 mL of distilled water. The control solution was prepared only with 9 mL of distilled water and 1 mL of dye. Afterward, the test-solutions were mixed and incubated at 28 ° C±1 for 240 hours. Samples were taken every 24 hours, centrifuged for 20 minutes at 5000 rpm and analyzed by UV-VIS spectrophotometry from 800 to190 nm in 5 mm quartz cuvette.

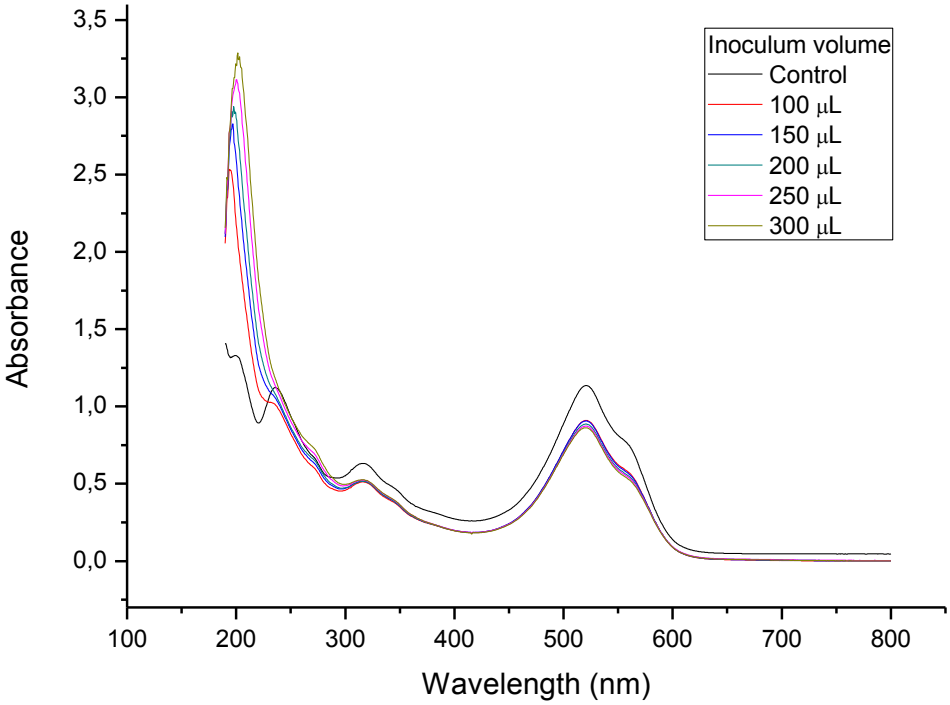
The biodegradation was evaluated by observing the absorbance spectra and analyzing the absorbance ratio according to Glenn and Gold (1983). The absorbance ratio values were determined by the ratio between the absorbance at two different wavelengths corresponding to the chromophore group (521 nm) and the azo group (320 nm) (SILVERSTEIN *et al*., 1994), i.e. A_{521}/A_{320}.

5.4 Results and discussion

5.4.1 Biosorption test

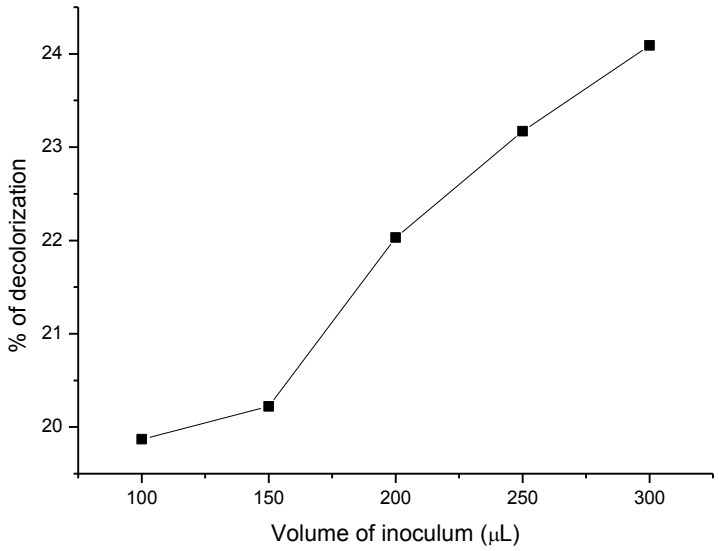
Figure 4 shows the spectrum of DR75 dye after two hours of treatment with the inoculum. It can be seen that dye was removed of the solution, in other words, the dye was adsorbed by the microorganisms. The absorbance between 250 and 200 nm increased due to the addition of the inoculum that has higher concentration of organic material than the dye solution. However, the difference among samples cannot be noticed because the values were close, as seen in Figure 5. The percentage of decolorization was relatively low and there is little variation among samples.

Figure 4 – DR75 spectrum after 2 hours of treatment with the inoculum



Source: Made by the author.

Figure 5 –Decolorization percentage of DR75 in relation to the volume of inoculum



Source: Made by the author.

Almeida (2008) evaluated the Colony-forming unit (CFU) present in the inoculum of this digester and the estimated number for bacteria was 8.45 x 10^7 CFU/mL. Fungi and yeasts have not reached a minimum value for the count. The prevalence of bacteria occurs due to the characteristics of anaerobic and acidic environment. Therefore, considering a large number of CFU/ml of bacteria, it is expected that the biosorption and biodegradation processes are favored.

Besides the microorganisms, there are other dissolved and suspended compounds, which can be composed of undigested remnants in the digester that have passed through the sieve. Especially the compounds in suspension, may have contributed to the removal, adsorbing the dye molecules. Thus, the percentage of decolorization increases as the volume of inoculum added increases. The higher the concentration of compounds capable of adsorbing the dye, the greater will be the dye removal of the solution.

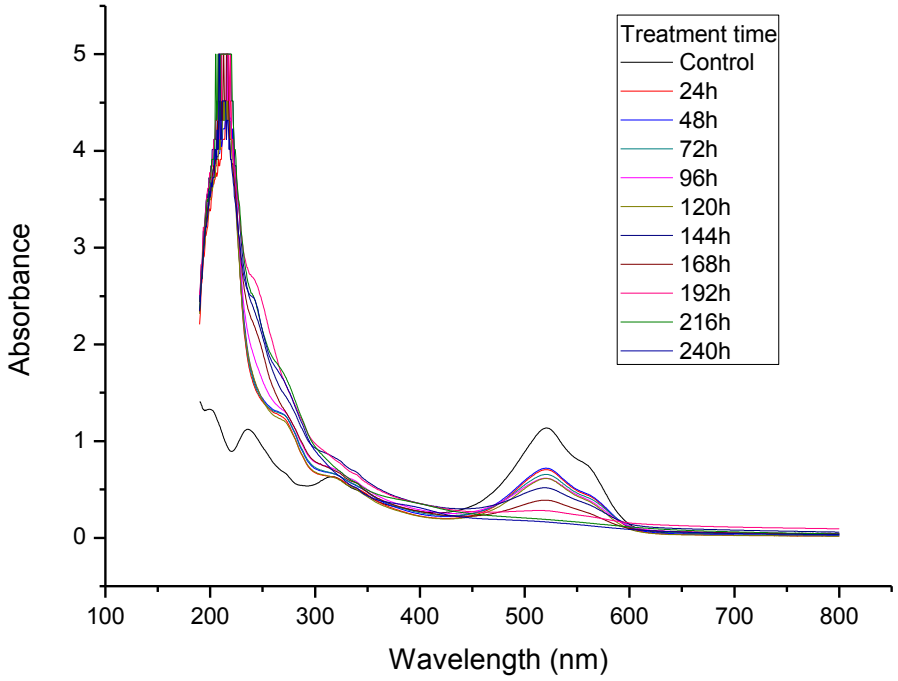
Entire molecules of dye can be removed of the solution using the biosorption technique. Thus, there is no fragmentation and generation of possible toxic compounds. The dye molecules may be removed more easily of the effluent by decantation or centrifugation after being adsorbed. Therefore, the contamination problem would be reduced with a shorter treatment time and the adsorbents attached to the dye could be processed later.

5.4.2 Biodegradation test

By using UV-VIS spectrophotometry it was possible to assess whether there were signs of biodegradation.

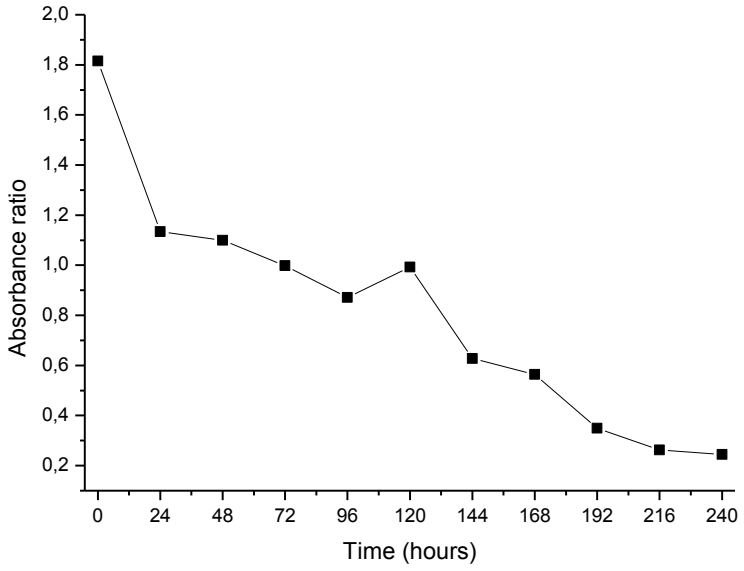
The spectrum of the dye treated with the inoculum decreased each day of treatment as it can be seen in Figure 6. Therefore, there has been dye removal of the solution. However, to observe signs of degradation it is necessary to assess the absorbance ratio values, as proposed by Glenn and Gold (1983) shown in Figure 7.

Figure 6 –DR75 spectrum after 240 hours of treatment with the inoculum



Source: Made by the author.

Figure 7 – Absorbance ratio each 24 hours initiating by the control (0 hour) after DR75 dye treatment by the inoculum



Source: Made by the author.

According to Glenn and Gold (1983), a compound presents biodegradation signals when there is a wide variation in the absorbance ratio values of the treatments regarding to the control absorbance ratio. These signals appear because the absorbance of the samples at the wavelengths considered do not decrease to the same extent, indicating that, probably, the dye links may have been broken. However, in the case of absorbance ratio to remain constant, the predominant process is the biosorption, i.e., the absorbance decreases proportionately, with no change in the dye structure.

5.5 Conclusion

The search for new wastewater treatment methods that are efficient and low-cost, encourages industries to improve their systems and, thus, will reduce their pollution potential.

Use of industrial wastes and microorganisms for this type of treatment has been evaluated and showed good results. Therefore, the cost is reduced and the wastewater treatment may have a greater efficiency.

The biodegradation and biosorption are advantageous processes to reduce contamination by dyes. In the present study, the DR75 dye was removed from the solution by biosorption and, probably, the mixed culture present in the inoculum was able to break the dye bonds, breaking up the molecule.

Tests with greater hydraulic retention time (HRT) are needed, besides different methods of analysis to evaluate the occurrence of biodegradation. This study evaluated 10 days of treatment using UV-VIS spectrophotometry. The biodegradation evidence was also found using UV-VIS spectrophotometry. In later work, analysis will be performed using other equipment such as LC-MS.

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6 CHAPTER 3

Evaluation of the Direct Red 75 biodegradation process in anaerobic bioreactor and fixed bed bioreactor using different support materials and rice husks as source of microorganisms

6.1 Abstract

Biological treatment has been widely studied as an eco-friendly and low cost textile wastewater treatment. Anaerobic and aerobic processes are applied in order to degrade dyes and other persistent compounds. Moving Bed Biological Reactor (MBBR) technology seems to be efficient in the biodegradation studies. It is based on the biofilm growth on a plastic carrier that is kept suspended in the reactor by aeration or stirring. However, it is still an expensive technique. Rice husks as carrier in biological treatment could be a low cost alternative, since it is an agricultural waste. Thus, the present study aims to evaluate the DR75 dye biodegradation using two different systems (anaerobic and anaerobic/aerobic) and different carriers (MBBR and rice husks). Besides, it aims to examine the aromatic amines as intermediate metabolites. The systems assembled were a Bioreactor (Bioreactor A) filled with MBBR and a Fixed Bed Bioreactor (Bioreactor B) composed by three anaerobic bioreactors (B1, B2 and B3) filled with rice husks plus one aerobic bioreactor (B4). Rice husks were the source of microorganisms to Bioreactor B while the rinse water of rice husks was the source of Bioreactor A, glucose and yeast extract were tested as a carbon source and three different HRT. The Bioreactor B fed with yeast extract culture medium presented the best decolorization rates reaching, more than 90% of decolorization, and the best COD removal, 71.76% with 48 hours of HRT while Bioreactor A removed 19.55% with the same HRT. The decolorization using glucose as carbon source was not as efficient as the yeast extract. The aromatic amines were evaluated by LC/MS, GC/MS and HPLC; however the attempt did not succeed well due to the high percentage of unknown compounds in the dye composition. Still, the rice husks can be a commendable alternative as a support material in textile wastewater treatment due to its large surface area and also to be a source of microorganisms.

Keywords rice husks, MBBR, azo dye, amines, glucose, yeast extract

6.2 Introduction

The textile industries are among the ones which use the most water in their processes, besides, it contains pollutants from different phases of dyeing and finishing, as well as from other processes. The pollutants found in the textile effluents are mainly persistent organic substances, such as dyes, which confer low biodegradability to the effluent (FU *et al*., 2011).

The azo dyes group is the one with the greater number of representatives (60 a 70%); therefore, they constitute most common dyes of the effluents of textile industries and are the most studied (HUNGER, 2003, VAN DER ZEE *et al*., 2003). The azo dyes have one, two or more azo groups (–N=N–) attached to aromatic rings. Besides to the textile application, this class is also used in the pharmaceutical, food, leather and cosmetics industries (TELKE *et al*., 2008).

According to Wijetunga *et al*. (2010), the dyes are continuously being upgraded and replaced by compounds with improved stability and resistant to natural degradation. For this reason it is necessary to develop new technologies which can improve the textile wastewater treatment.

The composition of the effluents from textile industry is highly variable due to the existence of different cloths, the huge variety of dyes available commercially and fashion trends of each season (SANTOS and BOAVENTURA, 2015).

Industries are looking for an eco-friendly treatment system that uses the least amount of chemicals and saves energy of nature (Almeida and Corso, 2014). It is possible to find these characteristics in the biological treatment. Furthermore, besides being environmental friendly, the biological treatment may be implemented with low investment (AHMAD *et al*., 2015).

There are different methods of treatments; however, none is completely effective and some require a combination of techniques to be efficient. For instance, the conventional aerobic processes have been insufficient to degrade most azo dyes (IŞIK and SPONZA, 2008, ALMEIDA and CORSO, 2014). A lot of investments have been made in research in order to improve the quality of the industrial wastewater treatment in an effort to minimize the polluting and toxic potential of the residues.

The biological treatment is the most common and widely used in the treatment of effluents, being used for more than 150 years. Biological methods have been applied to remove organic compounds and the color of textile effluents, due to their low cost, simple operation and maintenance (HUNGER, 2003, DAFALE *et al*., 2008, FU *et al.*, 2011). Bacteria and fungi are the most common microorganisms used in these treatments (CORSO and ALMEIDA, 2009, CERVANTES and DOS SANTOS, 2011, SARATALE *et al*., 2011, CORSO *et al*., 2012, SANTOS and CORSO, 2014).

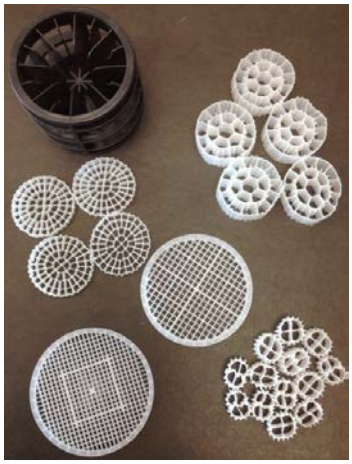
According to Zollinger (1991), degradation of azo dyes starts with reductive cleavage of the azo group, releasing aromatic amines as metabolites. Aromatic amines are toxic and carcinogenic to the organisms present in the environment (SCHNEIDER *et al*., 2004, IŞIK and SPONZA, 2008, ALMEIDA and CORSO, 2014). The aromatic amines can be formed by anaerobic process by means of reductive cleavage of the azo bond, leading to decolorization, and then it can be degraded in aerobic environment (WESENBERG *et al*., 2003, VAN DER ZEE and VILLAVERDE, 2005, HOSSEINI KOUPAIE *et al*., 2011, BAÊTA *et al*., 2015).

The complete mineralization of the dyes would be ideal to minimize the pollution by textile effluents; however, the synthetic dyes are compounds difficult to mineralize.

Different methods to improve the biodegradation process have been evaluated by researchers all over the world. For instance, methods that use only pure culture or mixed culture (YANG *et al*., 2009, CORSO *et al*., 2012, SANTOS and CORSO, 2014); using bioreactors as UASB (WIJETUNGA *et al*., 2010, BAÊTA *et al*., 2012) and recently they are using Moving Bed Biological Reactor technology (MBBR) to evaluate dye biodegradation (CALDERÓN *et al*., 2012).

MBBRs can be operated as anaerobic or aerobic phases with floating plastic carriers which increase the protected surface area for biofilms growth (CALDERÓN *et al*., 2012). Actually, according to Borkar *et al*. (2013), there are two forms of biomass in the MBBR: suspended flocks and a biofilm attached to carriers. It has a high-density population of microorganisms, it can be operated at high organic loads and it is less sensitive to hydraulic overloading and starving periods (SIPMA *et al*., 2010, BORKAR *et al*., 2013). Some examples of MBBR can be seen in Figure 1.

Figure 1 – Examples of MBBR



Source: Made by the author.

Hosseini Koupaie *et al*. (2011) and Hosseini Koupaie *et al*. (2012) have been evaluated this technology. However, MBBR solutions are often connected to patent and it is expensive when compared with other biological methods. Besides that, there are only a few researches in this field.

Due to the cost of treatment, waste biomasses and low cost materials can be used as carrier and also as alternative adsorbent for the removal of heavy metals and organic pollutants (SAFA and BHATTI, 2011, BORKAR *et al*., 2013). However, the materials used as carrier need to be quite inert so as not to add organic load into the water.

Some waste products from industrial or agricultural operations have potential as inexpensive adsorbents (CHUAH *et al*., 2005). For instance, the rice husks are an agricultural waste available in excess, obtained from the rice mills after the separation of rice from paddy and it has been used as adsorbent of dyes (CHUAH *et al*., 2005, VADIVELAN and KUMAR, 2005, LAKSHMI *et al*., 2009, SAFA and BHATTI, 2011). Moreover, the rice husks seem to carry microorganisms capable of dye degradation (TÜRGAY *et al*., 2011, FORSS *et al*., 2013). The microbial diversity in a continuous system based on rice husks for biodegradation of the azo dyes was evaluated by Forss *et al*. (2013).

Thus, the rice husks could be used as carrier in textile wastewater treatment in developing countries, since it is an inexpensive material often available in these countries. According to Schneider *et al*. (2004), the use of azo dyes has been drastically reduced in Europe due to national regulations, but may still be a problem in non-European countries.

Focusing on the use of rice husks as source of microorganisms, the present study aimed to evaluate the biodegradation of the azo dye Direct Red 75 (DR75) in anaerobic and anaerobic/aerobic bioreactors with MBBR and rice husks as support material, besides to check the aromatic amines as intermediate metabolites.

6.3 Materials and methods

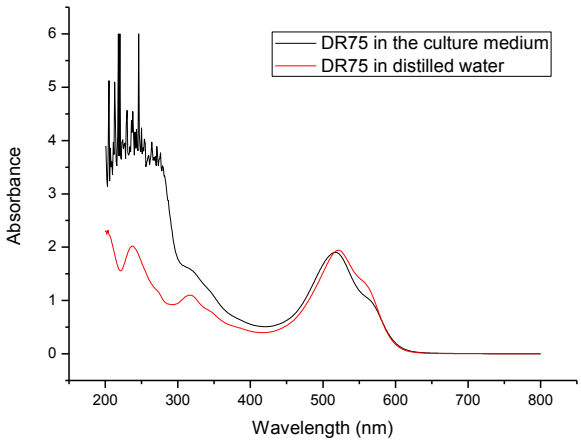
The experiments were performed at Linnaeus University, Växjö, Sweden under supervision of the Prof. Dr. Ulrika Welander and Dr. Jörgen Forss.

6.3.1 Dye

The dye Direct Red 75 (CAS 2829-43-8 and Colour Index 25380) was acquired from Sigma-Aldrich Chemical Company, Inc. The maximum wavelength (λ_{max}) used in this study was 517 nm. DR75 was used without additional purification.

The dye used in the studies of chapters 1 and 2 is the same as one used in the present chapter and has the same lot number. However, the λ_{max} analyzed in previous chapters, by spectrophotometry, were 522 nm and 521 nm for chapters 1 and 2, respectively, while in this chapter was 517 nm. The dye stock solution was diluted in the culture medium (pH around 8). The difference in the λ_{max} could be explained by the composition of the culture medium with yeast extract detailed later. Since the yeast extract is yellowish, the chromophore peak of DR75 was slightly shifted as it can be seen in Figure 2.

Figure 2 – Spectrum UV-VIS of DR75 diluted in distilled water and diluted in the culture medium with yeast extract



Source: Made by the author.

The stock solution of DR75 1000 mg/L was prepared by dissolving 1g of dye in 1L of distilled water. This solution was stored in a refrigerator.

6.3.2 Microorganisms

Rice husks from Uberlândia - Minas Gerais, Brazil were used as source of microorganisms.

The mixed culture used in Bioreactor A was obtained from a rinse water of the rice husks.

The rinse water was prepared with 250 mL of distilled water and 12g of rice husks under shaking for 30 minutes at 300 rpm. Thus, the microorganisms in the rice husks could be released into the water.

No rinse water was used in the Bioreactor B. The microorganisms present in this system were microorganisms indigenous from the rice husks, in other words, the rice husks were used without any preparation in the Bioreactor B.

6.3.2.1 Culture medium with yeast extract

- Yeast extract 2 g/L
- NaCl 0.9%
- DR75 dye 100 mg/L
- Distilled water

NaOH 1M was used to correct its pH to 8 because after some hours of treatment, the pH decreased, due to the anaerobic reactions of microbial metabolism, which hindered the microbial reproduction and survival.

The yeast extract is a water soluble compound that provides amino acids, nitrogenous compounds, carbon, sulfur, trace nutrients, vitamin B complex, other vitamins and other important growth factors, which are essential for the growth of diverse microorganisms (ACUMEDIA, 2011).

6.3.2.2 Culture medium with glucose

- Glucose 3 g/L

- KH_2PO_4 0.054 g/L
- $NaNO_3$ 0.169 g/L
- NaCl 0.9%
- DR75 dye 100 mg/L
- Distilled water
- 0.1 M Sodium phosphate buffer (pH 7.5)

The concentration of $NaNO_3$ and KH_2PO_4 was determined considering the ratio C:N:P as 250:5:1 to anaerobic digestion as stated in the literature (METCALF & EDDY, 1991, AMMARY, 2004, THOMPSON *et al*., 2006, KE *et al*., 2011). Nitrogen and phosphorus are essential nutrients in stimulating microorganisms to perform their synthesis reactions (KE *et al*., 2011).

Sodium phosphate buffer was added to this culture medium, 2 days later of the beginning of the treatment, after having noticed that the pH inside the bioreactor A was dropping too fast due to the metabolizing of the medium by the microorganisms. Buffer was added to the Bioreactor B after 31 days because the pH took more time to decrease to dangerous levels for the microorganisms and, consequently, to the system.

6.3.2.2.1 *Sodium phosphate buffer*

- Na_2HPO_4 (Sodium phosphate dibasic), MW = 141.96
- NaH_2PO_4 (Sodium phosphate monobasic), MW = 119.98

Solution A (0.2 M Na_2HPO_4) was prepared dissolving 28.4 g in 1L of distilled water and solution B (0.2 M NaH_2PO_4) was prepared dissolving 27.6 g in 1L of distilled water.

Buffer was prepared by adding 840 mL of solution A and 160 mL of solution B in 1 L of distilled water. The culture medium chemicals dissolved in distilled water were added to the buffer solution.

The culture medium was autoclaved without the dye which was added, under sterile environment, after the medium cooled down.

6.3.3 Treatment systems

Two different treatment systems were used to analyze the azo dye biodegradation and they were called Bioreactor A and Bioreactor B and two different carbon sources were tested, glucose and yeast extract.

Both systems were fed continuously with a culture medium at the required rate using a peristaltic pump Watson Marlow 400, Sci-Q.

Two experiments were performed with the same settings for both bioreactors; however, the first one had the bioreactors fed with culture medium with glucose (glucose test) and the second one with culture medium with yeast extract (yeast extract test). The bioreactors A and B as the MBBR and tubing were washed and sterilized before setting up the system treatment using culture medium with yeast extract and new rice husks were added to the Bioreactor B

6.3.3.1 Anaerobic bioreactor (Bioreactor A)

The bioreactor A (Figure 3) is a compact benchtop fermenter of 2L. The pO_2, pH and stirring were controlled by the unit control Biostat B, B Braun Biotech. The temperature was measured by the unit control; however, the temperature of the bioreactor was maintained by a water bath at 30°C, since the thermostat was not working.

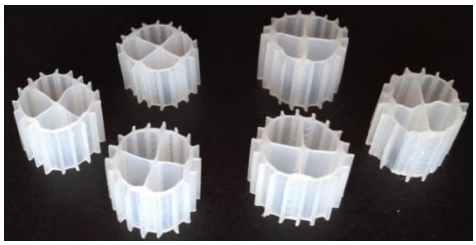
Figure 3 - Bioreactor A and unit control Biostat B

Source: Made by the author.

MBBR biofilm technology was applied in bioreactor A and had Kaldnes K1 (Figure 4) as carrier material, obtained from AnoxKaldnes™. The Kaldness K1 is a cylinder with a cross inside and fins on the outside; the length is 7 mm, the diameter is 10 mm, the specific surface

area is 690 m^2/m^3 total and 500 m^2/m^3 effective, the estimated surface area is 670 mm^2/piece total and 490 mm^2/piece effective (ØDEGAARD, 2000).

Figure 4 - Carrier Kaldnes K1 of AnoxKaldnes™



Source: Made by the author.

About 50% of the bioreactor effective volume was filled with the carriers. The process was anaerobic and the vessel had capacity for 2L of culture medium. Nitrogen was flushed into the vessel with culture medium at start up, to have an oxygen free environment.

The system was carried out with constant temperature at 30°C, pH around 6, stirring at 100 rpm and hydraulic retention time (HRT) of 24 and 48 hours to glucose test and 24, 48 and 12 hours to yeast extract test. Since the previous tests were performed with HRT of 24 hours, the HRT chosen to start the system was 24 hours. The HRT of 12 hours was tested after the HRT of 48 hours as a complementary study and that is why the results related to 12 hours of HRT are shown after 48 hours in the graphs.

The bioreactor was filled with MBBR, culture medium and rinse water. Afterwards, there was neither addition nor releasing of the culture medium in the system for 24 hours to favor the growth of microorganisms.

Since the system was fed continuously with culture medium at pH 8 in the yeast extract test and the anaerobic microorganisms were metabolizing the culture medium, the final pH measured by the unit control was about 6. The initial pH in the glucose test was 7.5, because the buffer while the final pH was about 7.0.

6.3.3.2 Fixed Bed Bioreactor (Bioreactor B)

The Bioreactor B consists of three anaerobic bioreactors (B1, B2 and B3) with capacity for 350 mL of culture medium and one aerobic bioreactor (B4) with capacity for 500 mL (Figure 5). The bioreactor B4 had an air stone attached to an air pump to provide oxygen to

the system. The anaerobic bioreactors were filled with ¾ of rice husks, approximately 42 g. The rice husks had a function of carrier material and source of microorganisms.

The process was performed without stirring in room temperature, pH around 6 in the anaerobic bioreactors as in Bioreactor A and around 8 in the aerobic bioreactor to the yeast extract test while to the glucose test the pH was around 7 in the anaerobic bioreactors as in Bioreactor A and around 8 in the aerobic bioreactor. The retention time was the same of the Bioreactor A for both tests, with glucose and yeast extract.

Figure 5 – Bioreactor B composed by the sequenced bioreactors B1, B2, B3 and B4 (aerobic)

Source: Made by the author.

When the HRT was changed from 48 hours to 12 hours, a new aerobic bioreactor, called C, was added to the Bioreactor B, between B3 and B4, in order to keep the HRT of the

aerobic process equal 48 hours. The decision to add this aerobic bioreactor was made based on the organic acids and COD analysis.

6.3.4 Batch experiment

The batch experiment was performed to identify the best carbon source to the microorganisms in order to achieve the DR75 dye biodegradation.

Rinse water of rice husks was used as source of microorganisms.

Forest residue wood chips from Sweden were also tested as a source of microorganisms. Microorganisms from the wood are able to degrade lignin and also dye. Rinse water of forest residue was added to the glucose medium culture instead rinse water of rice husks in order to check if the microorganisms from the forest residue would be able to biodegrade the dye in culture medium with glucose. Forss and Welander (2011) already evaluated the use of forest residues to the biodegradation of dyes using yeast extract as carbon and nitrogen source. They reported good rates of decolorization, about 90%, of the azo dyes Reactive Black 5 and Reactive Red 2 in the first reactor of 3, after 4 days and 23 h.

The experiment was prepared in 100 mL Erlenmeyer's flasks in triplicate. Glucose and yeast extract was evaluated as carbon source. The samples (100 mL) were kept in the oven at 30°C for 7 days and scanned by UV-VIS spectrophotometer (800-200 nm) in quartz cuvette each 24 hours.

6.3.4.1 Culture medium with Glucose

- Glucose 3 g/L
- $NaNO_3$ 0.1690 g/L
- KH_2PO_4 0.540 g/L
- NaCl 0.9%
- DR75 100 mg/L
- Distilled water

6.3.4.2 Culture medium with yeast extract

- Yeast extract 2 g/L

- NaCl 0.9%
- DR75 100 mg/L
- Distilled water

Samples with and without 0.1 M Sodium phosphate buffer (pH 7.5) were analyzed in order to verify the effect of pH changing in the decolorization process.

Table 1 shows the contents of each sample analyzed. The samples codes are related to the presence or absence of a carbon source, the buffer and a source of microorganisms. Thus, GBR means glucose, buffer and rinse water of rice husks; GBF means glucose, buffer and rinse water of forest residue; GB- means glucose and buffer without any rinse water; YBR means yeast extract, buffer and rinse water of rice husks; YB- means yeast extract and buffer without rinse water of rice husks; Y-R means yeast extract and rinse water of rice husks without buffer; Dye-R means dye solution and rinse water of rice husks without buffer and carbon source.

Table 1 – Samples composition of the batch experiment performed with DR75 dye solution 100 mg/L

Sample code	Glucose*	Yeast Extract*	Buffer*	Rinse water of rice husks*	Rinse water of forest residue*
GBR	+	-	+	+	-
GBF	+	-	+	-	+
GB-	+	-	+	-	-
YBR	-	+	+	+	-
YB-	-	+	+	-	-
Y-R	-	+	-	+	-
Dye-R	-	-	-	+	-

*+ indicates presence and – indicates absence

6.3.5 Chemical reduction

Chemical reduction was adapted from Cioni *et al*. (1999) to reduce the dye using sodium dithionite, allowing the evaluation of DR75 and its probable metabolites. Sodium dithionite ($Na_2S_2O_4$) is one of the most frequently used reducing agents for azo dyes

(AHLSTRÖM *et al*., 2005). Amines are usual products of the azo dye reduction by sodium dithionite (OH *et al*., 1997, PINHEIRO *et al*., 2004)

2 mL of sodium dithionite 200 mg/mL was added to 17 mL of the dye solution 100 mg/L and heated in a reaction vessel with cap at 70 ±2°C for 30 min. The samples were analyzed by HPLC, LC/MS and GC/MS.

Tests with sodium dithionite 100 mg/mL and not heated samples were also performed to check the effect of the sodium dithionite concentration and heating on the dye solution.

6.3.6 Analysis

All samples were centrifuged before the analyses in a centrifuge Sigma 3K10 in 7000 rpm for 7 minutes. The samples to HPLC and LC/MS analyses were filtered through 0.45 µm filters (Whatman, GE Healthcare).

6.3.6.1 Decolorization

According to Fernández *et al*. (2010), UV-VIS spectrophotometry can be used for the determination of the concentration of several dyes during the process of biodegradation.

The samples were analyzed by UV-VIS spectrophotometry using a LAMBDA 35 UV-VIS spectrophotometer Perkin-Elmer and the UV WinLab software. The samples were scanned from 200 nm to 800 nm three times a week in 5 mm quartzo cuvettes.

The absorbance of the chromophore group was used to monitor the decolorization. The chromophore wavelength to the medium culture with DR75 was 517 nm. The decolorization of the dye was evaluated using the equation 1:

$$\%\,decolorization = \frac{A_{517nm(\lambda\,\max)}\,culture\ medium - A_{517nm(\lambda\,\max)}\,sample}{A_{517nm(\lambda\,\max)}\,culture\ medium}\ x\ 100 \quad (1)$$

6.3.6.2 COD, Organic acids, Nitrogen, Phosphate and Orto-phosphate

Chemical Oxygen Demand (COD), organic acids, nitrogen, phosphate and orto-phosphate concentrations were determined using Hach-Lange DR 2800 spectrophotometer, Hach-Lange LT 200 thermostat and Hach-Lange colorimetry test cuvettes as follows:

- COD: LCK 014 (1000 – 10000 mg/L O_2), LCK 114 (150 – 1000 mg/L O_2)

- Organic acids: LCK 365 (50 – 2500 mg/L CH_3COOH and 75 – 3600 mg/L CH_3H_7COOH)

- Nitrogen: LCK 238 (5 – 40 mg/L TN)

- Phosphate: LCK 350 (2 – 20 mg/L PO_4-P and 6 – 60 mg/L PO_4)

- Orto-phosphate: LCK 049 (5 – 90 mg/L PO_4)

COD and Organic acids analysis was monitored weekly while nitrogen, phosphate and orto-phosphate were carried out with the medium culture and the last samples collected from the bioreactor A and the aerobic bioreactor (B4) outlet.

6.3.7 Amines evaluation

6.3.7.1 HPLC

The HPLC analysis was performed by HP 1100 HPLC system, Hewlett-Packard equipped with a G1314A deuterium lamp detector and a Fortis C18 (3µm , 250mm X 4.6mm) column, Fortis Technologies, using a mobile phase of Methanol HPLC gradient grade (A) and buffer (B) (Ammonium Formate 3.03 nM in Formic acid 50 mM), pH 2.8. Two methods were tested:

- *Method 1*: The mobile phase gradient started with 20% (A) and 80% (B). After 20 min of elution the gradient increased linearly to 100% (A) for 25 min. The post phase continued at 20% (A) and 80% (B) for 15 min. The wavelength analyzed was 240 nm; the flow rate was 0.5 mL/min, injection volume was 20µL and the samples were analyzed once a week.

- *Method 2*: The mobile phase gradient started with 80% (A) and 20% B. After 20 min of elution the gradient increased linearly to 100% (B) for 25 min. The post phase continued at 80% (A) and 20% (B) for 15 min. The wavelength analyzed was 240 nm; the flow rate was 0.5 mL/min, injection volume was 20µL and the samples were analyzed once a week.

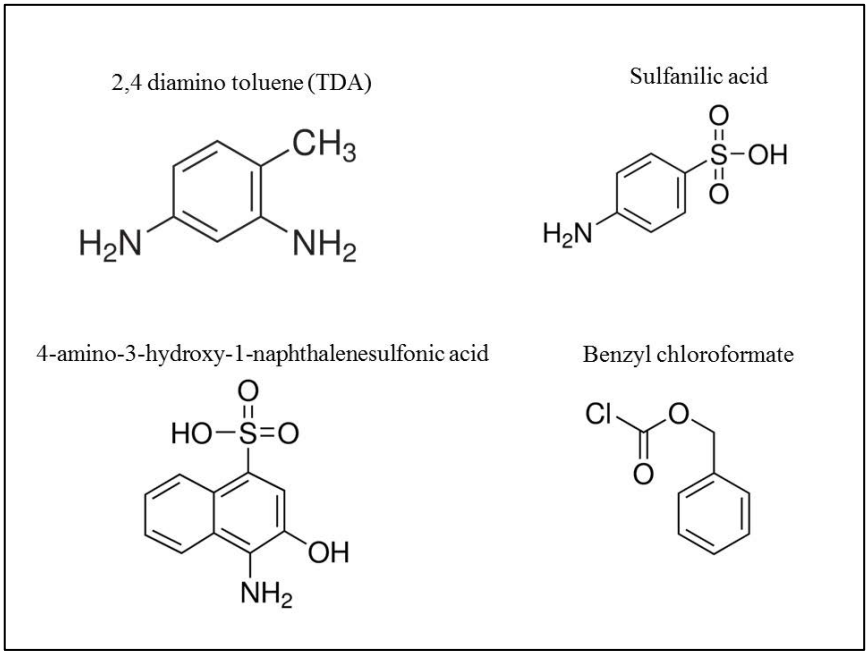
6.3.7.2 LC-MS

The LC/MS and LC/MS/MS analyses were performed by SP Technical Research Institute of Sweden in order to detect aromatic amines possibly generated in the dye biodegradation process. The samples were stored in the freezer at -20°C before the analysis.

Samples collected from the outlets of the bioreactors A, B1, B2, B3 and B4 in the 5th week and samples from A and B4in the 9th and 11th week of treatment were evaluated by LC/MS technique. The dye solution (100 mg/L) and samples from the chemical reduction process were also analyzed.

Three aromatic amines were analyzed before to evaluate the other samples. The reference substances, 2,4-toluene diamine (TDA), sulfanilic acid and 4-amino-3-hydroxi-1-naphthalenesulfonic (Figure 5) acid were used for establishing of reaction and detection conditions and DR75 was used as reference azo dye. The reference substances were derivatized by the addition of benzyl chloroformate (Figure 6) in order to detect the substrate by parental ion scanning, specifically for detection of the benzyl group.

Figure 6 - Reference substances of aromatic amines (TDA, sulfanilic acid and 4-amino-3-hydroxi-1-naphthalenesulfonic) and benzyl chloroformate used to derivatization and analysis by LC/MS



Source: Made by the author.

6.3.7.2.1 *Derivatization*

4 µL of benzyl chloroformate was added to 100 µL 1 ppm amines in 1:2:2 water: methanol:acetonitrile solution. The reaction mixture was kept at room temperature for 5 min even though the reaction is almost instant. No further dilution was performed before analysis by LC/MS.

Two different methods were performed, method A and method B:

- *Method A*: The samples were separated by a 3 min linear gradient from 95% mobile phase A (95% H_2O + 5% Acetonitrile +0.1% NH_3) to 95% mobile phase B (Acetonitrile) with 0.8 ml/min flow rate at 70°C on a Acquity UPLC BEH C18 (1.7µm, 2.1 x 50mm) column.
- *Method B*: The samples were separated by a 2 min linear gradient from 95% mobile phase A (95% H_2O + 5% Acetonitrile +0.1% FA) to 95% mobile phase B (Acetonitrile) with 0.8 ml/min flow rate at 70°C on a Acquity UPLC CSH C18 (1.7µm, 2.1 x 50mm) column.

The detectors used were a PDA scanned from 230-350 nm (Waters) and Xevo QT of G2-S (Waters) online with the LC separation unit. The mass spectrometer was operated in both positive and negative ion mode and electrospray was used for ionization.

6.3.7.3 GC/MS

Only the samples from the chemical reduction were analyzed by GC/MS as the equipment is not able to measure molecules with molecular weight above 650 and DR75 has 990.79. The samples were analyzed on a Varian Saturn 2000 GC/MS equipped with a Varian CP 3800 GC. Agilent DB-5MS Ultra Inert column (30 m×0.25 mm ×0.25 μm) was used to chromatographic separation. The injection was carried out by SPME fiber coating Polyethylene Glycol (PEG) 60μm (Supelco) and SPME fiber coating 100μm polydimethylsiloxane (PDMS) (Supelco). The oven temperature was kept constant at 50°C for 5 min at the moment of injection, then increased to 110 ◦C, with a temperature increment of 20°C/min and held for 2 min, then increased to 160 ◦C, with a temperature increment of 10°C/min and held for 2 min, then increased further to 250 ◦C (at 20 ◦C/min increment) and held for 5 min. Helium was used as a carrier gas, with a flow rate of 1 ml/min and the injector temperature was 240 ◦C. The mass range analyzed was 40–650 m/z and a scan rate of 1 scans/s.

6.3.8 Rice husk surface analysis

Surface area of rice husks was measured using Brunauer–Emmett–Teller (BET) method and the porosimetry method was applied to analyze the porous of the husks.

Rice husks before treatment, after treatment washed with distilled water and washed with ethanol 70% were investigated.

After treatment, certain amount of husks were washed with water in order to clean the surface while another amount of husks were washed with ethanol to clean the surface as well, but also to sterilize the surface and to try to remove microorganisms attached to it.

6.3.8.1 Brunauer–Emmett–Teller (BET)

BET analysis provides specific surface area evaluation of different materials. The BET theory is based upon the phenomenon of physical adsorption of gases on the external and

internal surfaces of a porous material. A material is surrounded by gas, nitrogen is recommended, that has a certain temperature and relative vapor pressure, adsorbs physically a certain amount of gas (FAGERLUND, 1973, KALDERIS *et al*., 2008).

Samples of rice husks before and after the treatment were grounded and dried under vacuum at 85°C overnight in Micrometrics VacPrep 061. The analysis of specific surface area was done using Micrometrics TriStar 3000 Analyzer. The measurements were performed under N_2 adsorption at liquid nitrogen temperature.

6.3.8.2 Porosity of Rice husks

Mercury intrusion porosimetry is widely used to evaluate the porosity of industrial catalysts as other porous materials, for instance, rice husks ash.

The porosity of the rice husks was characterized by mercury intrusion porosimetry method using an Autopore IV 9500 (Micrometrics) INC. The rice husks were dried at 85°C before the analysis. The porosity was measured using a 5 bulb solid penetrometer of 1 mL, stem volume 0.392ml, penetrometer constant 10.79 ul/pF and pressure 0-30000 psia.

6.4 Results and Discussion

In the present study, the biodegradation of DR75 azo dye was carried out by two different treatment systems. The first parameter to be evaluated is the percentage of decolorization.

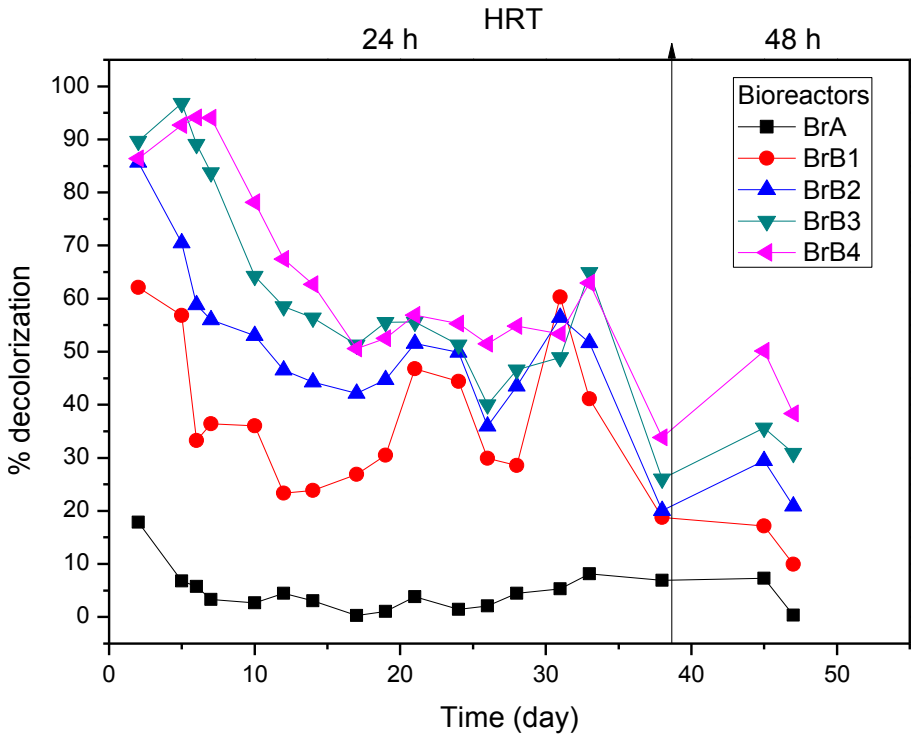
6.4.1 Decolorization

The dye biodegradation can be analyzed by different methods. The decolorization of dye solution is an important parameter to evaluate the capacity of dye removal by the microorganisms.

6.4.1.1 Bioreactor A and Bioreactor B fed with glucose culture medium

The microorganisms in bioreactor A were not able to decolorize the culture medium since the percentage of decolorization was fewer than 10% over the treatment as it can be seen in Figure 7.

Figure 7 – Percentage of decolorization in the Bioreactor A and bioreactors B1, B2, B3 and B4, fed with glucose culture medium, over the time and HRT of 24 and 48 hours



Source: Made by the author.

This treatment system fed with glucose culture medium was not efficient because the decolorization rates were lower than expected.

The low decolorization rates in Bioreactor A could be influenced by the presence of glucose or buffer in the culture medium.

The glucose could interfere due to the glucose effect. According to Madigan and Martinko (2006), in glucose effect the syntheses of a variety of enzymes, primarily catabolic, are repressed when cells grow in a medium that contains glucose. Actually, in some microorganisms, other carbon sources can cause the same effect and then it is called catabolite repression. However, the buffer was added to the system because the pH was decreasing fast which means that the microorganisms, in fact, were able to metabolize the glucose releasing organic acids in the medium that caused the pH shift.

The decolorization in the first analysis of Bioreactor A is 17%. There was no flow of culture medium in the first 24 hours in order to favor the microorganisms' growth and

adaption into the bioreactor A. Then, the highest percentage of decolorization to this system was reached before adding buffer.

After the addition of buffer in the culture medium, more 150 mL of rice husks rinse water was added to the system to ensure the presence of live microorganisms and adapted to the new culture medium.

Rai *et al*. (2014), evaluated the decolorization of four dyes (congo red, brilliant blue, crystal violet and malachite green) by the enzyme extract of *Aloe barabadensis* with 3 different buffers (citrate, acetate and phosphate buffers). The test was performed at 30ºC for 1 h. There was decolorization of just three dyes. Congo red presented the best decolorization rate, 27.73%, with citrate buffer while for brilliant blue the best rate was 15% with acetate buffer and for malachite green was 10 % with phosphate buffer. However, there was no decolorization for crystal violet with any buffer.

There is little information about the effect of buffers in dye degradation process. More research should be done in order to understand the positive or negative effect of buffers in dye biodegradation. The results reported by Rai *et al*. (2014) and the present study can indicate that the buffer may have an effect in the biodegradation of some dyes such as crystal violet and DR75.

Bioreactor B showed better decolorization rates than Bioreactor A. About 96% was reached by the bioreactor B3 at the begging of the treatment. The bioreactors B1, B2 and B3 were filled with rice husks; therefore, the dye was, initially, adsorbed by the rice husks before the degradation started. Then, the percentage of decolorization decreased to less than 70%.

The buffer was added to the Bioreactor B after 31 days because the pH took more time to decrease than in Bioreactor A. However, the decolorization was still decreasing. Then, the HRT was changed from 24 to 48 hours.

The change in the HRT did not seem to influence the decolorization in Bioreactor A since the percentage remained similar to the decolorization with HRT of 24 hours. The same happened in the bioreactor B1. However, the decolorization in the bioreactors B2, B3 and B4 increased in the first measure and decreased in the last one. In fact, it was not possible to confirm the influence of the HRT on these systems because only two samples with 48 hours of HRT were analyzed before the end of the treatment.

Since the decolorization rates were not good enough, the system was stopped and a new system was set using yeast extract in the culture medium.

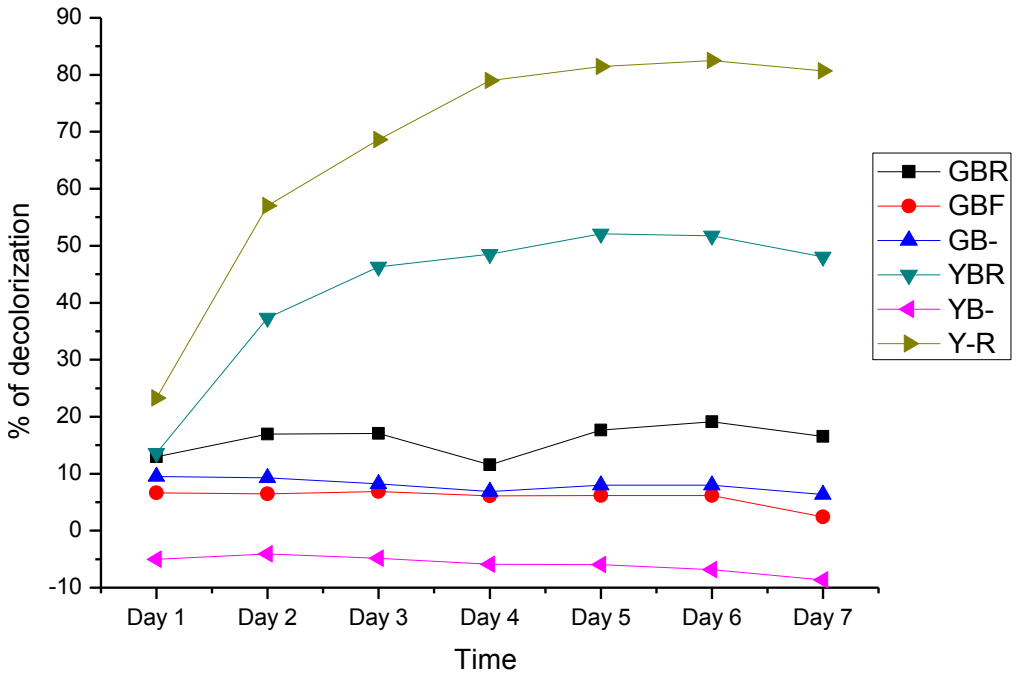
6.4.1.2 Batch experiment

This experiment was performed before to stop the treatment system with the Bioreactors A and B using glucose as carbon source, since the decolorization rates were not good enough and the yeast extract was planned to replace the glucose.

Besides the different carbon source, the effect of buffer was also analyzed.

Figure 8 shows the percentage of decolorization of the samples over the time and it can be seen that the sample with yeast extract, rinse water of rice husks and without buffer (Y-R) presented the best decolorization rate, about 80%.

Figure 8 – Percentage of decolorization of the batch experiment samples over the time. glucose + buffer + rinse water of rice husks (GBR); glucose + buffer + rinse water of forest residue (GBF); glucose + buffer - rinse water of rice husks (GB-); Yeast extract –buffer + rinse water of rice husks (Y-R); yeast extract + buffer - rinse water of rice husks (YB-); yeast extract + buffer + rinse water of rice husks (YBR)



Source: Made by the author.

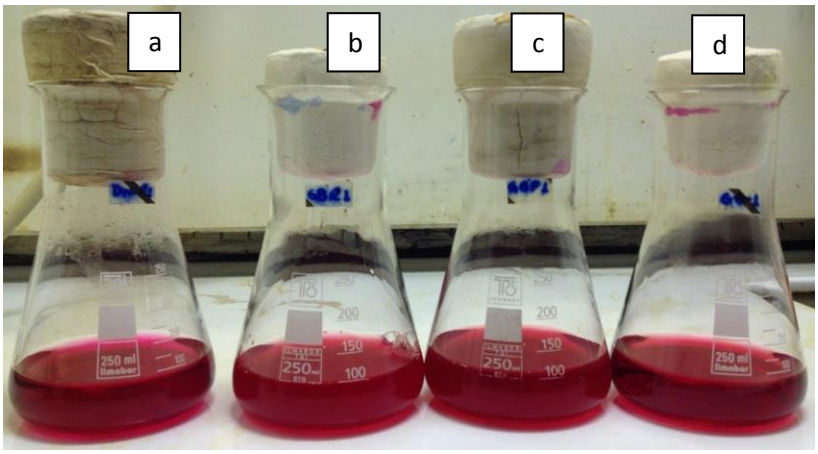
The percentage of decolorization of the sample with yeast extract, buffer and rinse water of rice husks (YBR) was 50%, approximately, less than Y-R, though it was even better than the samples with glucose. It is possible that the sodium phosphate buffer interfered somehow in the biodegradation process of the DR75 dye, as mentioned before (section 1.4.1.1), because the bioreactor A fed with glucose culture medium and buffer did not show good rates of decolorization as GBR and YBR.

The sample with yeast extract and buffer without rinse water (YB-) presented negative decolorization rates. In fact, the difference between the absorbance of the dye control solution and YB- could be attributed to the presence of yeast extract that has a yellowish hue. Thus, the dye plus the yeast extract presented higher absorbance than the dye solution or the dye with glucose which is colorless when in solution. Moreover, the absence of microorganisms to degrade the dye kept the decolorization in negative levels over the days.

Among the samples prepared with glucose culture medium, the sample with glucose, buffer and rinse water of rice husks (GBR) presented the best percentage of decolorization; however it was too low compared with the yeast extract samples. The sample with glucose and buffer without rinse water of rice husks (GB-) even without a source of microorganisms was able to decolorize almost 10%. The percentage remained constant over the days, which likely indicate the occurrence of adsorption by the molecules. The coagulation of the compounds in the sample may have occurred as well, and clots were removed from the solution after centrifugation. The sample with glucose, buffer and rinse water of forest residue (GBF) maybe does not have many microorganism or they did not adapt in the medium, so they were not able to decolorize the solution.

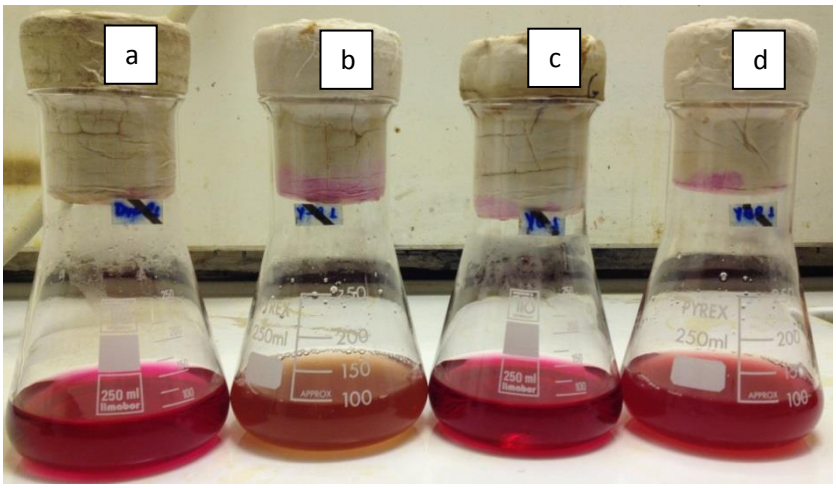
The dye decolorization can be observed in pictures of the samples after 7 days of treatment. Figure 9 shows Dye-R and the samples with glucose culture medium while figure 10 shows Dye-R and the samples with yeast extract culture medium.

Figure 9 – Visual analysis of the dye solution and the samples prepared with glucose culture medium. Glucose - buffer + rinse water of rice husks (Dye-R) (a); glucose + buffer + rinse water of rice husks (GBR) (b); glucose + buffer + rinse water of forest residue GBF (c); glucose + buffer - rinse water of rice husks (GB-) (d)



Source: Made by the author.

Figure 10 – Visual analysis of the dye solution and the samples prepared with yeast extract culture medium. Glucose + rinse water of rice husks (Dye-R) (a); Yeast extract –buffer + rinse water of rice husks (Y-R) (b); yeast extract + buffer - rinse water of rice husks (YB-) (c); yeast extract + buffer + rinse water of rice husks (YBR) (d)



Source: Made by the author.

Azo dye reduction is possible because of the oxidation–reduction reactions that have the azo dye acting as final electron acceptor (OZDEMIR *et al.*, 2013). Since the culture medium

contained the azo dye DR75, it was essential to provide a co-substrate responsible for the electron donor.

Glucose and yeast extract were tested as carbon source. Another source of nitrogen and phosphorus had to be added to the culture medium prepared with glucose whereas the culture medium with yeast extract had nothing added because, as mentioned before (section 1.3.2.1), the yeast extract provides not only carbon, but also other important growth factors for microorganisms (ACUMEDIA, 2011).

Alex (2009) investigated the nutrition requirements that could improve the textile dyes decolorization and among the compounds analyzed were glucose and yeast extract. Six and five different concentrations of glucose and yeast extract, respectively, were evaluated in the Acid orange II aerobic treatment with a mixed culture isolated from a textile effluent. After 96 h, decolorization rates around 60% was reached with 3 and 5 g/L of glucose and 2 and 5 g/L of yeast extract. Thus, 3 and 2 g/L of glucose and yeast extract, respectively, were used in the present study, since these concentrations have demonstrated to be enough to promote the growth of microorganisms and to achieve satisfactory decolorization rates.

Both carbon sources are electron donors and, when metabolized, the products of the catabolism act as redox mediators enhancing the dye decolorization (ALEX, 2009, VAN DER ZEE and CERVANTES, 2009, BAÊTA *et al*., 2012, SUN *et al*., 2013).

Redox mediators are able to accelerate the electron transfer from an electron donor to a final electron acceptor, which can increase the reaction rates and speed up the biodegradation process (DOS SANTOS *et al*., 2005).

Sun *et al*. (2009) reported the use of glucose and Baêta *et al*. (2012) reported the use of yeast extract as co-substrate for redox mediators. According to Sun *et al*. (2009), at identical dye concentrations, the presence of glucose greatly increased the dye decolorization of Active brilliant red X-3B as compared to the control without extra organic carbon sources. On the other hand, Baêta *et al*. (2012) analyzed the decolorization of reactive blue HFRL in UASB reactor, in operational phases, using only glucose, only yeast extract and both. The decolorization in presence of glucose was lower than decolorization in presence of yeast extract. Hence, the absence of yeast extract led to a decrease in decolorization rates, proving that the use of yeast extract improved the dye degradation.

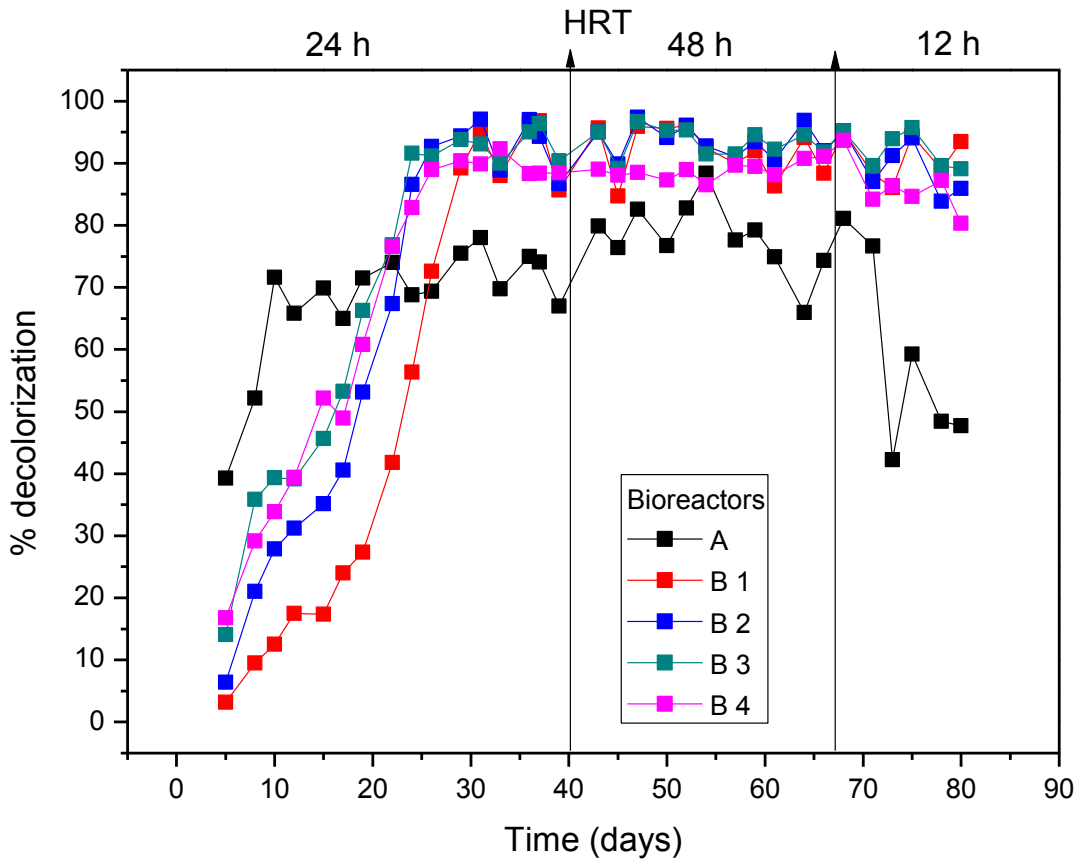
Yeast extract is the source of the redox mediator riboflavin that plays a role in the azo bond reduction. The riboflavin may be reduced by electron carriers, for instance NADH, at the bacterial cell surface and then be re-oxidized by the azo dye (BAÊTA *et al*., 2012).

Therefore, the results presented in this study may also suggest that yeast extract is a better carbon source for dye biodegradation than glucose.

6.4.1.3 Bioreactor A and Bioreactor B fed with yeast extract culture medium

The Bioreactor B showed better rates of decolorization than the Bioreactor A, as it can be seen in Figure 11 which shows the percentage of decolorization in both systems over the days with HRT of 24, 48 and 12 hours.

Figure 11 - Percentage of decolorization in the Bioreactor A and bioreactors B1, B2, B3 and B4, fed with yeast extract culture medium, over the time with HRT of 24, 48 and 12 hours



Source: Made by the author.

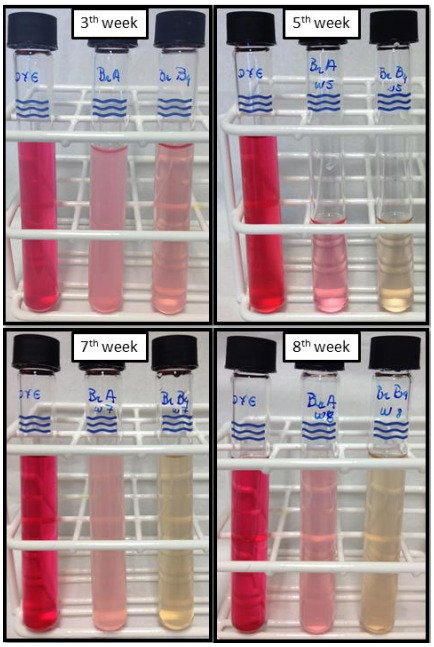
The third point of the Bioreactor A with 12 hours of HRT in the Figure 11 is shifted. The shift occurred due to a problem with the inlet tubing (from the bottle with culture medium to the bioreactor). The tubing broke and culture medium leaked out over the night. The tubing was replaced as soon as the problem was noticed, in the morning. The flow was stopped for a while until the volume of wastewater has been recovered.

The decolorization in Bioreactor A stabilized after the 10th day of treatment. After changing the HRT to 48 h, the percentage of decolorization increased from about 67.9% to 78.3%, when compared with 24 hours, afterwards it decreases with 12 hours.

The Bioreactor B took more time to stabilize. Since the Bioreactor B was filled with rice husks, the initial process of decolorization occurred by adsorption of the dye molecules and about 25 days the percentage of decolorization to B1, B2 and B3 and B4 was increasing. On the other hand the percentages of decolorization in these bioreactors were higher than Bioreactor A after 22 days.

The decolorization can also be observed in the visual analysis of the the DR75 100 mg/L, the samples of Bioreactor A and bioreactor B4 (Bioreactor B) after 3, 5, 7 and 8 weeks of treatment (Figure 12). 3, 5, 7 and 8 weeks correspond to 21, 35, 49 and 56 days of treatment.

Figure 12 – Visual analysis of the DR75 100 mg/L, the samples of Bioreactor A and bioreactor B4 (Bioreactor B) after 3, 5, 7 and 8 weeks of treatment



Source: Made by the author.

Both systems have different microbial activity since one is anaerobic and the other one is anaerobic/aerobic. The setup is also different, for example, different carrier material was used in the treatments as well as the temperature. However, some parameters can be compared in order to find a better setup for a future treatment system encompassing the best characteristics of each process. For instance, the temperature of the bioreactor A was maintained at 30°C whereas the Bioreactor B was at room temperature, 22°C approximately. It was expected that the decolorization in Bioreactor A would be higher than Bioreactor B because the temperature. According to Angelova *et al*. (2008), that evaluated the temperature effect on bacterial azo bond reduction, the decolorization rates increase as the temperature also increases until a certain limit for bacterias. On the other hand, the decolorization rates of Bioreactor B were better than Bioreactor A, indicating that even with lower temperature, the Bioreactor B is more efficient to decolorization.

Another difference between the bioreactor A and the Bioreactor B is the effect of the HRT in the decolorization. The HRT was changed twice in order to evaluate the biodegradation process over the time and to verify which HRT would be more advantageous to these treatment systems. The system was initiated with 24 hours of HRT, then it was changed to 48 hours after 40 days, approximately, and it was changed once more to 12 hours after almost 70 days of the treatment beginning.

When the HRT was increased to 48 hours and afterwards decreased to 12 hours, the decolorization in bioreactors B1, B2 and B3 did not have an expressive variation unlike the bioreactor A. Thus, the system A seems to be more sensitive to HRT changes than Bioreactor B. Therefore, despite it took a longer time to stabilization; the Bioreactor B was more efficient to decolorize the dye solution than Bioreactor A. As the textile industries are concerned with the color removal of the wastewater, it is interesting for them to use a system of wastewater treatment that will be able to remove the color, as much as possible, even if the system needs a certain time to stabilize.

Another advantage of the Bioreactor B is the HRT/bioreactor. The HRT of 24 and 48 hours is for the whole system. However, if each bioreactor is analyzed separately, the HRT will be 6 and 12 h/bioreactor. The bioreactor B1, for instance, showed decolorization rates above 90%. Moreover, B1, B2 and B3 could work together in order to reach higher rates of decolorization. Although, after 30 days, B1 showed high decolorization rates as well as B2 and B3, indicating that B1 was the main responsible for decolorization of the Bioreactor B in

this period, after system stabilization. Furthermore, B1 was the first bioreactor to receive the culture medium rich in nutrients, which likely favored its microorganisms.

Forss *et al*. (2013) also analyzed the biodegradation of dyes in two bioreactor filled with rice husks. 80% of decolorization was reached after 28.4 hours of HRT, i.e., 14.2 hours for each bioreactor. Therefore, with HRT of 6 hours, the Bioreactor B has proven to be an interesting alternative to the dye biodegradation and it should be further assessed, so this technology can be possibly applied in the future for textile wastewater treatment.

6.4.2 Chemical analyses

The success of a textile wastewater treatment should not be only based on decolorization. There are several parameters to be evaluated and the present study could verify some factors in the biodegradation studies.

Besides the decolorization, COD (Chemical Oxygen Demand) is also noteworthy because it allows measuring the amount of organic matter susceptible to be oxidized by chemical reactions in a liquid sample. Hence, the COD was measured over the weeks to evaluate the organic matter degradation by the microorganisms.

Nitrogen and phosphorus are also important since they are related with the capacity of the microorganisms to grow and biodegrade the dye. The concentration of total nitrogen, phosphate and orto-phosphate must be in the proper proportions to enable cell growth without nutrient limitation.

The chemical analysis of the biodegradation process in Bioreactor A and Bioreactor B fed with yeast extract culture medium showed that the conditions for microbial growth were favorable and also that the aerobic process was more efficient for COD removal than the anaerobic process.

Since the treatment in Bioreactor B is combined anaerobic/aerobic, it is interesting to measure the organic acids concentration as well. Organic acids are produced during the anaerobic digestion, in this case, during the process of dye biodegradation, and they are consumed in aerobic conditions. Therefore, organic acids concentration is higher in samples from the anaerobic reactors than those from the aerobic reactor and medium. Table 2 shows

the percentage of COD removal and concentration of organic acids, total nitrogen, phosphate and orto-phosphate of the samples.

Table 2 COD removal and organic acids concentration in the dye biodegradation process

Bioreactor	COD removal (%)			Organic acids [a] (mg/L)		
	HRT 24h	**HRT 48h**	**HRT 12h**	**HRT 24h**	**HRT 48h**	**HRT 12h**
A	15.38	19.55	12.89	625.40	527.25	697.00
B1	7.33	8.36	17.28	690.00	727.00	710.00
B2	18.41	14.89	17.21	703.00	666.00	796.00
B3	19.28	25.61	13.78	634.00	559.50	748.00
B4	55.62	71.76	65.94 [b]	304.00	152.48	242.43[b]

a) Organic acids concentration for the medium culture is 280 mg/L.

b) COD removal and Organic acids with HRT of 12 hours were calculated using the average between the bioreactors B4 and C.

The percentage of COD removal for the bioreactor A was lower than Bioreactor B. The highest value of COD removal in bioreactor B was in B4 that is the aerobic bioreactor. Observing Figure 10, it can be noticed that there are good rates of decolorization. However, the percentage of COD removal is unsatisfactory in the anaerobic bioreactors.

B4 showed better rates of COD removal when compared with the anaerobic bioreactors. According to Dafale *et al*. (2010), the usual low efficiency of COD removal by conventional techniques have been overcome by the development of anaerobic/aerobic system and the results achieved in this research support this statement.

The HRT could also influence the COD removal. Possibly, better rates of COD removal could be reached with a longer HRT as it can be seen in Table 2.

There was only one aerobic bioreactor (B4) at the beginning of the treatment with the HRT of 12 hours. The COD removal decreased and the organic acids concentration increased with a lower HRT. In face of this changes, another aerobic bioreactor (C) was added to the

system, increasing the HRT to 48 hours only to the aerobic treatment while the anaerobic treatment remained with 12 hours, in order to enhance the COD removal.

The color removal was provided by the reductive cleavage of the dye bond, which used a carbon source as electron donor through anaerobic decolorization under reductive conditions in bioreactors (LIM *et al*., 2014). The carbon sources in the present study were yeast extract and glucose; however only the COD removal using yeast extract was analyzed. DR75 could be a source as well despite being deficient as carbon source.

According to van Haandel (2012), the theoretical COD of a compound can be calculated from stoichiometric considerations and it is possible to know if the organic material was completely oxidized. Using the Theoretical COD (COD_t) equation (2) proposed by van Haandel (2012), it is possible to deduce that the yeast extract was provided for the main COD contribution in the medium while the dye only contributed to a small portion of the COD .

$$CODt = \frac{8\times(4x+y-2z)}{(12x+y+16z)} \text{ g COD/g } C_xH_yO_z \qquad (2)$$

The contribution of DR75 was around 6.4%, considering the dye concentration of 100 mg/L and the COD_t value in the medium. Since the dyes were not fully degraded under anaerobic conditions, only decolorized due to cleavage of the azo bond, the COD removal was to a large extent attributed to the consumption of yeast extract, while the presence of dye metabolites contributed to the residual COD (JONSTRUP *et al*., 2011).

Decolorization means that the azo bonds of DR75 were broken, thus color was removed from the wastewater. However, subproducts of the reductive cleavage were not degrading by the anaerobic treatment. Therefore the COD removal was lower in the anaerobic treatment. Analyzing azo dye treatment, Ozdemir *et al*. (2013) observed that the aeration of the last of four compartments of the anaerobic baffled reactor (ABR) increased the COD removal efficiency from approximately 89% to 97%. This result was reached due to aerobic degradation of the residual COD (OZDEMIR *et al*., 2013) as occurred the B4 aerobic bioreactor (Table 2).

Other important factor that interferes in the COD removal is the HRT. According to Işık and Sponza (2008), about 80% of COD and 91% color removal efficiencies were obtained at a HRT of 100 h. After decrease the HRT to 6 h, the COD removal efficiencies

decreased to 29.4%. Considering the HRT of 24 h, decolorization about 95% in bioreactor A and 80 % in B and COD removal about 15% in A and 65% in B, the COD removal should be better with a bigger HRT.

The bond molecules were broken by the action of the microorganisms, but it does not mean that they were degraded, but just that the molecules are smaller than before.

After noticing that the organic acids concentration was lower when the HRT was 48 hours and higher in the first week of treatment with 12 hours, around 512 mg/L, one more aerobic bioreactor, called C, was added between B3 and B4. This bioreactor had the capacity for 2 L of effluent and HRT of 48 hours, while the HRT to the other bioreactors was 12 hours. The concentration of organic acids decreased to 156 mg/L in Bioreactor C and 59.3 in B4.

Regarding the organic acids concentration, it was higher in samples from the anaerobic reactors than those from the aerobic reactor, which was already expected. The organic acids concentration in Bioreactor B decreased about 45% with HRT of 24 hours, 23.4% with HRT of 48 hours and 32.3% with HRT of 12 hours after the aerobic treatment.

The organic acid concentration is higher in anaerobic bioreactors because a large fraction of substrate carbon is converted to products and a smaller fraction, less than 30%, is converted to cell mass while in aerobic fermentation, about 50% of substrate carbon is incorporated into cells and about 50% of it is used as an energy source (JONSTRUP *et al*., 2011). Furthermore, the aromatic amines generated by the reductive cleavage of azo bonds, as sulfanilic acid, can be oxidized to organic acids (SHULER and KARGI, 2002).

The orto-phosphate is directly available to the microorganism. It is important to be sure that there will be enough phosphorus to support the microbial growth. The concentrations of phosphate and orto-phosphate at the begging of the treatment were 8.63 mg/L 24.8 mg/L, respectively. After the treatment, the phosphate concentrations were 20.9 mg/L in bioreactor A and 20 mg/L in bioreactor B and the orto-phosphate concentrations were 28.6 mg/L in bioreactor A and 27.6 mg/L in bioreactor B. Therefore, the concentrations after treatment were higher than before, which means that there was enough phosphorus to the microorganisms or even excess phosphorus. Moreover, the higher concentration could be due to the presence of dead microorganisms that released phosphorus from their DNA.

Nitrogen and phosphorus are related to the capacity of the microorganisms to grow. The total nitrogen and phosphate concentration was 226 mg/L and 20.9 mg/L in bioreactor A and 191.4 mg/L and 20 mg/L in Bioreactor B, respectively. Considering the rate C:N:P as 250:5:1 in anaerobic treatment, 100:5:1 in aerobic treatment (AMMARY, 2004, THOMPSON *et al*., 2006) and the medium culture COD of 2347 mg/L, the concentrations of carbon, nitrogen and phosphate were in the proper proportions for enable microbial growth without nutrient limitation.

Nitrogen is incorporated into cell mass in the form of proteins and nucleic acids and phosphorus is present in nucleic acids and in the cell wall of some gram-positive bacteria besides to be a key element in the regulation of cell metabolism (SHULER and KARGI, 2002). Yeast extract was the source of organic nitrogen in the bioreactors. Therefore, the nitrogen and phosphorus of the medium was used by the microorganisms.

Observing the final nitrogen concentration, it is possible to propose that besides the nitrogen that is consumed by the microorganisms; a fraction of microorganisms' residue was released into the wastewater.

6.4.3 Amines evaluation

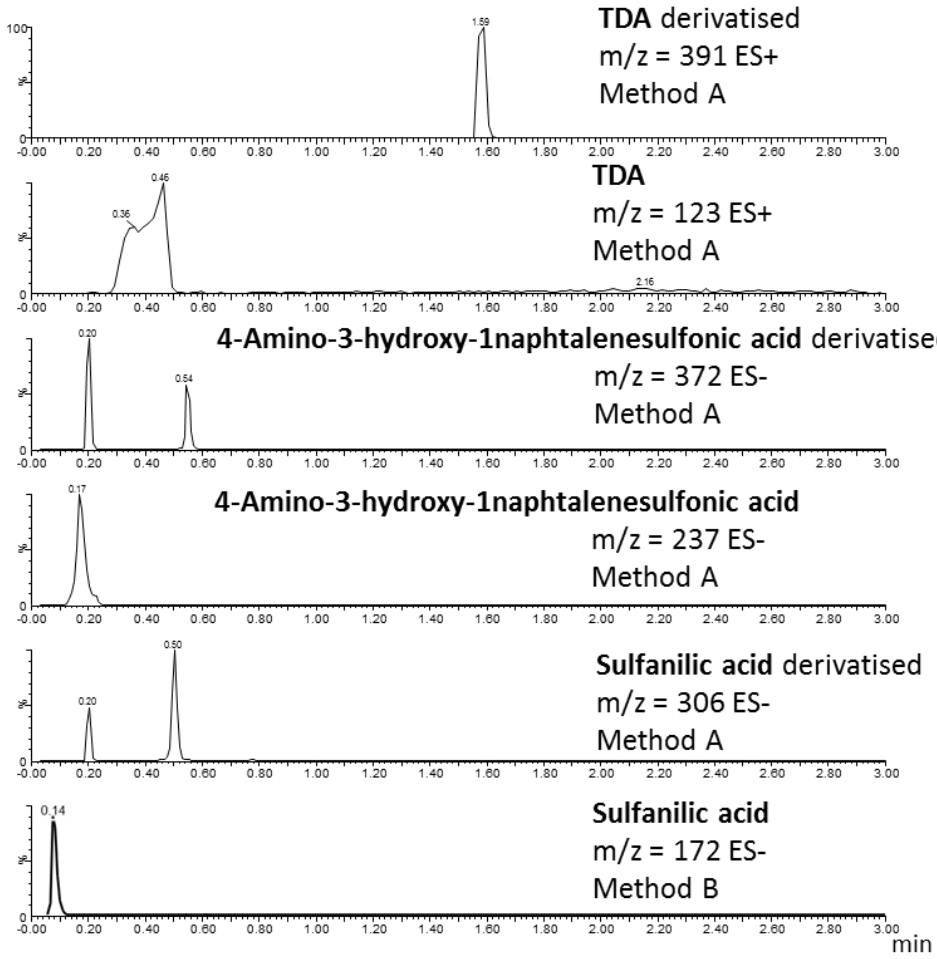
6.4.3.1 LC-MS analysis

The analysis by LC/MS and LC/MS/MS showed a range of degradation products; however, it was not possible to identify those products. Identification needs an extensive effort and it can even be impossible based only on MS-data. Selective derivatization of aromatic amines, or at least amines, will aid the detection or confirmation of absence of these degradation products.

6.4.3.1.1 Derivatization of aromatic amines

The reactions using TDA, sulfanilic acid and the naphthalenesulfonic acid showed full conversion to the benzylformate derivative as it can be noticed in Figure 13. No remaining of the starting material was detected in the derivatized samples. The benzylformate-derivatives were not quantified and the total yield was not calculated but approximated to be close to 100%.

Figure 13 - Ion chromatograms of the standard amines before and after derivatization.



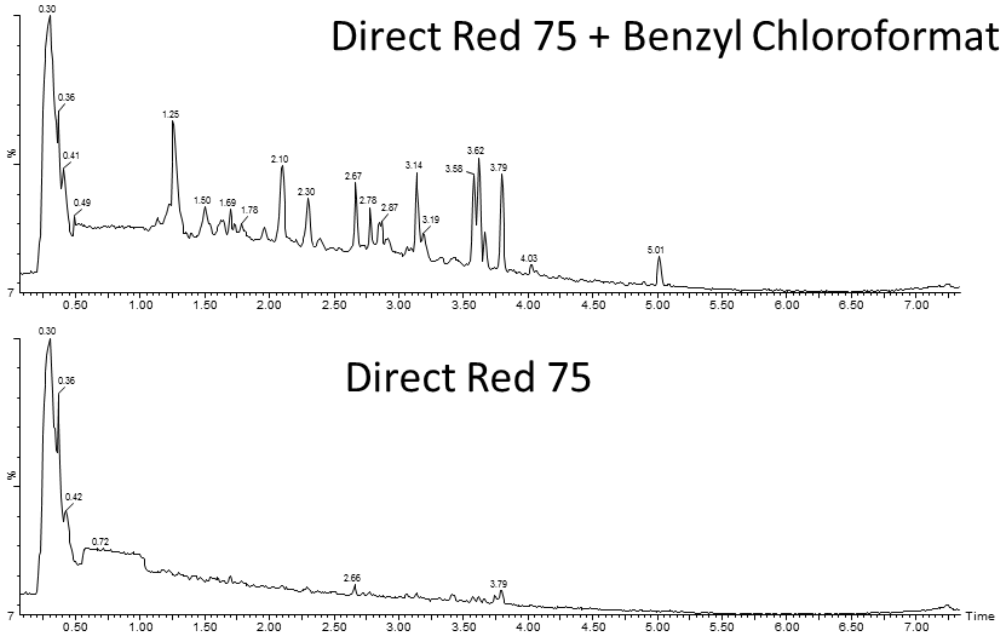
Source: SP Technical Research Institute (2015) .

The double signals in the derivatised samples, showed in Figure 13, are explained by the analysis of acidic components using a basic eluent causing some of the analyte to interact with the column material.

6.4.3.1.2 *Derivatization of DR75 solution*

The analysis of derivatized DR75 resulted in a range of ions observable in the total ion chromatogram (Figure 14), probably, due to degradation of the starting material during the derivatization reaction.

Figure 14 - Comparison of total ion chromatograms of DR75 and derivatized DR75



Source: SP Technical Research Institute (2015).

The derivatized material gives rise to multiple products which was not characterized. No further work was focused on the material since the aim was to detect aromatic amines in the degradation products rather than the dye.

6.4.3.1.3 *Derivatization of chemically reduced and treated samples*

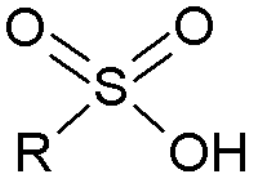
Samples of the Bioreactor A and Bioreactor B analyzed (5^{th}, 9^{th} and 11^{th} weeks of treatment) were also derivatized before analysis. The samples were considered too weak for further dilution and the reaction was kept in water with no addition of organic solvents. Since the reaction proceeded, a precipitate was observed on top of the solutions. The precipitate was not soluble in methanol or acetonitrile.

The samples contained other compounds from the culture medium such as yeast extract, which might interfere with the derivatization reagent. The lack of organic solvents might also affect the solubility of formed products. A strategy of precipitate proteins before reaction and using the addition of organic solvents should be tested later, in order to avoid the precipitation.

The chemically reduced DR75 sample was derivatized and showed a series of unidentified new ions in the total ion chromatogram. The ions were detected in negative ion

mode indicating the presence of sulfonic acid (Figure 15) in the product. Since DR75 has four sulfonate groups in its structure, the sulfonic acid may be a degradation product of the dye.

Figure 15 – Molecular structure of Sulfonic acid



Source: Made by the author

The samples analyzed by LC/UV/MS, for the detection of DR75 and monitoring of degradation products showed no signals. No ions were observed from the samples either before or after derivatization. The DR75 were not observed, possibly explained by severe fragmentation in the ionization step. The samples did not indicate any degradation products. Samples containing chemically reduced DR75 were also analyzed; however with no or very limited number of ions detected. In all cases, the analytes concentrations used were less than detectable by the UV-detector.

Further optimization of the derivatization method might be needed since hydrochloric acid is expected to be produced during the reaction. The acid can potentially cause further degradation of the components in the sample. To prevent degradation, the acid needs to be neutralized, possibly by the addition of a non-amine containing base.

Therefore, the method to identify aromatic amines from the DR75 azo dye by using LC/MS technique still needs to be optimized to be able to detect the degradation products.

6.4.3.2 HPLC and GC/MS analysis

It was not possible to analyze the DR75 dye and to identify the amines in the treated samples by HPLC. A lot of effort was put into testing different methods, however without success. Unfortunately, the same occurred with the chemical reduction analysis by GC/MS.

DR75 has 30% of dye molecules and the 70% left is unknown which may have interfered with the identification of metabolites. Little information was found about DR75 being analyzed by HPLC, LC/MS or GC/MS which makes it difficult to evaluate the dye molecule and the metabolites after degradation. The researchers have been investigated the

adsorption and/or the biodegradation of this dye by UV-VIS spectrophotometry (WAWRZKIEWICZ, 2011, BENZINA *et al*., 2012, HADDAD *et al*., 2013, KHALFAOUI *et al*., 2014, SAROJ *et al*., 2014).

Wesenberg *et al*. (2003) also reported the difficulty to monitor complex transformations of dyes by means HPLC. The identification of metabolites is labor intensive and it may be possible for some individual dyes, but clearly not for all of them.

6.4.4 Rice husks surface area

Rice husks surface area before the dye treatment, rice husks after treatment washed with water and with ethanol 70% were evaluated and the results are presented in Table 3.

Table 3 – Surface area and pore analysis of rice husks before and after biological treatment.

Sample	Surface Area (m^2/g)	Total pore area (m^2/g)	Average pore diameter (nm)
Rice Husk before treatment	1.00 ± 0.03	23.78 ± 0.78	74.10 ± 5.23
Rice husk washed with H_2O	1.58 ± 0.22	19.73 ± 0.44	86.25 ± 6.86
Rice husk washed with ethanol 70%	2.06 ± 0.11	16.76 ± 0.76	103.55 ± 6.72

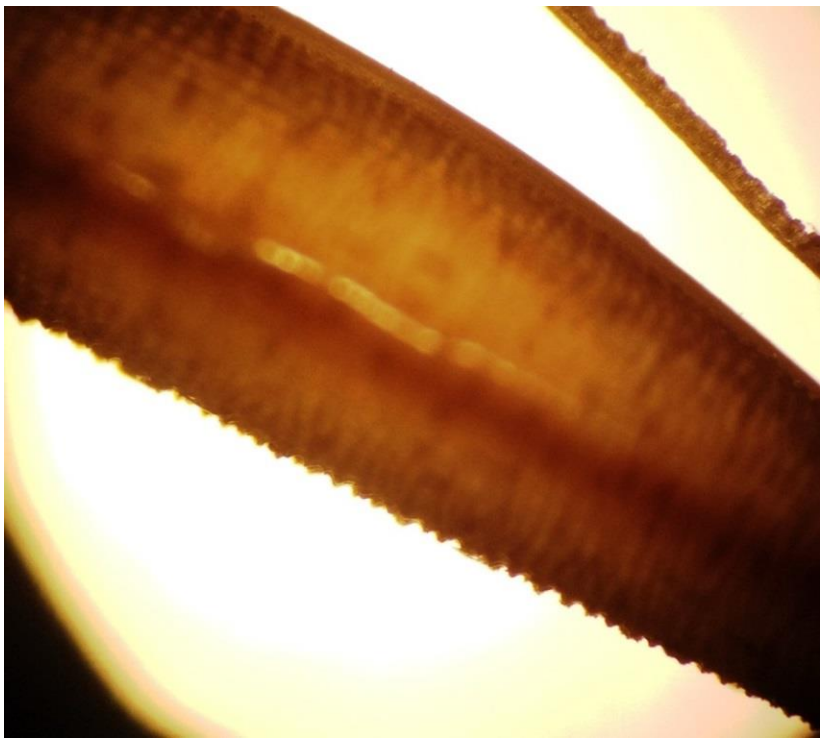
The rice husks evaluated in this research are from Brazil and have surface area of 1.0 m^2/g. It is lower than the surface area of rice husks from Thailand, measured by Paethanom and Yoshikawa (2012), that was 2.2 m^2/g. The surface area of rice husks can, normally, vary according to the characteristics of the cultivated rice.

The difference among the samples could be assigned by the microorganisms action. According to Kumar *et al*. (2010), the rice husks are composed by several compounds such as cellulose, hemicellulose and lignin. Paethanom and Yoshikawa (2012) also investigated the rice husks and characterized them regarding the content of carbon, nitrogen and hydrogen in weight percentage (wt%) dry basis. Wt% is used to show the relative proportions of elements. Thus, 37.9 wt% of C, 0.4 wt% of N and 3.9 wt% of H were measured in those rice husks.

Hence, the rice husks could likely be metabolized by the microorganisms, decreasing the total pore area and increasing the surface area of the rice husks.

A rice husk before treatment was observed in an optic microscope at 40x mag and it can be visualized in Figure 16. It is possible to see the pores ate the surface of the husk.

Figure 16 - A rice husk before treatment observed in optic microscope at 40x mag



Source: Made by the author.

6.4.5 Carrier evaluation

MBBR process uses plastic carriers as support material where biomass can attach and grow forming a biofilm. Biofilm is formed when microorganisms start to adhere to the a surface that may be solid, such as a support and microorganisms, or liquid (HABOUZIT *et al*., 2014).

Rice husk is an organic material not designed for the wastewater treatment. It is an agricultural waste that has been investigated by Forss *et al*. (2013) and Türgay *et al*. (2011) as carrier and source of microorganisms in the dye biodegradation process.

Processes by using biofilm to decolorization of colorful solutions and wastewater have shown satisfactory results (RATCLIFFE *et al*., 2006, VERHAGEN *et al*., 2011, CALDERÓN *et al*.,

2012, ZHU *et al*., 2015). Moreover, microorganisms that grow fast forming biofilm are favored by the presence of carrier at the beginning of the treatment (VERHAGEN *et al*., 2011).

The decolorization rates were better in Bioreactor B fed with both culture medium. Since the systems had different carriers, the decolorization capacity can be related to the difference between the nature and surface area of the support material and the microorganisms that formed the biofilm.

According to Habouzit *et al*. (2014), the nature of the carrier may promote adhesion of specific microorganisms groups. Therefore, the microorganisms present in Bioreactor A can be different from those ones in Bioreactor B, since the support material is completely unlike. Future evaluation of the microbial community in both systems will try to prove it.

Regarding the surface area, more surface area means that there is more space for the microorganisms to attach and grow forming biofilm. Consequently, more biofilm probably means more adsorption and more microorganisms acting in the dye biodegradation.

The total specific surface area of carriers used to MBBR process is 690 m^2/m^3 and the density is close to 1 g/cm^3 (10^6 $g/m^{3)}$ (ØDEGAARD, 2000). Considering the density of the polyethylene carrier close to 10^6 g/m^3, it is possible to convert the total specific surface area to 0.00069 m^2/g. The surface area for rice husks is 1 m^2/g. Furthermore, Bioreactor A was filled with, approximately, 132g of carriers and each bioreactor of Bioreactor B was filled with, approximately, 42 g of rice husks. Therefore, Bioreactor B had the largest surface area of the systems evaluated, providing a larger area for biofilm growth.

Besides that, as the rice husks are organic material and the MBBR process uses polyethylene carriers, the microorganisms probably consumed the husks as carbon and nitrogen source. This situation favors the growth of microorganisms and, consequently, the reductive cleavage which broke the azo bonds causing the dye biodegradation and the decolorization of Bioreactor B in higher rates.

6.5 Conclusion

The setup of Bioreactor A was different from the Bioreactor B. However, it is important to evaluate the difference between them and try to build up a new system with the best characteristics of each one.

Therefore, the sequenced anaerobic/aerobic treatment system using rice husks as carrier and source of microorganisms presented the best decolorization rates. COD was removed easily by the aerobic bioreactor when compared to the anaerobic system also proving that the wastewater can be better treated by a sequenced anaerobic/aerobic system.

The choice of a good carbon source and co-substrate is essential to favor the reductive cleavage of the azo bond and, consequently, to enhance the dye decolorization. Yeast extract revealed to be a better choice for the treatment systems proposed by this study.

Different support material was also evaluated. The rice husks prove to be an interesting material to be applied by the textile industries for the wastewater treatment since they have shown a satisfactory efficiency in the dye biodegradation. The possibility to build up a low-cost system should be considered as well, because the rice husks are cheaper than MBBR. Moreover, the surface area of rice husks is larger than MBBR carrier and this will provide a largest area for biofilm formation.

Besides acting as carrier material, the rice husks are also the source of microorganisms.

The presence of aromatic amines could not be proved by using the analytical techniques applied. LC/MS, GC/MS and HPLC were not efficient to this purpose. However, the difficulty seems to be in the dye composition. DR75 is composed by 30% of dye molecules and 70% of other compounds not specified. This large amount of unknown compounds is likely to interfere in the analysis. Thus, these techniques cannot be a good option to analyze the amines formation in actual textile wastewater as there are several compounds and dyes in the wastewater composition.

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7 FINAL CONSIDERATION

In order to develop an efficient, eco-friendly and low-cost wastewater treatment system, several studies have been performed and a great effort has been made in this research area. There is a long way to go before the ideal treatment system can be built; however, each research on the wastewater treatment has contributed to enhance the biological treatment and this study intends to represent one step forward.

Over the three chapters, the biodegradation of the DR75 azo dye by different microorganisms and methods was evaluated. The chapters exposed the improvement of the methods. The way of thinking about biodegradation and to analyze the process evolved as knowledge was acquired after the conclusion of each experiment.

This is what happens in science, the simplest ideas are developed into the most complex systems.

Thus, the biodegradation of an azo dye, DR75, was evaluated in order to understand the decolorization process and to find a low-cost option for the colorful wastewater treatment.

The treatment systems proposed by this study have been analyzed from the pure cultures of microorganisms until the use of mixed cultures from different sources. The use of a mixed culture has more advantages over the use of pure cultures since it has several microorganisms with different skills and it can be more effective both for dye biodegradation and for degradation of some compounds that compose the wastewater of these industries.

The rice husks are agricultural waste that proved to serve as an alternative source of microorganisms and support material, which may reduce the costs of a textile wastewater treatment.

Despite finding a potential source of microorganisms capable of acting in the dye degradation, it is essential to provide a co-substrate which acts as electron donor for enhancing the decolorization process. Meanwhile, the dye acts as a final electron acceptor in order to favor the reductive cleavage of the azo bond. The yeast extract is a source of riboflavin, a redox mediator that will act as an electron donor.

The degradation products, generated by the reductive cleavage of the azo bond, can be further degraded under aerobic conditions. Therefore, a sequenced anaerobic/aerobic treatment system appears to be an attractive choice for the dye mineralization.

Therefore, it is crucial not to stop the investigation of wastewater treatment because new sources of microorganisms can be applied, new alternative materials can be tested, everything in order to find new eco-friendly and low-cost technologies. Thus, the industries will be more attracted by the biological treatment instead the chemical treatment.