CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

DYE DECOLORIZATION BY YEASTS: INSIGHTS ON PATHWAYS TOWARDS AN INNOVATIVE SOLUTION FOR TEXTILE EFFLUENTS

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology – with specialization in Environmental Science and Engineering

By

Marta Sofia de Almeida Mendes

August 2023

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Marta Sofia de Almeida Mendes

Under the supervision of Professora Doutora Paula Maria Lima Castro

Under the co-supervision of Professora Doutora Maria Manuela Estevez Pintado

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To all my family, pets and friends

ABSTRACT

The textile industry produces high amounts of effluents containing synthetic dyes that are recalcitrant, toxic, potentially hazardous to the environment, and difficult to treat by conventional methods. Biotechnological approaches are generally considered more environmentally friendly and biological methods for dye degradation need to be further investigated. The work described in this PhD thesis aimed to develop a yeast-based solution to decolorize textile dyed effluents that can be safely discharged into the environment.

Yeast strains *Candida parapsilosis* (HOMOGS20B), *Yarrowia lipolytica* (HOMOGST27AB) and *Candida pseudoglaebosa* (LIIIS36B), isolated from wastewater treatment plants, were tested for their ability to decolorize textile dyes. A total of 32 commercial textile synthetic dyes and simulated textile effluents were tested with the target yeasts (single strains and consortia) and the decolorization was evaluated spectrophotometrically for 48-72 hours. Yeasts were able to perform decolorization through adsorption and biodegradation for 28 of the dyes and simulated effluents to extents higher than 50%. *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) presented true decolorization of reactive dyes, above 90% at 100 mg/L, and of simulated effluents at 5 g/L of concentration. Enzyme production was evaluated: oxidoreductase was found in the three yeasts, whereas tyrosinase was only found in *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B).

The potential toxicity of the dyed simulated effluents, before and after treatment, was investigated. The toxicity of the yeast-treated effluents was assessed using three different assays to represent different trophic levels. Mutagenicity was also evaluated. *C. pseudoglaebosa* (LIIIS36B) was the most effective strain, completely decolorizing four of the five simulated effluents. Degradation products, possibly aromatic amines, were detected in the supernatant. Although the toxicity of the original simulated effluents was considered high, treated effluents demonstrated a decrease in toxicity and none exhibited mutagenicity.

A yeast-based solution for decolorization of textile industrial wastewater was assessed. The three yeast strains previously selected for their dye decolorization capacity were freeze-dried. Additionally, *Y. lipolytica* (HOMOGST27AB) was also spray-dried. Skim milk powder and maltodextrin were used as cell protectors, and the freeze-dried products were stored at cold (4 °C) and room temperature for 210 days. The viability of the yeast cells and their decolorization capacity over time were assessed. Dried yeast cells maintained their viability and decolorization capacity for at least 90 days of storage after spray- and freeze-drying with both cell-protecting agents. The dried yeast-based solution for decolorizing textile industrial wastewater combines stability, efficiency, and convenience of production for application in real industrial facilities.

A sequencing batch reactor (SBR) inoculated with activated sludge and successfully bioaugmented with the dye-decolorizing yeast strain – *Y. lipolytica* (HOMOGST27AB) was

assembled. The bioaugmented AGS-SBR was operated for the treatment of a synthetic saline wastewater (12 g/L) intermittently fed with the reactive textile dye (Navy Everzol ED) at 25, 15, and 7.5 mg/L. Dye degradation did not occur although some dye adsorbed to the granules. AGS-SBR performance in removing carbon and nitrogen was effective and was not affected by the dye addition. The AGS core microbiome gathered essentially microorganisms from the *Proteobacteria* and *Bacteroidetes* phyla. The microbial profile showed a dynamic microbiome established at Phase I of the operation, with a high decrease in abundance of *Ignavibacterium* from the initial biomass to the granules formed and an increase of *Actinobacteria*, *Cytophagia*, *Flavobacteria*, and *Alphaproteobacteria* in the remaining phases of the bioreactor operation. The bioaugmented yeast remained present throughout the entire reactor process.

Y. lipolytica (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) are a potential biotechnological tool for dye degradation in textile wastewaters, especially those containing reactive dyes and a promising tool to integrate in bioremediation solutions, contributing to eco sustainability in the water sector, but further operational scenarios need to be researched.

Keywords: Decolorizing yeasts; Biodegradation; Textile dye decolorization; Stream's toxicity; Cell's preservation methods; Yeast-bioaugmented aerobic granular sludge.

RESUMO

A indústria têxtil produz grandes quantidades de efluentes contendo corantes sintéticos que são recalcitrantes, tóxicos, potencialmente perigosos para o meio ambiente e são difíceis de tratar pelos métodos convencionais. As abordagens biotecnológicas são geralmente consideradas mais ecológicas e métodos biológicos para degradação de corantes precisam ser mais investigados. O trabalho descrito nesta tese de doutoramento teve como objetivo desenvolver uma solução à base de levedura para remoção de cor de efluentes têxteis corados para que possam ser descartados com segurança no meio ambiente.

As estirpes de levedura *Candida parapsilosis* (HOMOGS20B), *Yarrowia lipolytica* (HOMOGST27AB) e *Candida pseudoglaebosa* (LIIIS36B), isoladas de estações de tratamento de águas residuais, foram testadas quanto à sua capacidade de remover cor de corantes têxteis. Um total de 32 corantes têxteis sintéticos comerciais e efluentes têxteis simulados foram testados com as leveduras-alvo (estirpes individuais e consórcios) e a remoção de cor foi avaliada espectrofotometricamente por 48-72 horas. As leveduras foram capazes de realizar a descoloração por adsorção e biodegradação para 28 dos corantes e efluentes simulados em mais de 50%. As estirpes *Y. lipolytica* (HOMOGST27AB) e *C. pseudoglaebosa* (LIIIS36B) apresentaram descoloração verdadeira de corantes reativos, acima de 90% a 100 mg/L, e de efluentes simulados a 5 g/L de concentração. A produção enzimática foi avaliada: a oxidoredutase foi encontrada nas três leveduras, enquanto a tirosinase foi encontrada apenas na *Y. lipolytica* (HOMOGST27AB) e *C. pseudoglaebosa* (LIIIS36B).

A potencial toxicidade dos efluentes simulados corados, antes e depois do tratamento, foi investigada. A toxicidade dos efluentes tratados com levedura foi avaliada usando três testes diferentes para representar diferentes níveis tróficos. A mutagenicidade também foi avaliada. A estirpe *C. pseudoglaebosa* (LIIIS36B) foi a mais eficaz, removendo a cor completamente a quatro dos cinco efluentes simulados. Os produtos de degradação, possivelmente aminas aromáticas, foram detetados no sobrenadante. Embora a toxicidade dos efluentes simulados originais tenha sido considerada alta, os efluentes tratados demonstraram uma diminuição na toxicidade e nenhum exibiu mutagenicidade.

Uma solução à base de levedura para a remoção de cor de efluentes industriais têxteis foi produzida e avaliada. As três estirpes de leveduras previamente selecionadas pela sua capacidade de descoloração de corantes foram liofilizadas (freeze-dry). Além disso, a estirpe *Y. lipolytica* (HOMOGST27AB) foi também seca por pulverização (spray-dry). Leite em pó desnatado (Skim milk) e maltodextrina foram utilizados como protetores celulares, e os produtos liofilizados foram armazenados a frio (4 °C) e a temperatura ambiente durante 210 dias. A viabilidade das células de levedura e a sua capacidade de descoloração ao longo do tempo foram avaliadas. As células de levedura mantiveram sua viabilidade e capacidade de descoloração por pelo menos 90 dias de armazenamento após secagem por pulverização e

liofilização com ambos os agentes protetores de células. A solução à base de levedura seca para descoloração de efluentes industriais têxteis combina estabilidade, eficiência e conveniência de produção para aplicação em instalações industriais reais.

Um reator inoculado com lamas ativadas e bioaumentado com sucesso com a estirpe de levedura descolorante *Y. lipolytica* (HOMOGST27AB) foi montado. O reactor bioaumentado foi usado para o tratamento de um efluente salino sintético (12 g/L) alimentado intermitentemente com o corante têxtil reativo (Navy Everzol ED) a 25, 15 e 7,5 mg/L. A degradação do corante não ocorreu, embora algum corante tenha adsorvido aos grânulos. O desempenho do biorreactor na remoção de carbono e azoto foi eficaz e não foi afetado pela adição de corante. O microbioma central dos grânulos aeróbios (AGS) reuniu essencialmente microrganismos dos filos *Proteobacteria* e *Bacteroidetes*. O perfil microbiano mostrou um microbioma dinâmico estabelecido na Fase I da operação, com uma grande diminuição na abundância de *Ignavibacterium* desde a biomassa inicial até aos grânulos formados e um aumento de *Actinobacteria*, *Cytophagia*, *Flavobacteria* e *Alphaproteobacteria* nas demais fases da operação do biorreator. A levedura bioaumentada permaneceu no reactor durante todo o processo.

As estirpes *Y. lipolytica* (HOMOGST27AB) e *C. pseudoglaebosa* (LIIIS36B) são uma potencial ferramenta biotecnológica para a degradação de corantes em efluentes têxteis, especialmente aqueles que contêm corantes reativos e uma ferramenta promissora para integrar em soluções de biorremediação, contribuindo para a eco sustentabilidade no setor da água, mas outros cenários operacionais necessitam de ser investigados.

Palavras-chave: Leveduras descolorantes; Biodegradação; Descoloração de corantes têxteis; Toxicidade dos fluxos; Métodos de preservação de células; Grânulos aeróbios bioaumentados por leveduras.

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LIST OF ABBREVIATIONS

1N2A4S	1-naphthol-2-amino-4-sulfonic acid
4A1NS	4-amino-1-naphthalenesulfonic acid
AGS	Aerobic granular sludge
AGS-SBR	Aerobic granular sludge - Sequencing batch reactor
AOB	Ammonia-oxidizing bacteria
BLAST	Basic Local Alignment Search Tool
BOD	Biochemical oxygen demand
CFU	Colony-Forming Unit
COD	Chemical oxygen demand
d	Day
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
GAO	Glycogen-accumulating organism
h	hour
HOMOGS20B	*Candida parapsilosis* (HOMOGS20B)
HOMOGST27AB	*Yarrowia lipolytica* (HOMOGST27AB)
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography with photodiode-array detection
ITS	Internal transcribed spacer
LD50	Median Lethal Dose

LIIIS36B	*Candida pseudoglaebosa* (LIIIS36B)
LiP	Lignin independent Peroxidase
MDM	Minimum Decolorization Media
MDX	Maltodextrin
MGA	Minimal Glucose Agar
MiP	Manganese independent Peroxidase
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MnP	Manganese dependent Peroxidase
n.d.	Not detected
NADH-DCIP	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H) + Dichlorophenol Indophenol (DCIP)
NDA	No Decolorization by Adsorption
NDM	Normal Decolorization Media
NE	Navy Everzol ED
NGS	Next-generation sequencing
NSDM	Normal Solid Decolorization Media
NTD	No True Decolorization
OD_{550nm}	Optical density at 550 nm
OTU	Operational taxonomic unit
PAO	Polyphosphate-accumulating organisms
PCR	Polymerase chain reaction

RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S.D.	Standard deviation
SBR	Sequencing batch reactor
SKM	Skim Milk powder
SRT	Sludge retention time
SVI_5	Sludge volume index after 5 minutes of settling
SVI_{30}	Sludge volume index after 30 minutes of settling
TOC	Total Organic Carbon
TSS	Total suspended solids
Tyr	Tyrosinase
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

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CHAPTER 1

GENERAL INTRODUCTION

1.1. CHALLENGES FOR WASTEWATER TREATMENT

1.1.1. Environmental issue

Water is essential for life and is the most valuable natural resource on earth. Approximately 71% of the earth's surface is covered by water, totalizing about 1.4 billion km^3. However, of all the water available on the planet, 97% is salt water. Of the 3% of the world's freshwater, only less than 1% is accessible and available for human and animal consumption (European Environment Agency, 2018). The protection of this natural resource is of the utmost importance and should be a priority for world governments. The increase in population, and the need for water for agriculture and animal consumption, added to the higher industrialization, contribute to the increase in water demand. The rise in consumption, associated with severe droughts observed due to climate change, contributes to increased water scarcity. The disposal of untreated wastewater into water bodies results in environmental and human health effects. These polluted wastewaters commonly charged with nutrients, namely nitrogen, and phosphorus, create anoxic conditions that contribute to the eutrophication of the water bodies and are lethal to aquatic organisms. There is an enormous environmental problem concerning the contamination of freshwater due to the discharge of contaminated effluents and human health is indirectly affected due to toxic contaminants and pathogens present in untreated wastewater (Dellamatrice *et al.,* 2017).

1.1.2. Industrial wastewater

Industrialization is considered a key factor in the development of the global economy. Industry development is necessary to meet the needs and demands of a growing global population (Pinheiro, 2022). As a result, the production of wastewater from industrial activities is inevitable. Modern industrialization is responsible for the formation of a wide range of environmental pollutants and industrial wastewater generated by the textile industries is a major source of global water pollution (Ali, 2010). The textile sector is a multibillion-dollar market that consumes a large amount of water (60-400 L/kg of fabric) and produces high volumes of effluents that contain a complex mixture of synthetic dyes (Ali *et al.,* 2009; Dellamatrice *et al.,* 2017). It is estimated that about 280,000 tons of textile industrial dyed effluents are discharged each year worldwide (Jin *et al.,* 2007; Sen *et al.,* 2016; Singh *et al.,* 2015; Solís *et al.,* 2012). In India, one of the top textile producers, this consumption may reach 200-300 m^3 of water per ton of finished product (Dasgupta *et al.,* 2015). In Europe, this sector consumes between 50 and 240 m^3 of water per ton of finished product (Mata *et al.,* 2015). During the dyeing process, approximately 20 to 50% of the dyes do not fix to the fabrics and end up being discharged (Al-Tohamy *et al.,* 2020). Dyes are persistent and visible in the water at concentrations below 1 ppm. In general, a small amount of dye in an aqueous

solution can produce a vivid color (Varjani *et al.,* 2020). In order to enrich the final products with visual, physical, and aesthetic properties, diverse industrial textile dyeing processes are implemented. The processes usually start with pre-treatments using i.e., ammonia, solvents, and disinfectants; bleaching is also commonly carried out with i.e., hydrogen peroxide (H_2O_2), sodium hypochlorite (NaClO) and other bleaching agents; the coloring and the printing processes with i.e., dyes, salts, metals and formaldehyde; and the finishing with i.e., fixating agents, organic acids and oxidant/reduction agents. (Danouche *et al.,* 2021; dos Santos *et al.,* 2007). The wastewater composition depends on the dyes and chemicals used in the dry and wet textile industrial processes, in which the main contaminants are recalcitrant and toxic organic-colored compounds, salts, surfactants, and chlorine compounds (Chequer *et al.,* 2013). The dyeing processes involve complex combinations of chemicals which turn these effluents even more challenging to degrade (Croce *et al.,* 2017; Dellamatrice *et al.,* 2017). An example of an industrial dyeing process comprehends that in pre-treatment processes, agents are added to minimize fiber breakage in the weaving process. Subsequently, chemical compounds are removed from the fibers, as these can interfere with the dyeing process. In the bleaching process, chemical compounds such as sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) are often used to remove the intrinsic and undesirable color from fibers. Then, in the dyeing process, color is added to the fibers, normally requiring the use of high volumes of water. However, it is the finishing process that usually most significantly influences the volume of effluents produced, representing about 70-80% of all the water consumed in the process (dos Santos *et al.,* 2007). In order to produce 1 kg of textile fabric, more or less 200 L of water are necessary, depending on the process chosen (Ghaly *et al.,* 2014). These wastewaters are characterized by extreme fluctuations in many parameters such as the chemical and biochemical oxygen demand (COD and BOD), pH, salinity, and color, which lead to the deterioration of several ecosystems (Chequer *et al.,* 2013; Sen *et al.,* 2016). Over the past few decades, the world has paid more attention to the potentially toxic, mutagenic, and carcinogenic effects of many chemical pollutants that cannot be readily degraded by nature. Many of these synthetic compounds such as pesticides, organochlorines, polycyclic aromatic hydrocarbons, synthetic polymers, dyes and preservatives, and polychlorinated biphenyls, among others, are extremely resistant to biodegradation and are responsible for much of the existing environmental problems (Ali, 2010; Dellamatrice *et al.,* 2017; Sen *et al.,* 2016).

Wastewater treatment plants (WWTPs) have shown to be very efficient in the removal of pollutants such as organic matter, nutrients, and phosphorus but are not very efficient in removing color from textile industries. Nutrients and organic compounds are removed during the step of biological treatment, which consists of the biological degradation according to the natural earth's elemental cycles, such as carbon, nitrogen, and phosphorous (Christensen *et al.,* 2015). However, the natural bacteria that occur in the activated sludge systems are not prepared for the new challenges that have emerged. Antibiotics and other pharmaceuticals, hormones, halogenated and fluorinated

compounds, micropollutants, illicit drugs, pesticides, cleaning, and personal care products, are examples of pollutants that appear in WWTPs (Chang *et al.,* 2009; Ely *et al.,* 2022). Wastewater treatment can be difficult because of the variable chemical composition of the streams originated from industrial facilities, due to the presence of recalcitrant pollutants and high content of salts, among others. Industrial wastewaters are a combination of several discharged streams from different industrial processes, that also depend on the sector of production. These industrial effluents are loaded with a wide variety of organic and inorganic compounds, some of them (and their possible intermediates) with extreme toxicity and poorly biodegradable (Oliveira, 2020). Innovative investigation needs to be conducted for the development of more sustainable solutions for the treatment of these textile-dyed effluents.

1.2. DYES

Dyes can be of natural or synthetic origin. They can be used in several different materials such as textile fiber, paper, leather, animal fur, plastic material, foodstuff, the petroleum industry, and pharmaceutical and cosmetic products, among others (Sen *et al.,* 2016; Sudha *et al.,* 2014). For centuries, natural dyes were used for various purposes (from body paintings in tribal ceremonies to decoration), since they could be extracted from different parts of plants but also from some other organisms (algae, fungus, and bacteria). However, since the 19th century, when the first synthetic dye was reported, its use has increased remarkably (Pinheiro *et al.,* 2022). In fact, the use of natural dyes has been almost entirely replaced by synthetic ones because of their less expensive and much faster production, easiness of use, stability, and variety of colors, compared with natural dyes. It is estimated that more than 10,000 dyes are commercially available nowadays and the annual world production exceeds 7x10^5 tons of dyestuffs (Kasiri and Safapour, 2014; Robinson *et al.,* 2001).

A dye is a colored compound that has an affinity with the substrate to which it is being applied. The color of a dye depends on the ability of the substance to absorb light in the visible region of the electromagnetic spectrum (350-700 nm). To act as a dye, colored compounds should have a chromophore group and an auxochrome group. The chromophore group is responsible for producing color in the dye because of its ability to absorb light in the visible region. Common chromophores include the azo group (-N=N-), carbonyl group (=C=O), ethylene group (=C=C=), carbon-nitrogen group (C=NH, -CH=N-), nitrous (NO or N-OH), nitro group (-NO_2 or NO-OH) and carbon-sulfur (C=S). The auxochrome group is a substituent group that, when introduced into a molecule, enhances its color. Some auxochromes include amino (–NH_2), carboxyl (-COOH), sulphonate (-SO_3H), and hydroxyl (-OH). These groups also have the important property of providing a higher affinity to the fiber. In the presence of an auxochrome, stable chemical bonds are created to attach the dye to the target fiber. The chromogen, which is an aromatic structure (normally benzene, naphthalene, or anthracene ring), is part of a chromogen-chromophore

structure (Püntener *et al.,* 2003; Pinheiro *et al.,* 2022; Sudha *et al.,* 2014). Figure 1.1 shows an example of an azo dye molecule with the respective chromophore and auxochromes signaled.

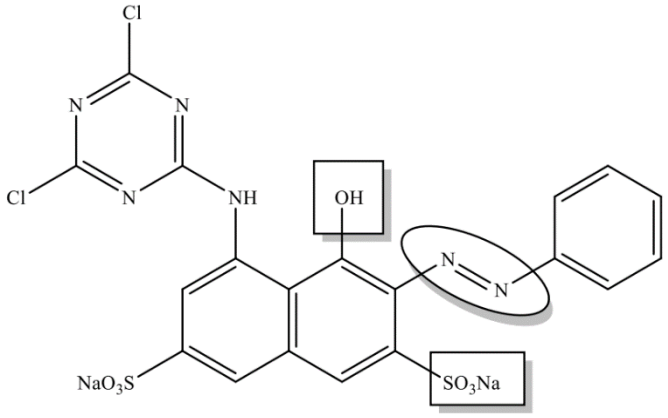


Figure 1.1 - Example of an azo dye molecule of Reactive Red 2. Chromophore in oval shape and auxochromes in square shape. Adapted from dos Santos *et al.* (2007).

Synthetic dyes have a significant structural diversity due to the presence of the chemical structure of the chromophore. Some of the most important groups for the textile industry are anthraquinones, phthalocyanines, indigoids, and benzodifuranones. However, the majority of synthetic dyes in use are azo dyes derivatives (Pinheiro *et al.* 2022). Dyes are grouped into different classes and the selection of the class to be used depends on its application, meaning that its choice depends on the type of material to be colored and its affinity for the dye. The classes of dyes frequently used in the textile industry are acid, basic, direct, disperse, vat, and reactive, among others. Acid dyes are anionic, soluble in water, and are used in nylon, wool, silk, paper, and leather. Basic and direct dyes are cationic, soluble in water, and are used for paper, nylon, polyester and cotton, paper, leather, and nylon, respectively. Disperse dyes are mostly non-ionic, insoluble in water, and are used in polyester, polyamide and acrylic fibers. Vat dyes are insoluble in water and need to be applied by an alkaline bath, usually with sodium hydrogen sulfite; they are used to color cotton and wool. Reactive dyes form covalent bonds with the fibers of cotton, wool, silk and nylon (Püntener *et al.* 2003). Table 1.1 shows some examples of dyes with different classifications, applications and possible toxicity.

Table 1.1 - Classification of Textile Dyes. Adapted from Jorges (2016) modified from the European Commission (2003).

Classification	Chemical groups	Examples	Application	Toxicity	Losses in process
Acid	Azo, anthraquinone, triphenilmetane, phthalocyanine	Acid Violet 19, Acid Blue 92	Wool 25-30%, Polyamide 70-75%	Generally non-toxic. Some could have allergenic effect	5-20%
Basic	Quaternary amino group. Sometimes positively charged with an atom of oxygen or sulphur	Basic Blue 3, Basic Red 18	Acrylic fibers, polyamides and fiber combination	Many are toxic: B Blue 3, 7, 81; B Red 12, B Violet 16 and C Yellow 21	0-5%
Direct	Azo, stilbenes, oxazines, phtalocyanines. Acid and carboxylic hydroxyl groups	Direct Red 81, Direct Green 23, Direct Brown 44, Direct Yellow 12	Cotton, rayon, flax, jute, silk and polyamide	Condensation products of formaldehyde with amines, polynuclear aromatic phenols or dicyandiamide, cyanamide. Direct Orange 62	5-30%
Disperse	50% simple azo, 25% anthraquinones, 25% metyl, naphthoquinone and nitryl	D Red 1, 11, 15, 17; D Blue 1, 3, 7, 26, 35, 102, 124; D Orange 1, 3, 76	Polyester, cellulose, polyamides and acrylics	Aromatic amines. Allergenic effects in D Red 1, 11, 15, 17; D Blue 1, 3, 7 26, 35, 102, 124; D Orange 1, 3, 76; D Yellow 1, 9, 39, 49, 54, 64	5-20%

				adsorbed in sludge	
Complex with metals	Chromophore mono-azo 1:1 Or 1:2 metal complexes. Carboxyl, hydroxyl or amine groups. Metals: chromium, cobalt, nickel and copper	Acid Violet 56, Acid Blue 158	Proteins, polyamides 30% or 60% wool	50% can be eliminated with biological processes. Contain organic halogens, heavy metals (Cr, Cu, Co, Ni) and inorganic salts	2-10%
Chrome	Acids complexed with metals. Joined in potassium dichromate salts		Protein (silk and wool)	Chromium release	5-10%
Naphthol	Similar to azo but without a sulfonic group. Quick salts of coloring: aniline, toluidine blue, ortho or meta anisidine, diphenyl amine	Naphthol (P, L), Naphthol-G (P), Naphthol-ITR (P,L), Naphthol-D (P,L)	Cellulose, flax and polyester	Aromatic amines, nitroanilines, cloroanilines, β-naftilamines	10-25%
Reactive	Link-chromophore and a reactive group. Covalent bonds with cellulose fibers	Reactive Black 5, Remazol, Drimalan Erofix, Lanasol, Levafix	Cellulose, cotton, wool and polyamides	Difficult to eliminate through phyto and biological treatments. Contaminants: organic halogens and dispersant additives	<5%

Sulphur	Combinations of sulphur or sulphites with amines and phenols. Insoluble in water but soluble in alkaline conditions		Cotton, cellulose, synthetic fibers (polyamides and polyester), silk	Eliminated in activated sludge. Contaminants: dispersive additives	10-40%
Vat	Anthraquinoid, indigoid and insoluble in water but soluble in alkaline conditions	Vat Blue 1, 4; Vat Orange 3	Cotton and cellulose. Polyester and polyamides	Easy to eliminate because is insoluble. Contaminants: dispersive additives. Heavy metals (Cu, Fe, Mn, Ba, Pb)	20-30%

Unfortunately, for many of the commercial dyes there's no knowledge available about their chemical structure, possibly due to industrial secrecy. According to EPA (1996), about 60% of dyes are of unknown structure, wherein 40 to 55% are considered toxic, 13% have uncertain toxicity and 32% are considered safe.

Synthetic dyes are mostly produced by petrochemical and chemical processes with serious environmental impact. Reactive dyes are one of the most frequently used class of dyes in the textile industry. They are characterized by being typically azo-based chromophores combined with different types of reactive groups, e.g., vinyl sulfone, chlorotriazine, trichloropyrimidine, and difluorochloropyramidine. It is assumed that there are more than 2,000 different azo dyes. These meet most of the requirements of manufacturing: good linkage to the fabrics, keep the colors for a long time, good stability, fastness to light, high fixation, simple to synthetize and apply, low energy consumption for production, stability to chlorine and repeated washing, and exists in almost all colors that are appealing to the consumer (Gupta and Suhas, 2009; Kasiri and Safapour, 2014).

Among all dyes used in textile industry, azo dyes are currently the most widely used. Chemical structure of the azo reactive dyes is characterized by the presence of one or more –N=N– (azo) bonds, typically conjugated to aromatic rings. This structure allows the displacement of the electrons, in transitions in the ultraviolet-visible (UV-Vis), absorbing the light in the zone of the

visible. They may further contain various substituent groups such as chlorine (-Cl), methyl (-CH_3), amino (-NH_2), hydroxyl (-OH) and carboxyl (-COOH), resulting in different dyes. Within this class, depending on the number of azo bonds in the molecule, there are monoazo, diazo, triazo and poliazo dyes, the latter being the least important at industrial level (Saratale *et al.,* 2011; Varjani *et al.,* 2020).

Textile effluents comprise a mixture of different synthetic dyes in their constitution, hence it becomes essential to search for solutions that decolorize mixtures of dyes. Azo dyes are the most used and therefore are present in higher concentrations in industrial effluents (Solís *et al.,* 2012; Varjani *et al.,* 2020). Due to its high stability, azo dyes are recalcitrant and difficult to eliminate from the environment and the products resulting from the breaking of the azo bond could be toxic, carcinogenic and mutagenic to ecosystems and human health. Therefore, due to the consequences that the discharge of textile effluents could cause in the environment, there is a great need to develop and to implement new technologies that must be effective and affordable, without harming the associated economic activity and that comply with governmental legislation that, in more developed countries, has been increasingly restrictive (Pinheiro, 2022). In recent years, due to the toxic potential of these compounds, some azo dyes and the aromatic amines that are released, have been regulated and even prohibited and governmental control agencies were created to monitor and to ensure regulations compliance. As an example, in 2006 the European Union (EU) created a regulation entitled REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals). This regulation lists 24 types of aromatic amines formed after azo dyes degradation that are considered hazardous and prohibits the use of azo dyes in products that may have direct and prolonged contact with skin if they produce 30 mg/kg or more of these amines. Also, the EU restricts other colorants in concentrations above 0.1% of its weight, and if it's for foodstuff, the limit is 0.01% mg/kg. Over the years, several countries in Asia, such as India (1997), China (2005), South Korea (2010), Taiwan (2011), Egypt (2012) and Japan (2014) also started to regulate some chemicals, including azo dyes. India was the first Asian country to regulate chemicals and have banned the use of about 112 dyes and aromatic amines. Even though these countries have their own regulations, they are similar to the EU. The United States doesn't have specific laws related with azo dyes but have restrictions on aromatic amines derived from them. Individual states can have their own specific laws. Some other countries like Canada, Brazil, Pakistan, Morocco and Turkey, have restrictions only for the color of the effluents (Pinheiro *et al.,* 2022).

1.3. TOXICITY OF DYES AND THE RESULTING DEGRADATION PRODUCTS

Biological treatment methods are generally considered more economic and environmentally friendly because they can result in complete mineralization of organic pollutants (Pandey *et al.,* 2007). They can also remove COD, BOD and suspended solids and toxicity of dyes and/or the resulting degradation products. In fact, today there's no universal method of treatment because the dye removal efficiency depends on the physical and chemical characteristics of the dye, as well as the selected treatment method (Jorges, 2016). Biological treatment methods are performed in aerobic or anaerobic conditions or a combined aerobic/anaerobic condition, but the removal efficiency depends on the adaptability of the microorganisms to the process. Microbial biodegradation of azo dyes has been reported to involve three main mechanisms: a) under anaerobic conditions, the cleavage of the azo bond through reduction by the action of azoreductase, thus originating potentially cytotoxic and mutagenic aromatic amines; b) under aerobic conditions, the direct oxidation of the azo bond, giving rise to highly electrophilic diazonium salts azo dyes; and c) metabolic oxidation other than the azo bond, specially of free amine groups (Saratale *et al.,* 2011). Treatment with activated sludge is common and anaerobic treatment depends on an oxidation-reduction reaction with hydrogen, formation of methane, hydrogen sulfide, carbon dioxide and other compounds. The increase of free electrons reduces the bounds and leads to decolorization, but this process is limited by the carbon source (i.e., glucose) (Zaharia and Suteu, 2012). Discharge of dyes can have a toxic effect on the aquatic ecosystem and can introduce a potential bioaccumulation hazard, which can eventually affect humans through their transport across the food chain (dos Santos *et al.,* 2007; Saratale *et al.,* 2011). Most azo dyes are water soluble and are rapidly absorbed through skin contact and inhalation, causing risk of cancer, nausea, bleeding, allergic reactions, are irritating to the eyes and highly toxic if inhaled or consumed, causing irreversible damage to the kidneys, livers and reproductive and central nervous systems (Sudha *et al.,* 2014). Besides the environmental problems affecting photosynthetic activity, the reduction of the absorption and reflection of sunlight and consequently reduction of the dissolved oxygen concentration, creates anoxic conditions that are lethal to aquatic organisms (Kunz *et al.,* 2002).

Dye toxicity can be directly related to: a) the molecular weight and lipophilicity that influences the diffusion through the cell membrane, thus controlling bioavailability; b) the presence of free amines and N-acetylated amines, which may undergo metabolic activation due to the formation of nitrenium ions, thus being able to bind to DNA and RNA resulting in genotoxicity; and c) enzymatic or non-enzymatic degradation resulting in potentially toxic by-products. The formation of amines, particularly of aromatic ones, mainly through the anaerobic pathway, are produced by the reduction of the azo bond and can result in their conversion into electrophilic groups, which have the ability to covalently bond to DNA, resulting in DNA damage and possibly into mutagenic and carcinogenic events, having a higher degree of toxicity than the dye of origin (Khan *et al.,* 2013; Rawat *et al.,* 2016). Some studies revealed at least 22 aromatic amines with potential carcinogenic effect (Pinheiro *et al.,* 2004). Some of these amines and aromatic amines formed from azo dyes have been banned or restricted at European level by the 19th amendment to Directive 76/769/EEC (Pinheiro *et al.,* 2004). In the last few decades, scientists, regulators and policymakers have been studying ways to overcome these problems by modifying the structures of many dyes by adding certain functional groups such as alkyl, carboxyl and sulphonic acid, in order to increase hydrophilicity, intermolecular hydrogen bonding and steric hindrance. These modifications aim to reduce metabolic activation of amine groups and diffusion through cell membranes. Also, they lead to a direct reduction in toxicity under laboratory test organisms. However, this could result on the necessity of altering the textile effluents treatment methods currently used (Pinheiro *et al.,* 2004; Rawat *et al.,* 2016). Several research reported the study of the toxicity of some azo dyes and respective metabolites formed by biodegradation. As an example, Franca *et al.* (2015) stated that the breakdown of the azo bond in Acid Red 14 resulted in two aromatic amines, 4-amino-1-naphthalenesulfonic acid (4A1NS) and 1-naphthol-2-amino-4-sulfonic acid (1N2A4S). 4A1NS has a LD50 value of 1g/kg for rat and no mutagenicity was detected with *Salmonella typhimurium* and *Escherichia coli*. Also, although dyes like Acid orange 7 and Reactive Black 5 do not present toxicity themselves, the resulting amines from dye degradation presented phytotoxicity to *Cucumis sativus*, genotoxicity to *E. coli* and cytotoxicity towards *Vibrio fischeri* (Bay *et al.,* 2014; Gottlieb *et al.,* 2003).

1.4. TRADITIONAL AND BIOLOGICAL TREATMENTS

1.4.1 Conventional methods

A wide variety of technologies have been developed for the removal of synthetic dyes from waters and wastewaters to decrease their negative environmental impact. Remediation is based on color removal but also in degradation and mineralization of dye molecules. In fact, decolorization occurs when dye molecules are removed from the solution or when the chromophore is broken, but the main molecule or major fragments remain intact (Jorges, 2016). Classic treatment methods are based on physical and chemical processes, occasionally in conjunction with biological treatments (Ali, 2010; Lalnunhlimi and Veenagayathri, 2016). Some of the classic processes include

precipitation, flotation, membrane filtration, flocculation with Fe(II)/$Ca(OH)_2$, adsorption, chemical oxidation and reduction, electrochemical techniques among others (Table 1.2). These methods have been shown to be most of the time little effective, expensive and can even generate a significant amount of toxic by-products or high amounts of residues that have to be treated separately, increasing substantially the cost and complexity of these processes (Nigam *et al.,* 1996; Saratale *et al.,* 2011; Mahmoud, 2016; Kurade *et al.,* 2017).

Table 1.2 - Advantages and limitations of some of the traditional methods for removing dyes from textile effluents (adapted from Chacko and Subramaniam, 2011; Vandevivere *et al.,* 1998).

Physical/Chemical Methods	Stage	Advantages	Limitations
Fenton Reagent	Pre-treatment	Effective decolorization of soluble and insoluble dyes; Low costs	Waste production
Ozonation	Pos-treatment	Application in the gaseous state, not altering the volume	Short half-life (20 minutes)
Photochemical	Pos-treatment	No waste production	Formation of more toxic products; Low decolorization rate; Slow process
Reverse Osmosis	Pos-treatment	Decreased organic load of effluents	High cost; Slow process
Electrochemical Destruction	Pre-treatment	Not dangerous resulting products	High cost and consumption of electricity; Low decolorization rate
Activated Charcoal	Pre- or Pos-treatment	Good removal of various dyes	High cost; Generates a large amount waste

Silica Gel	Pre- or Pos-treatment	Effective in removing basic dyes	Secondary reactions occur that inhibit the commercialization
Filtration Membrane	Main- or Pos-treatment	Removal of all types of dyes	Generates a large amount of highly concentrated waste
Ion Exchange	Pos-treatment	Regeneration – no loss of adsorbent	High cost; Not effective in removing all dyes
Irradiation	Pos-treatment	Effective oxidation at lab scale	Requires a high volume of oxygen

Alternatively, industry must look for "green" processes that are less problematic (Gupta and Suhas, 2009; Ali, 2010; Khandare and Govindwar, 2015; Pezzella *et al.,* 2016).

1.4.2. Biological methods

Biological methods are generally more environmentally friendly since they can lead to complete mineralization of organic pollutants at a low cost (Pandey *et al.,* 2007). The biodegradation of recalcitrant compounds can occur through mechanisms of biosorption or enzymatic degradation, or even a combination of them. The success of these treatments depends on the adaptation of the selected organisms as well as the characteristics of the effluents, including the composition and structure of the dyes (Solís *et al.,* 2012; Khan *et al.,* 2013). The microorganisms involved in these treatments are diverse and have been studied both individually and in consortia (Das en Mishra, 2017).

1.4.2.1. Microbial degradation

Over several years, numerous species of organisms have been tested for the decolorization and degradation of several dyes, including filamentous and non-filamentous bacteria and fungi, yeasts and algae (Saratale *et al.,* 2011; Solís *et al.,* 2012). Bacteria are the most studied, together with filamentous fungi, because of their enzymatic toolbox. Algae and yeasts, that are usually present

in treatment plants, may play an important role at treating textile effluents with several advantages (Moreira *et al.,*2001; Kalyani *et al.,*2009; Imran *et al.,* 2015; Martorell *et al.,* 2017). Yeasts have an added value in bioremediation and in the process of dye decolorization since they have "the best of 2 worlds": they are easy to grow and maintain like bacteria, and are resistant and adaptable to adverse environments, and are capable of removing color through the production of a wide variety of enzymes such as laccases, azoreductases, manganese peroxidases (MnP), lignin peroxidases (LiP), NADH-DCIP reductases, among others, such as fungi (Zaharia and Suteu, 2012; Khan *et al.,* 2013; Danouche *et al.,* 2021). Yeasts are present in the activated sludge of wastewater treatment plants with a great diversity of strains but in small numbers when compared to other environments. Similar to microalgae, yeast decolorization mechanisms are still poorly understood and may involve adsorption, enzymatic degradation, or a combination of both (Solís *et al.,* 2012). Some yeasts have been studied in decolorization of different azo dyes due mainly to their high adsorption capacity of dyes and heavy metals such as lead and cadmium (Solís *et al.,* 2012; Khan *et al.,* 2013). However, some strains have been also tested for the capacity to mineralize the aromatic amines resulting from the process of dye degradation (Kurade *et al.,* 2015). Various studies revealed that some species of yeasts are successful in the treatment of effluents containing high concentrations of dyes (Jafari *et al.,* 2014). Examples of some yeast species and their respective decolorized dyes are shown in Table 1.3.

Table 1.3 - Examples of some yeasts studied and respective decolorized dyes.

Microorganism	**Dye**	**Reference**
Galactomyces geotrichum	Remazol Red, Brown 3 REL, Scarlet RR, Direct Red 2B, Malachite Green; Amido Black 10B, Methyl Red, Orange HE 2R	Kurade *et al.* (2017) Jadhav *et al.* (2008)
Saccharomyces cerevisiae	Ramazole Blue, Remazol Black B, Remazol Red RB	Mahmoud (2016) Aksu (2003)
Trichonosporon beigelli	Yellow 4R-HE,	Saratale *et al.* (2009)

Trichosporon multisporum ***Trichosporon laibachii***	Red 7B HE, Blue RR-BB, Green RR-4B Navy Blue HER, Red HE7B, Golden Yellow 4BD, Green HE4BD, Orange HE2R, Malachite Green, Crystal Violet Methyl violet	Pajot *et al.* (2007)
Debaryomyces polymorphus	Reactive Black 5, Procion Scarlat H-E3G, Procion Marine H-EXL, Reactive Brilliant Blue K-NR, Reactive Yellow M-3RE, Reactive Brilliant Red K-2BP, Reactive Red M-3RE	Yang *et al.* (2003)
Scheffersomyces spartinae	Acid Scarlet 3R	Tan *et al.* (2016)
Candida tropicallis	Basic Violet Reactive Black 5, Reactive Brilliant Blue K-NR, Reactive Red M-3BE Acid Red B Remazol Blue	Solís *et al.* (2012) Yang *et al.* (2013) Li *et al.* (2015) Dönmez (2002)
Candida rugopelliculosa	Reactive Blue	
Candida boidinii	Reactive Black 5	Martorell *et al.* (2017)
Candida oleophila	Reactive Black 5	Lucas *et al.* (2006)

Some studies indicate that dye degradation by yeasts is highly associated with the yeast growth process and its primary metabolism. Furthermore, yeast cells do not grow without glucose or an easily metabolizable source of carbon and energy, and the decolorization process requires a carbon source. Moreover, the production of oxidases and reductases such as MnP, Tyr and NADH-DCIP reductase by yeasts, appears to be induced by the presence of azo dyes (Solís *et al.,* 2012).

1.4.2.1.1. Aerobic granular sludge reactor and bioaugmentation

Wastewater treatments face several problems due to the complex and variable chemical composition of the streams, often with recalcitrant compounds, salts and other pollutants from chemical, pharmaceutical, textile and agro-food industries, among others (Adav *et al.,* 2008). Biological treatments with activated sludge are one of the most used processes for the treatment of textile effluents (Vilaseca *et al.,* 2010). However, they have some disadvantages such as lack in the degradation of complex compounds, high biomass production, low flexibility to fluctuating stream rates and need for large areas for building tanks and settlers (Beun *et al.,* 1999). The recent technology of Aerobic Granular Sludge (AGS) developed in the 90's contributes for a more sustainable and environmental-friendly wastewater treatment solutions (Adav *et al.,* 2008). This technology, based on the biofilm structure, together with storage polymers produced by microorganisms, lead to the production of aerobic granules (Morgenroth *et al.,* 1997). Aggregates are considered AGS if observed the following characteristics: "the presence of active microorganisms in a biopolymer matrix, with the minimum diameter of 0.2 mm (limit adjusted per case or granule type), with a sludge volume index after 5 minutes settling (SVI_5) comparable to activated sludge after 30 minutes of settling (SVI_{30}), and non-coagulation under low hydrodynamic shear" (Morgenroth *et al.,* 1997). Simplifying, AGS are compact microbial aggregates with defined boundaries, which form with a matrix of extracellular polymeric substances (EPS), and which have much better settling properties and resistance to chemical toxicity and toxic loads than activated sludge flakes (de Kreuk *et al.,* 2005). Thus, AGS have also many advantages such as the high compact/dense structure – higher than the conventional activated sludge; regular, smooth and almost round shape; high settling properties; wide range of microbial species with high biomass retention; ability to withstand at high organic loading and tolerance to toxicity. In addition, the AGS technology was also used for treating wastewaters loaded with nitrogen, phosphorus, organic and toxic substances (Adav *et al.,* 2008). Since AGS presents all these advantageous characteristics, some full-scale plants were already successfully implemented in different industrial sectors, engineered to commercially application as Nereda® Technology (Giesen *et al.,* 2013).

AGS stable granulation starts when activated sludge is submitted to a sufficiently high sedimentation selective pressure, allowing the selective washout of the slowest settling biomass and the retaining of the faster settling sludge (de Kreuk *et al.,* 2005). Additionally, it is known that the production of EPS that are the main constituent from the granules' matrix, along with the

selection of slow growing bacteria, improve granulation (de Kreuk *et al.,* 2005). EPS matrix is composed of a variety of high molecular weight polymers, such as proteins, humic acids, polysaccharides, nucleic acids and lipids resulting from the metabolism and/or cell lyses (Flemming *et al.,* 2007). As most of the EPS components are hydrophilic, it contributes for the water retention of the hydrogel matrix. Proteins and polysaccharides are the main components of EPS; humic substances are also an important part of EPS but are not secreted by microorganisms, they are adsorbed from the environment. Nucleic acids are mainly resulted from lysed cells and could also be important for eventually potentiating horizontal gene transfer between the microbial community in AGS (Flemming *et al.,* 2007; More *et al.,* 2014). EPS components establish a tertiary network structure with each other and with the surrounding environmental compounds through hydrogen bonds, electrostatic forces, attractive ionic forces and/or biochemical reactions, that are very important for adsorb substances from the environment, providing nutrient for the microbial community (Felz *et al.,* 2016). Another advantage of these EPS interactions is that they contribute for AGS tolerance to toxic chemicals and for the high biosorption capacity of the granules to other compounds such as pharmaceuticals, chemicals and heavy metals. This biosorption capacity varies with the biochemical composition of EPS and the amount of functional anionic groups (Adav *et al.,* 2008; More *et al.,* 2014).

Internal characterization of the granules could be achieved by advanced microscopy techniques such as scanning electron microscopy, light and confocal laser scanning microscopy with fluorescence *in situ* hybridization (Adav *et al.,* 2008). Different microbial communities are found in AGS, also depending on the culture medium where that are formed. Bacteria such as heterotrophic, nitrifying and denitrifying, phosphorous- and glycogen-accumulating bacteria have been identified in AGS (Adav *et al.,* 2010; de Kreuk *et al.,* 2005). These bacteria are able to remove organic carbon, nitrogen, phosphorous and toxic compounds such as metals, phenol and 2,4-dichlorophenol and also pharmaceutical compounds such as endocrine-disrupting chemicals and norfluoxetine, among others (Adav *et al.,* 2008; Amorim *et al.,* 2016; Ely *et al.,* 2022).

Although all the advantages that AGS technology promises, the microorganisms present in the AGS on bioreactors may not be able to degrade some pollutants. Bioaugmentation is a strategy that has been used to overcome this difficulty. Microorganisms with previously known capacities for degrading the pollutants of interest has been inserted in bioreactors with good results. Different strategies could be used for bioaugmentation. In an AGS-SBR system, bioaugmentation can be applied in the beginning of the bioreactor assembly so that the bioaugmenting microorganism could be integrated into the AGS at the granulation step or could be inserted from time to time throughout the reactor operation. Some studies reported single strains or microbial consortia to bioaugment AGS systems for degrading contaminants such as herbicides and organofluorines, among others (Duque *et al.,* 2011; Quan *et al.,* 2015; Oliveira *et al.,* 2021b). Also, a few studies regarding the use of bacterial strains with dye-decolorizing capacity using AGS in SBR with synthetic wastewater

were investigated (Lourenço *et al.,* 2015; Franca *et al.,* 2020; Maqbool *et al.,* 2020). In addition, some yeasts are also being used for bioaugmentation with promising results, to biofortify a pilot-scale wastewater treatment plant to treat high-salinity organic wastewater (Louhasakul *et al.,* 2019; Wen *et al.,* 2022).

Sequencing batch reactors (SBRs) are systems that are frequently used to produce AGS once the operational conditions and requirements for AGS formation are satisfied (Adav *et al.,* 2008). Briefly, in this system, during the first step that is the feeding step, the influent is provided from the bottom of the reactor and then, at the second step, the aeration begins. In this step, several removal processes are being accomplished by the bacteria present in the AGS. An oxygen diffusion gradient occurs within the AGS, meaning that the provided oxygen only could penetrate the outside layers of the granules, and only the inner layers could be subjected to anoxic and anaerobic conditions. This is an advantage since this allows efficient and simultaneous carbon and nutrient removal. The next step of settling (after the aeration stopped) allow the granule to quickly settle at the bottom of the reactor, letting the efficient withdrawn of the treated effluent (last step). According to the application of the SBR system, all these operational conditions can be altered and adapted (Morgenroth *et al.,* 1997). The SBR system has numerous advantages over traditional systems since, due to its high efficiency in carbon and nutrient removal, added to the fast granules' settling time which permits a faster withdrawn of the treated effluent, all in the same system, allowing the saving in required space and energy costs. In addition, due to its faster processes, higher amounts of effluents could be treated in the same period of time, when compared with other traditional biofilm technologies. An example of AGS (under a magnifying glass) provided from a lab-scale SBR (scheme) are represented in Figure 1.2.

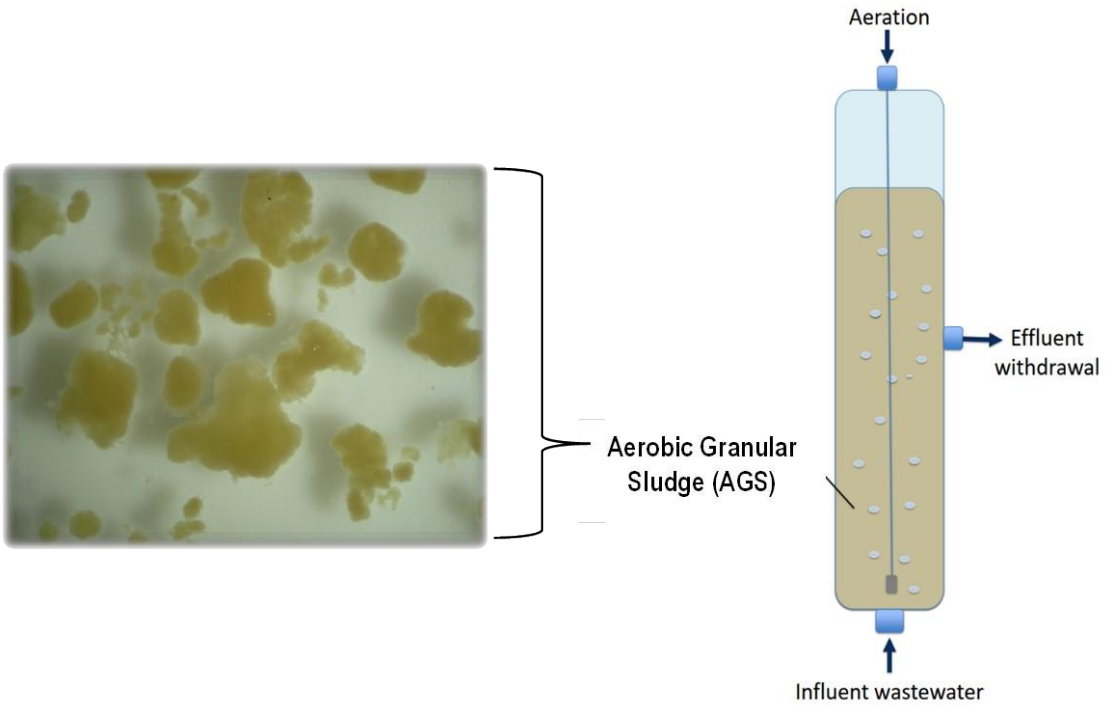


Figure 1.2 – Example of AGS (under a magnifying glass) provided from a lab-scale SBR.

In addition, the AGS-SBR combination has a simple and compact layout with good operational flexibility and also has been reported that could result in a 1-2 log reduction of pathogens (Kassab *et al.,* 2010; Lourenço *et al.,* 2001).

Effluents from the textile industry could benefit from this technology since some studies have reported color and COD removal at high levels. Furthermore, the greater tolerance to toxics and higher effluent loads that could contribute to generate greater microbial diversity, make AGS-SBR a promising alternative to flocculent biomass (Franca *et al.,* 2015; Mata *et al.,* 2015).

1.4.2.2 Formulations for use in biological processes

Preserving the integrity of microbial cells is crucial to the usefulness of microorganisms in real industrial/wastewater facilities. Formulations that contain microorganisms for use in industrial facilities need to be simple to use and easily stored for a long time, while maintaining the microbial integrity and viability. Also, these products need to be stable, safe and easy to apply (Abadias *et al.,* 2001). In this particular topic, preserving strategies that also maintain microbial decolorization capacity are in demand. In order to preserve and maintain the viability of microorganisms, several methods were developed overtime.

Freeze and spray-drying are the methods that are usually employed to preserve microbial cultures for application in modern industries such as lactic and wine industries (Połomska *et al.,* 2012). Freeze-drying is often considered to be the most straightforward and effective method of preserving microorganisms (Abadias *et al.,* 2001; Połomska *et al.,* 2012). This method is a low-temperature process, which removes water in a previously frozen sample, under reduced pressure, by sublimation, with almost nonchemical alteration (Barbosa *et al.,* 2015). This fact is a great advantage over other cell preserving processes, especially for biological materials and thermal-sensitive products such as enzymes. In addition, freeze-dried products can be easily rehydrated and recover its functions (Barbosa *et al.,* 2015; Castro *et al.,* 1997). Unfortunately, freeze-drying is a time-consuming process which makes it an expensive process when compared to other methods. Spray-drying is a simple and rapid method with lower costs, which justify being frequently chosen for industrial processes. This process transforms a sample solution directly into a dried powder: the sample solution is sprayed into a chamber with hot dry air and evaporates rapidly into a spray-dried powder. However, the high temperatures used in this process may result in a decrease of cell viability and loss of some chemical properties, also the presence of sugar molecules can cause stickiness causing operating difficulties. Even so, spray drying is a less expensive, rapid, simple, and continuous process widely used in industry (Barbosa *et al.,* 2015).

High temperatures used in spray-drying (ranging between 130 ºC to 170 ºC) and the low temperatures used in freeze-drying (-20 ºC to -80 ºC) processes could cause several damages to the cell wall of microorganisms. Also, damages in cell and cytoplasmatic membranes could result in lethal cell damages (Jesus and Maciel Filho, 2014; Tang *et al.,* 2020). To overcome some of the problems associated with the drying processes, many conventional cryoprotecting agents such as glycerol, DMSO (Dimethyl Sulfoxide), skim milk powder, maltodextrin, sorbitol and other sugar-based protecting agents, have been extensively used as a suspending agent before drying processes (Tang *et al.,* 2020). They are introduced into the sample solution to minimize stickiness and cellular lysis, preventing the crystallization of water molecules, improving cell survival throughout the drying processes (Prakash *et al.,* 2020). Cryoprotecting methods provide the protection to preserve the cells as they can replace water molecules without disturbing cell membrane during drying processes. These protecting agents also provide stability and protection against denaturation of sensitive proteins and other inactivating mechanisms (Barbosa *et al.,* 2015; Castro *et al.,* 1997; Połomska *et al.,* 2012; Tang *et al.,* 2020). Another possible form of microbial immobilization involves encapsulation in a matrix of sodium alginate and calcium chloride. The addition to a sodium citrate bath allows the capsules to retain their core liquid, facilitating exchanges between liquid and the entrapped microorganisms. The main advantages of encapsulation are the protection of the microorganisms against the external environment and consequently greater resistance during the treatment process (Qi *et al.,* 2005; Sousa *et al.,* 2015; Holkem *et al.,* 2016). New techniques for microbial preservation and novel cryoprotecting agents are being explored. Cell

Alive System (CAS)-freezing technique, electrospinning and electrospraying, gelatine disk method and entrapping methods with alginate and Acacia-gum, as well as Galacto-oligosaccharides (GOS), are examples of emerging microbial protecting methods and agents (Prakash *et al.,* 2020).

1.5. SCOPE AND OBJECTIVES OF THIS THESIS

It is known that the textile industry sector consumes a large amount of water and produces high volumes of effluents that contain a complex mixture of synthetic dyes that could be released into the environment causing several pollution and health problems. The increased pressure of civilization development, as well as the tap water scarcity, imposes on governments have led to the increase of the search for new wastewater treatments. Innovative biotechnological solutions are being investigated to decontaminate, valorize and even reutilize water from wastewater treatment plants in a circular economy perspective.

The work described in this thesis intends to further the knowledge on the biological decolorization of textile dyes using selected yeasts and thus to develop potential innovative biotechnological solutions to solve issues concerning the presence of dyes in the textile industry effluents considering their environmental impact. Previously known yeasts with decolorization ability were used to select the most efficient at decolorizing a wide range of textile dyes and simulated effluents. Then, the end-products and the toxicity at different trophic levels of the resulting streams after yeast treatment were investigated. Potential strategies for the preservation and long-term storage of the microbial strains were studied: spray and freeze drying, and alginate capsules as an immobilization strategy. Furthermore, a laboratory scale reactor with a simulated textile wastewater (containing Navy Everzol ED dye and salinity conditions) and bioaugmented with the decolorizing yeast *Yarrowia lipolytica*, was tested for the capacity of degrading nutrients and dye color removal. Finally, the bacterial profile from the bioaugmented AGS was investigated to reveal the communities responsible for the reactor performance.

The specific objectives of this thesis were:

- To do a screening of selected yeasts, single and in consortia, at decolorizing a wide range of classes of dyes commonly used in textile industries and simulated textile effluents;

- To evaluate the toxicity of the resulting treated streams, containing the end-products of decolorization resulting from the yeast metabolism, to access their safety when discharged into the environment;

- To evaluate the viability and stability, as well as the decolorizing ability, of the selected yeast strains cells after being subjected to preservation processes;

- To access the ability of the yeast-bioaugmented AGS to remove an azo dye commonly used in the textile industry in saline environments, as well as the reactor performance for

main nutrient removal processes and the composition of the microbial community during reactor operation.

1.6. OUTLINE OF THE THESIS

This thesis comprises six chapters.

The current chapter (Chapter 1) comprehends a brief literature review concerning the environmental problematic of the release of textile wastewaters, specifying the role of dyes and the potential toxicity of the end-products, and describes traditional and biological wastewater treatment methods, including bioaugmented bioreactors. Also, microbial preserving processes are depicted.

Chapter 2, the first experimental part of the thesis, presents the screening and evaluation of the performance of selected yeasts, single and in consortia, at decolorizing a wide range of classes of dyes commonly used in textile industries and simulated textile effluents.

Chapter 3 presents the analysis of the toxicity of the yeast-treated streams containing the end-products of decolorization, in order to evaluate their safe environmental discharge.

In Chapter 4 the evaluation of the viability and stability of the yeast strains cells after being subjected to preserving processes as well as the decolorizing ability throughout storage time (at room and refrigerated temperature) are presented.

Chapter 5 describes the ability of the yeast-bioaugmented AGS to remove an azo dye commonly used in the textile industry in saline environments, as well as the reactor performance for main nutrient removal processes. The composition of the microbial community during reactor operation is also presented and discussed.

Finally, the last chapter, Chapter 6, corresponds to the main conclusions of this thesis and suggestions for future work.

The core of this thesis is composed of four articles already published in international peer-reviewed journals, according to the following list:

Chapter 2

Mendes, M., A.C. Cassoni, S. Alves, M.E. Pintado, P.M. Castro, and P. Moreira. 2022. *Screening for a more sustainable solution for decolorization of dyes and textile effluents using Candida and Yarrowia spp.* Journal of Environmental Management 307:114421

https://doi.org/10.1016/j.jenvman.2021.114421

Chapter 3

M. Mendes, A.C. Cassoni, S. Alves, P. Moreira, M.E. Pintado and P.M.L Castro. 2023. *Removing color while lowering toxicity: the case for decolorization of textile dyes and simulated effluents with yeasts*. International Journal of Environmental Science and Technology 1-12.

https://doi.org/10.1007/s13762-023-04969-8

Chapter 4

Mendes, M., A.C. Cassoni, S. Alves, P. Moreira, P.M.L Castro and M.E. Pintado. 2023. *Assessment of drying conditions for storage of a yeast-based decolorization solution for application in textile industrial wastewater treatment plants.* Fungal Biology 127:7-8, 1111-1117.

https://doi.org/10.1016/j.funbio.2023.06.002

Chapter 5

Marta Mendes, Irina S. Moreira, Patrícia Moreira, Manuela Pintado and Paula M.L Castro. 2023. *Bioaugmentation of Aerobic Granular Sludge with Dye-Decolorizing Yeast for Textile Industrial Wastewater.* Processes 11:6, 1654.

https://doi.org/10.3390/pr11061654

CHAPTER 2

SCREENING FOR A MORE SUSTAINABLE SOLUTION FOR THE DECOLORIZATION OF DYES AND TEXTILE EFFLUENTS

This chapter is published as: Mendes, M., Cassoni, A.C., Alves, S., Pintado, M.E., Castro, P.M., Moreira, P., 2022. “Screening for a more sustainable solution for decolorization of dyes and textile effluents using *Candida* and *Yarrowia* spp”. *Journal of Environmental Management* 307, 114421.

2.1. INTRODUCTION

The textile industry is one of the most important sectors of the world economy. However, industrial textile processes lead to the formation of pollutants with high environmental impact (Dellamatrice *et al.,* 2017; Sen *et al.,* 2016). These pollutants can contaminate air, water, and soil affecting the photosynthetic activity of the ecosystems, creating anoxic conditions that are lethal to aquatic organisms and affecting indirectly human health (Ali, 2010). The most frequently used dye classes are acid, basic, direct, disperse, vat, and reactive. Natural fibers such as cotton, wool, silk, and leather are generally colored by vat, reactive, and acid dyes. Basic, direct, and disperse dyes are generally used for synthetic fibers such as polyester, nylon, polyamide, and acrylic fibers (Püntener *et al.,* 2003). Replacement of synthetic dyes by natural solutions with low environmental impact urges, but in the meantime, efficient treatment systems of textile effluents containing synthetic dyes, affordable and complying with governmental legislation that is increasingly restrictive in developed countries, is needed to guarantee safe disposal (Paz *et al.,* 2017). Classic methods are based on physical and chemical-treatment processes, occasionally combined with biological treatments (Ali, 2010; Lalnunhlimi and Veenagayathri, 2016). However, most of them have been proven reduced efficiency, high costs, and can generate a significant amount of toxic wastes that have to be treated separately, increasing substantially the cost and complexity of these methods (Mahmoud, 2016). Alternatively, the industry must look for “green” processes that are less problematic and contribute to the circular economy and eco-sustainability in the water sector (Ali, 2010).

Biological methods are generally more environmentally friendly since they can lead to the complete mineralization of organic pollutants at a lower cost or by adsorption by living or dead biomass (Paz *et al.,* 2017). Bacteria and filamentous fungi, because of their enzymatic toolbox, have been the most studied microorganisms for dye degradation, but also algae and yeasts, which are present in treatment plants, started attracting the attention of researchers and might play a relevant role in treating textile effluents (Martorell *et al.,* 2017; Moreira *et al.,* 2001). Yeasts, like *Galactomyces geotrichum* (Kalyani *et al.,* 2009), *Saccharomyces cerevisiae* (Mahmoud, 2016), *Trichosporon spp.* (Pajot *et al.,* 2007; Saratale *et al.,* 2009), *Schizosaccharomyces pombe* and *Kluyveromyces marxianus* (Aksu and Dönmez, 2003), *Debaryomyces polymorphus* (Yang *et al.,* 2005) and *Candida* spp. (Martorell *et al.,* 2017; Yang *et al.,* 2013), have been recently studied for dye degradation. These microorganisms can be of added value in the process of dye decolorization as they are easy to grow and maintain in culture and are capable of removing color through the production of a wide variety of enzymes. Examples of the latter is laccase (Pajot *et al.,* 2007), azoreductase (Kalyani *et al.,* 2009), manganese peroxidase (MnP), lignin peroxidase (LiP) (Yang *et al.,* 2013), NADH-DCIP

reductase (Saratale *et al.,* 2009), among others (Khan *et al.,* 2013). The dye molecular composition/structure and the capacity of each yeast strain to produce mechanisms and enzymes can explain the differences in degradation, either true decolorization (biodegradation of the dye molecules) or adsorption, observed for each class of dyes (Khan *et al.,* 2013). Yeast strains isolated from wastewater treatment plants, which were previously investigated in our work group, exhibited the capacity of decolorization (Jorges, 2016; Silva, 2011). The main objective of this study was to evaluate the performance of three selected yeasts, single and in consortia, at decolorizing a range of classes of dyes namely, reactive, disperse, direct, acid, and basic, commonly used in textile industries, and simulated textile effluents.

2.2. MATERIAL AND METHODS

2.2.1. Yeast strains and culture conditions

Three properly identified yeast strains, previously isolated from activated sludge from wastewater treatment plants from the north of Portugal, were tested: *Candida parapsilosis* (HOMOGS20B), *Yarrowia lipolytica* (HOMOGST27AB) and *Candida pseudoglaebosa* (LIIIS36B) (Jorges, 2016; Silva, 2011). The consortia formed by the combination between them were also tested totalizing 4 consortia: combinations of yeast pairs (50% in cell concentration of each strain) and combinations of all three yeasts in equal parts. Normal Decolorization Media (NDM), modified by Pajot *et al.* (2007) was used for culture maintenance and decolorization assays (Pajot *et al.,* 2007). Analytical grade reagents were used: 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.13 g of $CaCl_2$ (Merck, Darmstadt, Germany); 5.0 g of KH_2PO_4; 2.5 g of $(NH_4)_2SO_4$; 20.0 g of glucose (Sigma-Aldrich, St. Louis, MO USA); 2.5 g of yeast extract (Biokar diagnostics, Beauvais, France). For solid media (Normal Solid Decolorization Medium - NSDM) 20.0 g/L agar (Biokar diagnostics, Beauvais, France) was added. Cultures grown overnight were adjusted at $OD_{550\ nm}$ = 0.5 of McFarland standard (1.5 x 10^8 CFU/mL) and tested isolated and in consortia. All tests were performed under sterile conditions and at 30 °C and growth was followed for 48h. Samples were collected every 12 hours and decimal dilutions were spread (100 µL) into Petri dishes with NSDM and incubated at 25 °C/ 48h (in duplicate). Colonies from each strain were visually identified through different morphology and quantified by colony counting.

2.2.2. Dyes

Fifteen different types of commercial synthetic dyes (kindly provided by AQUITEX, SA) from 5 different classes (Everacid, Procion, Everdirect Supra, Basic, Everzol) were tested - Everacid: Red N-2BL, Yellow MR H|C, Blue N-AFN; Procion: Red HE 7B, Yellow HE 4R, Navy HEXL;

Everdirect Supra: Red BWS, Yellow RL, Blue FFRL; Basic: Yellow 200% Itocryl e Blue GRL-F200% Itocryl; Everzol: Red ED-3B, Yellow LX, Navy ED e Blue L-ED. Table 2.1 shows the name of the simulated effluents tested and the respective acronyms used henceforth in the text of this chapter.

Table 2.4 - Types of dyes tested and its corresponding acronyms.

Dye	Name	Acronym
Everacid	Red N-2BL	Evd-Red
	Yellow MR H|C	Evd-Yellow
	Blue N-AFN	Evd-Blue
Supra Everdirect	Red BWS	SEd-Red
	Yellow RL	SEd-Yellow
	Blue FFRL	SEd-Blue
Basic	Yellow 200% Itocryl	B-Yellow
	Blue GRL-F200% Itocryl	B-Blue
Everzol	Red ED-3B	Evz-Red
	Yellow LX	Evz-Yellow
	Navy ED	Evz-Navy
	Blue L-ED	Evz-Blue
Procion	Red HE7B	P-Red
	Yellow HE 4R	P-Yellow
	Navy HEXL	P-Navy

Maximum absorbance wavelength was determined for each dye in NDM by performing a spectral scanning between 200 and 800 nm. Stock solutions were prepared by dissolving powdered dyestuff in distilled water up to a concentration of 4 g/L that were filtered (sterile 0.22 µm filter, Sartorius, Germany) to sterilize before its incorporation in culture media.

2.2.3. Simulated textile effluents

Seventeen different simulated effluents were prepared by the company AQUITEX, SA (Porto, Portugal) by simulating the industrial processes in a lab-scale fabric dying device for Everacid, Supra Everdirect, Basic, Everzol as well as Disperse and Samovat dye types, mimicking real industrial conditions. Table 2.2 shows the name of the simulated effluents tested and the respective acronyms used henceforth in the text of this chapter.

Table 2.5 - Types of simulated effluents tested and its corresponding acronyms.

Simulated Effluent	Name	Acronym
Everacid	Red N-2BL	Evd-Red
	Yellow MR H|C	Evd-Yellow
Supra Everdirect	Rubine BL	SEd-Rubine
	Yellow RL	SEd-Yellow
	Blue 4BL	SEd-BlueBL
Basic	Red 14	B-Red
	Yellow 28	B-Yellow
	Blue 41	B-Blue 41
Everzol	Red ED-3B	Evz-Red
	Yellow LX	Evz-Yellow
	Navy ED	Evz-Navy
Disperse	Red EFBL 200%	D-Red
	Yellow Brown	D-YellowB
	Navy	D-Navy
Samovat	Red	S-Red
	Yellow	S-Yellow
	Blue	S-Blue

Briefly, for the Everacid simulated effluents, a solution with 1 g/L of an equalizer agent together with acid donor agent (0.5-1.5 g/L; pH 6-6.5) was heated at 20 °C for 5 min. Then, a x% of dye was added and stand for more 5 minutes at the same temperature. Then the temperature was raised until 70 °C (1 °C/min), staying for 15 minutes and then raised until 98 °C (1 °C/min) for additional 30-40 min. Then, a wash step was applied at 50 °C for 10 min, followed by a cold

wash for 10 min. After that, a fixation step was followed: 2-3% of a fixer solution and an acid buffer (1 g/L; pH-4.5-5) at 70 °C for 20 min. Finally, a softening step was prepared with a solution of 2% of softening agent and an acid buffer (1 g/L; pH-4.5-5) at 40 °C for 20 min. For the simulated effluents of Everdirect Supra dyes, a solution with x% of dye and 0.2-0.5 g/L of an agent that slows down the dyeing was prepared and heated at 40-50 °C and then y g/L of salt was added and heated for 10 minutes at the same temperature. The next step was raising the temperature until 95-100 °C (2 °C/min) and maintained for 30-50 min, then decreased to 70-80 °C. After that, the process was followed by two cold washes, 10 minutes each. The next step was the fixation of the color (only for medium-dark colors): 1-2% of a fixer solution and an acid buffer (1 g/L; pH-4.5-5) was let at 60 °C for 10 min. For the simulated Basic effluents, a solution composed by an agent that slows down the dyeing process (1-2 g/L), 5% of sodium sulphate, a dispersant agent (1 g/L) and an acid buffer (1.5 g/L; pH 4.5-5) was heated at 50 °C for 5 min. Then a x% of dye was added during 5 minutes and let it stand for 5 min. Then, the temperature was raised up to 80 °C (4 °C/min) and then until 102 °C (0.5 °C/min), maintaining it for 30 min. After that, the temperature went down to 80 °C (1 °C/min). The next step was a reducing wash with a solution composed by 1 g/L detergent, 1 g/L of Na_2CO_3 and 1.5 g/L of sodium hydrosulphite heated at 60 °C for 15 min. Then, it was followed by two wash steps at 50 °C, 10 minutes each. For the Everzol reactive dyes simulated effluents, the first step included a solution with an equalizer (1 g/L), a wetting agent (0.2 g/L), a x% of dye and a y g/L of salt heated at 60 °C/15 min, then the addition of 5 g/L of sodium carbonate (Na_2CO_3) and NaOH 38 ºBé (z mL/L) at the same temperature for 60 min. The next step was a cold wash for 10 minutes followed by a neutralization step at 50 °C/10 minutes and a soaping step (1 g/L) at 100 °C/15 min. The final step was a cold wash. The simulated industrial process for the Disperse effluents included: a pre-wash step with detergent (1.5 g/L) and a solubilizer solution (1 g/L) at 75 °C for 20 min; then the dyeing step at 50 °C with a dispersant/equalizer agent (1 g/L), an acid buffer (1-1.5 g/L; pH-4.5 final solution) and x% of dye (for 5 min). Then, the temperature was raised in a gradient from 50 °C to 130 °C (2 °C/min – 30-45 minutes) and ended at 70-80 °C (1.5 °C/min). The next step was a reducing wash with sodium hydrosulphite (4 g/L), sodium hydroxide (NaOH) 38 ºBé (2 mL/L) and Toxal NP/5 (1 g/L), at 80 °C for 15 min. The final step was to wash and neutralize the solution. For the Samovat type of simulated effluents, a solution composed by a sequestering agent (1 g/L), a dispersant (1 g/L), an equalizer (0.5 g/L), x% dye, z mL/L NaOH 38 ºBé and a y g/L of sodium hydrosulphite was heated at 40 °C/10 min, then the temperature was raised up until 80 °C (1.5 °C/min) for 45 min, decreasing until 60 °C (2 °C/min) for 10 min, followed by a wash step. The next step was an oxidation step with H_2O_2 200 vol. (2 mL/L) and pH: 5-5.5 (with acetic acid) heated at 55-60 °C for 15 min. Then a soaping step with a solution with a dispersant agent (1 g/L) and sodium carbonate (2 g/L) was heated at 100 °C for 15 minutes and finally washed. Process diagrams

of the preparation of all simulated effluent are presented in Supplementary Material at the end of this chapter, for better comprehension.

2.2.4. Decolorization in solid media

NSDM plates were prepared and supplemented, at 45 °C, with sterilized dye stock solutions (100 mg/L – final concentration). Plates containing Everacid: Red 2-NBL, Yellow MR H|C and Blue N-AFN; Everdirect Supra: Red, Yellow RL, and Blue FFRL; Basic: Yellow 200% and Blue GRLF-F200 200%; Procion: Red HE 7B, Yellow HE 4R and Navy HEXL were prepared in duplicate. In each plate, 20 µL of fresh overnight inoculum of each isolated strain was inoculated in duplicate (6 areas per plate), incubated at 30 °C and examined for colony growth and decolorization throughout 72 hours of incubation.

2.2.5. Decolorization in liquid media

Decolorization tests were performed in microplates with 24 wells using 500 µL of NDM supplemented with 100 mg/L (final concentration) of each dye and inoculated with 3% v/v of the single yeasts and consortia (cultures pre-grown in NDM up to $OD_{550\ nm}$ = 0,5) in triplicate. Besides the pure dyes, 17 simulated effluents were tested, incorporating 1 g/L, 5 g/L or 20 g/L of effluent depending on the color intensity, in 500 µL of NDM. Microplates were incubated at 25 °C and 100 rpm for 36 hours for dyes and 48 hours for simulated effluents. Sampling (100 µL) was performed every 12 hours for dyes and every 24 hours for effluents; each aliquot was centrifuged at 14,000 rpm for 15 min, and the supernatant was collected prior to further analysis. Negative controls were performed at the same conditions without microorganisms. Supernatants obtained from each decolorization assay (in triplicate) were monitored at each dye's maximum absorbance wavelength in a Specord S600 (Analytik Jena, Germany) spectrophotometer. The percentage of decolorization was calculated using the equation:

$$\text{Decolorization (\%)} = \frac{Abs\ t0 - Abs\ tx}{Abs\ t0} \times 100$$

(Equation 1)

where Abs t_0 = Absorbance at the initial time and Abs t_x = Absorbance at sampling time. Additionally, observation of dye and effluent adsorption by the biomass was carried by naked eye in each resulting pellet.

2.2.6. Characterization of enzymatic activity

The three isolated yeast strains were incubated in NDM at 25 °C and 100 rpm for 24 hours and also incubated in NDM supplemented with Yellow Everzol dye (100 mg/L), chosen due to its light color avoiding interference with the measurements, to evaluate the possible enabling effect of the dye in the enzymatic metabolism. The extracellular extract was obtained by recovering the supernatant after centrifugation of the inoculum for 15 minutes at 5000 rpm. Characteristic enzyme activities were determined spectrophotometrically: Manganese independent Peroxidase (MiP) (Mester and Field, 1997), Manganese dependent Peroxidase (MnP) (Castillo *et al.,* 1994), Lignin independent Peroxidase (LiP) (Moreira *et al.,* 2006), Veratryl alcohol (Moreira *et al.,* 2006) and Laccase (Gomare *et al.,* 2009). The intracellular extract was obtained by cellular disruption of the fresh pellet after centrifugation of the inoculum for 15 minutes at 5000 rpm. Disruption of the cells was achieved by suspending the pellet in phosphate buffer (100 Mm, pH 7.4) and adding glass beads (500 µm; Sigma-Aldrich, St. Louis, MO USA) in a proportion of 1:1 (w/v). Five cycles of 2 minutes of disruption and 2 minutes of pause were conducted using Disruptor Genie (Zymo Research, USA). All the samples were kept on ice during this process. Finally, samples were centrifuged at 14,000 rpm for 3 minutes and the supernatant was characterized. Oxidoreductase (Kurade *et al.,* 2015), Azoreductase (Jadhav *et al.,* 2007), and Tyrosinase (Martorell *et al.,* 2012) activities were determined spectrophotometrically.

2.2.7. Statistical analysis

Results obtained in the experiments were expressed in terms of means (average) and standard deviation (S.D.). First, data were analyzed for normality distribution. Then, the homogeneity of variances was verified through Levene's test. When the assumptions were guaranteed, one-way ANOVA was performed and Tukey pos hoc test was applied to compare means within groups. Otherwise, Kruskal-Wallis test was carried out with subsequent Mann-Whitney tests. The level of significance was 0.05. Statistical analysis was performed using IBM® SPSS® Statistics 26 (SPSS Inc., IBM Corporation, NY, USA).

2.3. RESULTS AND DISCUSSION

2.3.1. Yeast's growth singly and in consortium

Yeast's growth curves (Figure 2.1) showed that when growing isolated *C. parapsilosis* (HOMOGS20B) and *C. pseudoglaebosa* (LIIIS36B) had a higher growth rate (8 Log CFU/mL) than *Y. lipolytica* (HOMOGST27AB) (7 Log CFU/mL). When in consortium with the three strains, *Y. lipolytica* (HOMOGST27AB) was inhibited after 24 h. This behavior was noted only in the consortium of *Y. lipolytica* (HOMOGST27AB) with *C. pseudoglaebosa* (LIIIS36B). This inhibition of *Y. lipolytica* (HOMOGST27AB) could indicate that its growth was affected by the presence of *C. pseudoglaebosa* (LIIIS36B) or eventually by some metabolites derived from its growth. Further studies will be needed to understand the causes behind it, as to the best of our knowledge, no information on literature was found about this subject for similar strains. These consortia were further tested for decolorization in liquid med

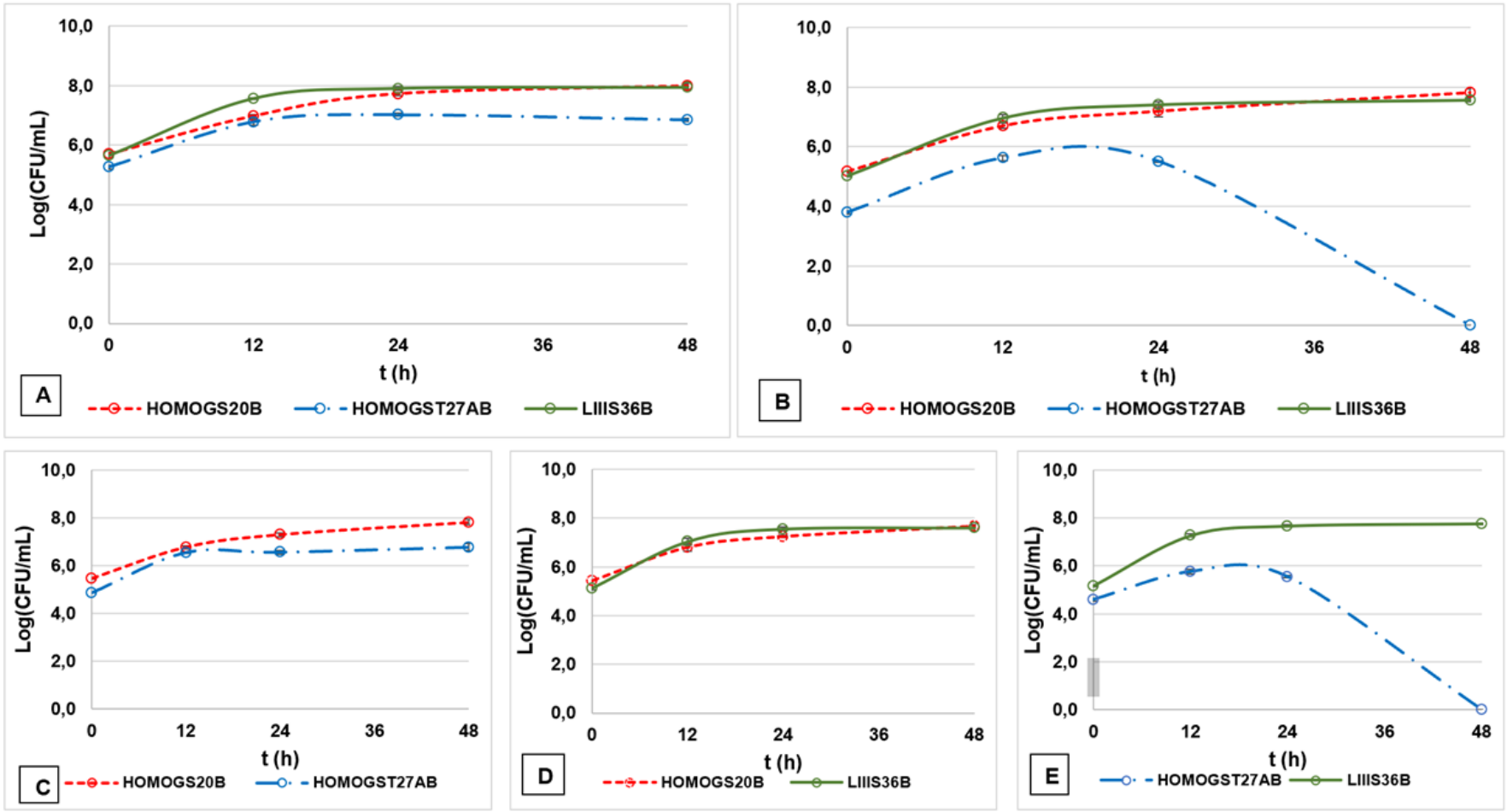


Figure 2.1 - Yeast's growth curves in NDM incubated at 25 °C for 48h.: A – Isolated strains; B – Triple consortium (HOMOGS20B+HOMOGST27AB+LIIIS36B); C - HOMOGS20B+HOMOGST27AB; D - HOMOGS20B+LIIIS36B; E - HOMOGST27AB+LIIIS36B.

2.3.2. Screening for decolorization ability

2.3.2.1. Yeast's ability to decolorize in solid media

Decolorization in solid medium was used as a qualitative method for a first screening of the capacity of the yeast strains to degrade and/or adsorb the target dyes. Decolorization was evaluated by naked eye observation within 72 hours of incubation (Figure 2.2).

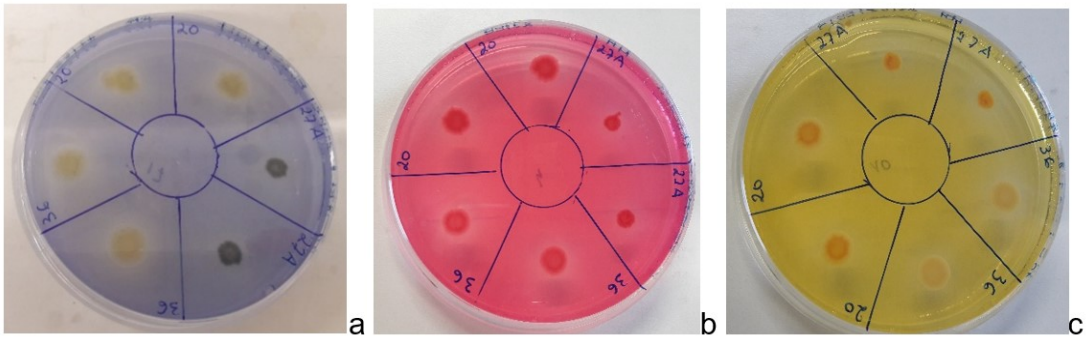


Figure 2.2 - Example of Petri dishes used for screening on solid medium for decolorization ability by selected isolated yeasts with NSDM supplemented with (a) Sed-Blue, (b) P-Red and (c) P-Yellow (i.e.). Colony colors and presence of halos indicate decolorization capacity.

The existence of halos without color around yeast colonies and colorless colonies were indicative of true decolorization ability, whereas colored colonies indicated adsorption and potential bioaccumulation of the dyes in cells. Results and examples are shown in Table 2.3. After 72 hours of incubation, all the strains managed to grow in colonies in each dyed medium (except for *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) for Basic Blue GRL-F200%). Strain *C. parapsilosis* (HOMOGS20B) showed low decolorization capacity for almost all the dyes tested, presenting small halos around colonies. This strain could only remove color at a higher extent for blue-dyed medium (except for the Basic group) whereas yellow from Everdirect Supra class and Red ED-3B from Procion were only partially removed. However, *C. parapsilosis* (HOMOGS20B) adsorbed most of the color that was removed from the medium, which could indicate that this strain does not perform true decolorization. Strain *Y. lipolytica* (HOMOGST27AB) had in general a low decolorization capacity of the dyes tested in solid medium. Even though this strain had the weakest growth, colony colors were strong, especially on the reverse side, with all the dyes tested, showing that cells also adsorbed the dyes. Strain *C. pseudoglaebosa* (LIIIS36B) showed a slightly higher decolorization capacity than the other strains. This strain showed that there was almost no color in the reverse and top of the colonies, which could be a sign of true decolorization.

Table 2.3 - Growth and decolorization by the three yeast strains in NSDM supplemented with different classes of dyes.

Dyes	Parameters	HOMOGS20B	HOMOGST27AB	LIIIST36B
Evd-Red	**Decolorization**	1	2	2
	Reverse colony color	3	4	2
	Top colony color	2	2	1
	Growth	4	2	4
Evd-Yellow	**Decolorization**	2	2	3
	Reverse colony color	3	4	3
	Top colony color	2	3	3
	Growth	4	2	4
Evd-Blue	**Decolorization**	3	2	3
	Reverse colony color	4	5	3
	Top colony color	3	2	3
	Growth	4	3	4
SEd-Red	**Decolorization**	1	1	1
	Reverse colony color	4	5	4
	Top colony color	2	1	1
	Growth	4	2	4
SEd-Yellow	**Decolorization**	3	2	3
	Reverse colony color	2	4	2
	Top colony color	3	3	1
	Growth	3	2	4
SEd-Blue	**Decolorization**	4	2	4
	Reverse colony color	1	4	1
	Top colony color	1	3	1
	Growth	4	3	4
B-Yellow	**Decolorization**	0	0	0
	Reverse colony color	3	4	3
	Top colony color	3	4	4
	Growth	3	2	3
B-Blue	**Decolorization**	2	0	0
	Reverse colony color	1	0	0
	Top colony color	1	0	0
	Growth	2	0	0
P-Red	**Decolorization**	3	2	3
	Reverse colony color	4	5	3
	Top colony color	3	1	2
	Growth	4	2	4
P-Yellow	**Decolorization**	2	2	3
	Reverse colony color	3	5	2
	Top colony color	2	4	1
	Growth	4	2	4
P-Navy	**Decolorization**	3	2	3
	Reverse colony color	4	5	3
	Top colony color	2	2	2
	Growth	4	3	4

0 - No decolorization/color/growth.

5 – Maximum or high decolorization/color/growth.

Species of yeasts such as *S. cerevisiae* (Aksu, 2003), *Candida* spp. (Dönmez, 2002; Li *et al.,* 2015; Martorell *et al.,* 2017), *Galactomyces* spp. (Jadhav *et al.,* 2008; Kurade *et al.,* 2017), *Debaryomyces* spp. (Yang *et al.,* 2005), are known to have the ability of bioaccumulation or biosorption of dyes in the cells and/or biodegrading capacity of the textile dyes, but our study included a far greater range of different classes and colors of dyes. In our study, *Y. lipolytica* (HOMOGST27AB) and especially *C. pseudoglaebosa* (LIIIS36B) showed higher decolorization capacity in solid media for the tested dyes. The differences in the behavior of the yeast strains towards the different dyes could be due to the different molecular structures of the dyes and the conditions used. The use of solid medium could limit the diffusion of nutrients and oxygen and the release of products and enzymes by the yeasts. The differences in color on top and reverse sides of the colonies could be due to the limited diffusion on agar but could also be related to the age and function of the cells in the colony (Jorges, 2016). In a liquid medium, it is expected not only a better homogenization of the nutrients but also of other conditions (i.e., better distribution of oxygen) when the culture is shacked (Váchová *et al.,* 2012). According to these assays in solid media, in general, the blue color seemed to be the easiest to be removed, followed by the red color. In addition, the strain *C. pseudoglaebosa* (LIIIS36B) showed better results on the removal efficiency of some of the dyes in study, with less adsorbed/accumulated dye in the cells, thus performing true decolorization.

2.3.3. Decolorization by yeasts and consortia in liquid media

2.3.3.1. Commercial synthetic dyes

Decolorization ability of the isolated yeast strains and consortia was tested in liquid medium for the synthetic dyes. After 36 h, decolorization of the different dyes was detected in most of the strains and consortia. Table 2.4 shows the decolorization by adsorption or bioaccumulation on yeast cells, while Table 2.5 shows true decolorization (biodegradation – cells without color). In general, for the majority of the dyes tested, color was removed mainly by biosorption or bioaccumulation. Table 2.4 shows that strain *C. parapsilosis* (HOMOGS20B) was the less effective strain in removing color since this strain acting alone presented lower percentages of decolorization than the other two isolated strains (below 85%) and the color removal was mainly by adsorption (except for Blue Supra Everdirect FFRL). Isolated strains *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) presented higher percentages of decolorization by adsorption with some values reaching near 90%. Furthermore, depending on the dye class, these strains also succeeded in removing color without accumulating it in cells, performing true decolorization for some dyes. Regarding the tested consortia, in general, the consortium formed between *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) presented better results in decolorizing at high extents (above 80%). Nevertheless, the consortium formed with *C. parapsilosis* (HOMOGS20B) and *Y. lipolytica*

(HOMOGST27AB) showed decolorization values significantly higher for the generality of the dyes and for a wider range of dyes, although they were removed by adsorption. Regarding the dye classes, in general, dyes from Everacid and Procion classes were removed by adsorption more efficiently by consortia, especially when *C. parapsilosis* (HOMOGS20B) and *Y. lipolytica* (HOMOGST27AB) were together. Everzol was the dye class that was removed more efficiently by true decolorization by consortia and especially by *C. pseudoglaebosa* (LIIIS36B), followed by the class of Everdirect Supra. Chandra (2015) stated that the dye degradation mechanisms by yeasts comprise processes such as biosorption, bioaccumulation, and biodegradation (Chandra, 2015). Mechanisms of biosorption or bioaccumulation occur when the yeast cells get the color of the dye tested. These processes of biosorption and bioaccumulation are probably due to the presence of some active groups on yeast cell surface such as acidic polysaccharides, lipids, amino acids, and other cellular components such as nitrogen-containing groups (i.e., peptidomannan and peptidoglucan) (Ashkenazy *et al.,* 1997). Das *et al.* (2010) studied the bioaccumulation properties of growing cells of *Pichia fermentans* MTCC 189 with synthetic dyes viz. Acid Blue 93, Direct Red 28 and Basic Violet 3 and reported that this microorganism was capable of bioaccumulating Basic Violet 3 up to 69.8% in the medium containing 10 mg/L of dye. Kumari and Abraham (2007) demonstrated that non-viable cells from *S. cerevisiae* and other microorganisms were able to remove some anionic reactive dyes through surface active groups. Das and Charumathi (2012) reported the removal of synthetic dyes from textile wastewater in a packed bed column with entrapped dead cells of *C. tropicalis* in a sodium alginate matrix through dye biosorption. Concerning true decolorization of the dyes (Table 2), in general, the yeast strains in consortia decolorized more dyes when compared to isolated strains. *C. parapsilosis* (HOMOGS20B) only removed color without adsorption for Blue Supra Everdirect FFRL, at 86%, and *Y. lipolytica* (HOMOGST27AB) for four dyes with decolorization above 90% (except for Basic Blue GRL-F200%). Strain *C. pseudoglaebosa* (LIIIS36B) was the strain that performed true decolorization for a wider range of dyes, especially for the Everzol class (above 90%). Consortium formed by *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) efficiently removed the color from more dyes than the other consortia, reaching very high values of true decolorization (above 80%), except for Basic Blue GRL-F200%. The other consortia could also remove efficiently the color of less dyes, with high percentages of true decolorization (above 82%). None of the studies in the literature were performed with the same strains and dyes studied in our research. Some strains of the yeast *Candida* spp. such as *C. tropicalis* (Yang *et al.,*2013), *C. oleophila* (Lucas *et al.,* 2006) and *C. krusei* G-1 (Yu and Wen, 2005) were reported to decolorize reactive dyes through biodegradation mechanisms. Aracagök and Cihangir (2013) reported that *Y. lipolytica* NBRC 1658 could effectively decolorize Reactive Black 5 through biodegradation. Dye decolorization by consortia is generally considered superior with advantages over the pure strains, complementing the

capacities of each isolated yeast (Patel *et al.,* 2012). Furthermore, different strains are believed to attack the dye molecule in different and/or complementary forms and positions, or strains may use decomposition products produced by the other strain for further dye molecule decomposition (Kurade *et al.,* 2015). Some consortia such as a bacterial-yeast consortium between *Brevibacillus laterosporus* and *Galactomyces geotrichum* have shown effectiveness in the biodegradation of Remazol Red (Kurade *et al.,* 2015). Our study suggests that for some dyes the use of a consortium resulted better than single strains, indicating possible combined decolorizing abilities. This happened for example with the consortium formed by *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) for Everacid Red N-BL. Biodegradation or true decolorization is considered a natural process of organic compounds transformation by living organisms, especially by microorganisms, which plays an essential role in the recycling of carbon and energy sources of ecosystems, through mineralization, generating less toxic products for the environment (Jadhav *et al.,* 2007).

Table 2.4 – Percentage of decolorization of the synthetic dyes by adsorption by the three yeast strains and consortia (100 mg/L) in NDM medium.

Dyes			Yeasts			Consortia			
Classes	Designation	λ_{max} (nm)	HOMOGS20B	HOMOGST27AB	LIIIS36B	HOMOGS20B + HOMOGST27AB	HOMOGS20B + LIIIS36B	HOMOGST27AB + LIIIS36B	HOMOGS20B + HOMOGST27AB + LIIIS36B
Everacid	Red N-2BL	511	64.0 ± 6,4^{b}	51.3 ± 10.3^{c}	66.7 ± 6.7^{b}	81.1 ± 10.5^{a}	NDA	85.1 ± 2.6^{a}	79.1 ± 7.9^{a}
	Yellow MR H|C	463	26.8 ± 8.8^{d}	67.9 ± 4.1^{b}	77.3 ± 1.4^{a}	74.2 ± 2.0^{a}	74.8 ± 6.8^{a}	NDA	56.2 ± 11.2^{c}
	Blue N-AFN	622	66.8 ± 5.8^{bc}	57.2 ± 13.7^{cd}	71.0 ± 10.4^{b}	67.0 ± 8.1^{bc}	54.3 ± 3.1^{d}	NDA	96.2 ± 1.0^{a}
Supra Everdirect	Red	532	73.4 ± 5.8^{bc}	71.2 ± 4.9^{c}	63.8 ± 6.4^{d}	78.1 ± 2.4^{b}	77.9 ± 4.8^{b}	84.4 ± 4.7^{a}	79.1 ± 7.2^{ab}
	Yellow RL	408	55.3 ± 4.9^{b}	NDA	NDA	96.8 ± 4.3^{a}	NDA	NDA	96.4 ± 2.8^{a}
	Blue FFRL	573	NDA	84.0 ± 3.9^{b}	NDA	89.6 ± 0.6^{a}	NDA	NDA	NDA
Basic	Yellow 200%	528	7.3 ± 0.7^{c}	1.9 ± 0.2^{e}	28.1 ± 8.1^{a}	8.1 ± 0.8^{c}	5.1 ± 0.5^{d}	15.9 ± 6.9^{b}	22.4 ± 2.2^{ab}
	Blue GRL-F200%	654	32.2 ± 3.2^{a}	NDA	5.0 ± 0.5^{c}	NDA	19.4 ± 1.9^{b}	NDA	NDA
Everzol	Red ED-3B	551	28.9 ± 6.2^{b}	99.7 ± 0.5^{a}	NDA	94.8 ± 6.8^{a}	NDA	94.5 ± 0.7^{a}	NDA
	Yellow LX	472	71.8 ± 0.6^{a}	NDA	NDA	NDA	NDA	NDA	NDA
	Navy ED	634	72.9 ± 2.9^{a}	NDA	NDA	NDA	NDA	NDA	NDA
	Blue L-ED	629	40.7 ± 5.7^{c}	90.8 ± 2.0^{b}	NDA	92.1 ± 5.5^{b}	99.3 ± 1.2^{a}	87.8 ± 1.6^{b}	86.3 ± 1.0^{b}
Procion	Red HE7B	551	68.8 ± 5.1^{de}	82.2 ± 4.2^{b}	78.5 ± 6.1^{bc}	94.6 ± 3.5^{a}	72.2 ± 8.0^{cd}	97.3 ± 1.0^{a}	62.9 ± 6.3^{e}
	Yellow HE 4R	455	85.9 ± 6.0^{ab}	91.3 ± 1.5^{a}	81.1 ± 3.4^{b}	91.3 ± 2.1^{a}	88.0 ± 6.6^{a}	NDA	86.9 ± 2.0^{a}
	Navy HEXL	622	45.3 ± 5.0^{d}	76.4 ± 2.3^{c}	96.1 ± 1.4^{a}	91.4 ± 1.8^{b}	90.7 ± 2.6^{b}	NDA	89.0 ± 0.9^{b}

In the "Designation" column, the original color of each dye is demonstrated before the decolorization tests, in order to compare with the resulting colors after the decolorization tests demonstrated along each corresponding row (lighter tone of the corresponding color). NDA – No Decolorization by Adsorption. Different superscript letters for significant differences within rows ($p < 0.05$).

Table 2.5 – Percentage of decolorization of the synthetic dyes by true decolorization by the three yeast strains and consortia (100 mg/L) in NDM medium.

Dyes			Yeasts			Consortia			
Classes	Designation	λ_{max} (nm)	HOMOGS20B	HOMOGST27AB	LIIIS36B	HOMOGS20B + HOMOGST27AB	HOMOGS20B + LIIIS36B	HOMOGST27AB + LIIIS36B	HOMOGS20B + HOMOGST27AB + LIIIS36B
Everacid	Red N-2BL	511	NTD	NTD	NTD	NTD	58.1 ± 4.8^{a}	NTD	NTD
	Yellow MR H|C	463	NTD	NTD	NTD	NTD	NTD	85.1 ± 1.1^{a}	NTD
	Blue N-AFN	622	NTD	NTD	NTD	NTD	NTD	91.7 ± 0.6^{a}	NTD
Supra Everdirect	Red	532	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Yellow RL	408	NTD	90.2 ± 2.1^{b}	81.8 ± 6.4^{c}	NTD	89.0 ± 0.7^{b}	99.3 ± 0.4^{a}	NTD
	Blue FFRL	573	86.3 ± 9.0^{a}	NTD	77.5 ± 3.1^{b}	NTD	86.7 ± 0.5^{a}	90.7 ± 2.8^{a}	90.0 ± 2.9^{a}
Basic	Yellow 200%	528	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Blue GRL-F200%	654	NTD	20.4 ± 2.2^{d}	NTD	91.2 ± 0.7^{a}	NTD	72.5 ± 4.1^{c}	82.2 ± 3.2^{b}
Everzol	Red ED-3B	551	NTD	NTD	95.2 ± 0.9^{b}	NTD	98.8 ± 1.4^{a}	NTD	88.7 ± 3.8^{c}
	Yellow LX	472	NTD	99.8 ± 0.3^{a}	94.1 ± 2.1^{b}	100.0 ± 0.0^{a}	100.0± 0.0^{a}	96.2 ± 2.4^{b}	95.1 ± 1.4^{b}
	Navy ED	634	NTD	89.5 ± 1.9^{cd}	91.7 ± 1.5^{b}	84.7 ± 5.1^{d}	98.4 ± 0.5^{a}	93.4 ± 2.7^{b}	90.4 ± 4.4^{bc}
	Blue L-ED	629	NTD	NTD	90.7 ± 1.3^{a}	NTD	NTD	NTD	NTD
Procion	Red HE7B	551	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Yellow HE 4R	455	NTD	NTD	NTD	NTD	NTD	96.6 ± 1.9^{a}	NTD
	Navy HEXL	622	NTD	NTD	NTD	NTD	NTD	91.2 ± 1.3^{a}	NTD

In the "Designation" column, the original color of each dye is demonstrated before the decolorization tests, in order to compare with the resulting colors after the decolorization tests demonstrated along each corresponding row (lighter tone of the corresponding color). NTD – No True Decolorization. Different superscript letters for significant differences within rows ($p < 0.05$).

2.3.3.2. Simulated effluents

Table 2.6 shows the decolorization of simulated effluents by yeast adsorption. *Y. lipolytica* (HOMOGST27AB) was the strain that could remove the color from a wider range of simulated effluents more efficiently with percentages of decolorization above 80%. The consortium formed by *C. parapsilosis* (HOMOGS20B) and *Y. lipolytica* (HOMOGST27AB) showed to be the most effective with percentages of decolorization above 85%. These higher percentages of decolorization by adsorption were observed mostly for Everacid Yellow MR H|C, Supra Everdirect Rubine BL and Blue 4BL; and Everzol Navy ED by isolated *Y. lipolytica* (HOMOGST27AB). Table 2.7 shows results by true decolorization. Everzol class and Basic Blue 41 were the more effectively decolorized effluents, with percentages of decolorization above 80%, in general. Strain *C. pseudoglaebosa* (LIIIS36B) reached 86-97% and the triple consortium reached 92-97% of decolorization. In fact, *C. pseudoglaebosa* (LIIIS36B) *and Y. lipolytica* (HOMOGST27AB) and some of the consortia that included these strains, seemed to perform true decolorization since they could remove color more efficiently. *C. pseudoglaebosa* (LIIIS36B) was the strain that inclusively could remove the color from the supernatant without adsorption of color in the cells, especially for the reactive dyes. Overall, yeasts and consortia were able to remove the color from the simulated effluents mainly by adsorption, which is similar to the findings of some authors with different yeast strains and effluents. Mahmoud (2016) reported the ability of *S. cerevisiae* to decolorize a real industrial effluent from textile industry in Egypt as well as a synthetic dye aqueous solution of Remazol Blue using a repeated-batch process (Mahmoud, 2016); a bacterial-yeast consortium composed by *B. laterosporus* MTCC 2298 and *G. geotrichum* MTCC 1360 was proposed to decolorize trough biodegradation two real textile effluents and a simulated synthetic effluent with promising results within 48 hours (Kurade *et al.,* 2017); Jadhav *et al.* (2010) studied the degradation of a textile effluent with reactive dyes by a bacterial consortium composed for *Pseudomonas* spp. in 48 hours (Jadhav *et al.,* 2010). Simulated dye effluents are used to mimic real industrial effluents. During the dyeing processes, there are losses of dyestuff and other chemicals, since not all dye can be attached to the fabric. Real textile effluents are very complex to degrade because they generally have strong colors, different pH and temperature, high salt concentrations, and quantities of suspended solids that could inhibit microorganisms (Solís *et al.,* 2012). High amounts of these dyed effluents are disposed into the environment causing serious problems directly to the aquatic life and this yeast-based approach could be potentially used in wastewater treatment plants to overcome this problem.

Table 2.6 - Percentage of decolorization of the simulated effluents by adsorption by the three yeast strains and consortia in NDM medium.

Effluents				Yeasts			Consortia			
Classes	**Designation**	**λ_{max} (nm)**	**Conc. (g/L)**	**HOMOGS20B**	**HOMOGST27AB**	**LIIIS36B**	**HOMOGS20B + HOMOGST27AB**	**HOMOGS20B + LIIIS36B**	**HOMOGST27AB + LIIIS36B**	**HOMOGS20B + HOMOGST27AB + LIIIS36B**
Everacid	**Red N-2BL**	517	20	NDA	43.5 ± 7.3^{a}	NDA	45.9 ± 4.5^{a}	NDA	NDA	16.7 ± 1.7^{b}
	Yellow MR H|C	455	5	87.0 ± 3.9^{a}	88.2 ± 1.6^{a}	64.2 ± 9.8^{b}	85.9 ± 5.1^{a}	57.6 ± 1.6^{bc}	58.4 ± 6.6^{bc}	53.8 ± 9.8^{c}
Supra Everdirect	**Rubine BL**	532	5	86.4 ± 7.6^{ab}	82,1 ± 4.0^{bc}	85.2 ± 4.3^{b}	94.0 ± 5.4^{a}	69.6 ± 10.0^{d}	78.8 ± 2.1^{cd}	69.9 ± 3.3^{d}
	Yellow RL	412	20	24.5 ± 8.7^{d}	45.8 ± 5.5^{c}	61.3 ± 2.3^{a}	47.0 ± 1.5^{c}	11.6 ± 2.7^{e}	51.1 ± 5.3bc	54.1 ± 1.7^{b}
	Blue 4BL	584	5	85.8 ± 2.7^{a}	84.2 ± 6.7^{ab}	66.4 ± 6.6^{e}	86.5 ± 2.7^{a}	77.8 ± 7.8^{bc}	69.1 ± 4.7^{de}	73.7 ± 5.9^{cd}
Basic	**Red 14**	559	1	NDA	NDA	NDA	NDA	NDA	NDA	NDA
	Yellow 28	499	5	3.9 ± 0.4^{d}	12.8 ± 5.5^{a}	4.5 ± 1.5^{d}	7.1 ± 2.5^{c}	7.6 ± 0.8^{bc}	2.5 ± 0.3^{e}	10.2 ± 2.7^{ab}
	Blue 41	654	1	NDA	NDA	NDA	NDA	NDA	NDA	NDA
Everzol	**Red ED-3B**	542	5	50.0 ± 3.2^{c}	70.5 ± 1.9^{b}	NDA	69.2 ± 1.4^{b}	80.0 ± 1.9^{a}	NDA	NDA
	Yellow LX	485	5	63.8 ± 2.2^{a}	68.1 ± 5.1^{a}	NDA	52.0 ± 7.7^{b}	NDA	NDA	NDA
	Navy ED	609	5	NDA	81.6 ± 3.2^{a}	NDA	NDA	NDA	86.5 ± 0.5^{a}	NDA
Disperse	**Red EFBL 200%**	434	20	12.2 ± 1.6^{a}	9.4 ± 0.9^{b}	1.7 ± 0.2^{d}	3.4 ± 0.3^{c}	1.7 ± 0.2^{d}	3.3 ± 0.3^{c}	3.1 ± 1.0^{c}
	Yellow Brown	433	20	0.4 ± 0.0^{e}	17.2 ± 2.3^{a}	4.7 ± 0.5^{d}	17.2 ± 3.8^{a}	12.7 ± 1.3^{b}	14.9 ± 1.5^{a}	9.5 ± 1.0^{c}
	Navy	654	20	8.0 ± 1.0^{bc}	8.8 ± 0.9^{b}	5.2 ± 0.5^{d}	14.1 ± 6.0^{a}	7.1 ± 0.5^{c}	2.4 ± 0.2^{e}	10.7 ± 1.1^{a}
Samovat	**Red**	392	20	NDA	18.5 ± 1.9^{b}	NDA	NDA	NDA	9.0 ± 0.9^{c}	30.9 ± 2.3^{a}
	Yellow	419	20	0.0 ± 0.0^{e}	11.4 ± 1.1^{d}	0.0 ± 0.0^{e}	17.0 ± 4.9^{c}	28.2 ± 5.7^{b}	12.3 ± 1.2^{cd}	52.5 ± 5.7^{a}
	Blue	433	20	0.0 ± 0.0^{e}	12.3 ± 3.6^{c}	0.0 ± 0.0^{e}	14.2 ± 5.6^{bc}	19.1 ± 3.5^{b}	4.4 ± 2.4^{d}	28.2 ± 3.7^{a}

In the "Designation" column, the original color of each dye is demonstrated before the decolorization tests, in order to compare with the resulting colors after the decolorization tests demonstrated along each corresponding row (lighter tone of the corresponding color). NDA – No Decolorization by Adsorption. Different superscript letters for significant differences within rows ($p < 0.05$).

Table 2.7 - Percentage of decolorization of the simulated effluents by true decolorization by the three yeast strains and consortia in NDM medium.

Effluents				Yeasts			Consortia			
Classes	**Designation**	**λ_{max} (nm)**	**Conc. (g/L)**	**HOMOGS20B**	**HOMOGST27AB**	**LIIIS36B**	**HOMOGS20B + HOMOGST27AB**	**HOMOGS20B + LIIIS36B**	**HOMOGST27AB + LIIIS36B**	**HOMOGS20B + HOMOGST27AB + LIIIS36B**
Everacid	**Red N-2BL**	517	20	6.6 ± 0.7^{c}	NTD	1.5 ± 0.2^{d}	NTD	13.7 ± 2.4^{b}	16.8 ± 1.7^{a}	NTD
	Yellow MR H|C	455	5	NTD	NTD	NTD	NTD	NTD	NTD	NTD
Supra Everdirect	**Rubine BL**	532	5	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Yellow RL	412	20	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Blue 4BL	584	5	NTD	NTD	NTD	NTD	NTD	NTD	NTD
Basic	**Red 14**	559	1	9.6 ± 3.2^{d}	14.6 ± 1.5^{c}	4.4 ± 0.4^{e}	30.9 ± 5.3^{a}	23.9 ± 3.1^{b}	27.5 ± 1.6^{a}	26.9 ± 4.9^{ab}
	Yellow 28	499	5	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Blue 41	654	1	87.6 ± 3.7^{d}	86.2 ± 2.4^{d}	89.5 ± 4.4^{cd}	98.5 ± 0.9^{ab}	99.9 ± 1.7^{a}	99.4 ± 1.1^{a}	92.9 ± 6.3^{bc}
Everzol	**Red ED-3B**	542	5	NTD	NTD	90.4 ± 2.4^{b}	NTD	NTD	76.1 ± 1.0^{c}	96.0 ± 2.6^{a}
	Yellow LX	485	5	NTD	NTD	86.1 ± 8.6^{a}	NTD	84.7 ± 5.3^{a}	62.7 ± 6.3^{b}	67.2 ± 4.0^{b}
	Navy ED	609	5	82.7 ± 2.5^{c}	NTD	97.1 ± 1.3^{a}	85.0 ± 2.8^{bc}	87.0 ± 0.7^{b}	NTD	97.3 ± 2.0^{a}
Disperse	**Red EFBL 200%**	434	20	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Yellow Brown	433	20	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Navy	654	20	NTD	NTD	NTD	NTD	NTD	NTD	NTD
Samovat	**Red**	392	20	3.4 ± 1.0^{c}	NTD	20.5 ± 2.1^{a}	16.4 ± 3.2^{b}	13.0 ± 4.1^{b}	NTD	NTD
	Yellow	419	20	NTD	NTD	NTD	NTD	NTD	NTD	NTD
	Blue	433	20	NTD	NTD	NTD	NTD	NTD	NTD	NTD

In the "Designation" column, the original color of each dye is demonstrated before the decolorization tests, in order to compare with the resulting colors after the decolorization tests demonstrated along each corresponding row (lighter tone of the corresponding color). NTD – No True Decolorization. Different superscript letters for significant differences within rows ($p < 0.05$).

2.3.4. Enzymatic activity

During this work it was possible to detect activity for the intracellular enzymes oxidoreductase and tyrosinase (Tyr), but no activity was detected for azoreductase. Yeasts *C. pseudoglaebosa* (LIIIS36B) and *Y. lipolytica* (HOMOGST27AB) presented activity for both oxidoreductase and tyrosinase and yeast *C. parapsilosis* (HOMOGS20B) only presented activity for oxidoreductase (Figure 2.3). Kurade *et al.* (2017) mentioned that intracellular tyrosinase was found in the bacterial-yeast consortium between *Brevibacillus laterosporus* MTCC 2298 and *Galactomyces geotrichum* MTCC 1360 and could be responsible for the biodegradation of two real textile effluents and a synthetic effluent. Also, Saratale *et al.* (2009) found that the yeast *Trichosporon beigelii* NCIM-3326 produced azoreductase and NADH-DCIP reductase and proposed that these enzymes would be responsible for the degradation of the reactive azo dye Navy blue HER. In our study, *C. pseudoglaebosa* (LIIIS36B) and *Y. lipolytica* (HOMOGST27AB) presented better dye decolorization capacity, when compared with *C. parapsilosis* (HOMOGS20B), which only presented activity for oxidoreductase enzyme. Nonetheless, we cannot conclude that the decolorization capacity of yeasts reported in the present study is related to these enzymes. None of the yeast strains in the study presented activity for the extracellular enzymes tested, namely Manganese independent Peroxidase (MiP), Manganese dependent Peroxidase (MnP), Lignin independent Peroxidase (LiP), veratryl alcohol and laccase. These extracellular enzymes are more frequently detected in filamentous fungi (Abadulla *et al.,* 2000; Heinfling *et al.,* 1998) and this fact may explain the obtained result. Nevertheless, some authors reported that some yeast strains are capable of decolorizing dyes through the production of such enzymes: Jadhav *et al.* (2008) and Martorell *et al.* (2017) found the production of LiP and MnP, among other enzymes, by *Galactomyces geotrichum* MTCC 1360 and *C. boidinii* MM 4035, which are possibly responsible for decolorizing Methyl Red and Reactive Black 5, respectively.

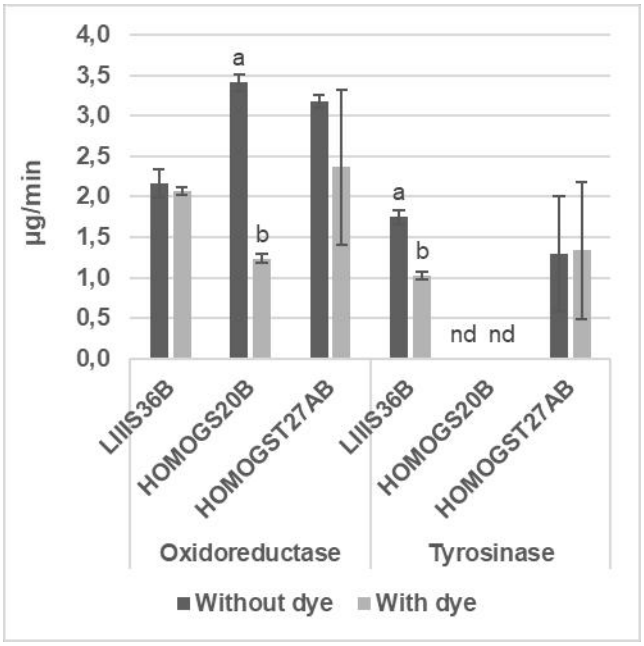


Figure 2.3 - Oxidoreductase and Tyrosinase enzymatic activities (µg/min) for the three yeast strains, grown with and without the presence of dye (Yellow Everzol, 100 mg/L). Different superscript letters for significant differences between dye conditions ($p < 0.05$). nd = not detected.

Chromophores from dyes are believed to be attacked directly by enzymes. Dyes like azo dyes are generally cleaved into aromatic amines by reductive enzymes such as azoreductase, occasionally produced by yeasts. The cleavage of the azo groups is often responsible for biodegradation. However, the success of the decolorization often depends on the complexity of the structure of each dye (Jadhav *et al.,* 2007). Still, in this experiment, incubation of the yeasts in the presence of the Yellow Everzol dye seemed to not stimulate the production of the enzymes tested, with only significant differences in the case of oxidoreductase for *C. parapsilosis* (HOMOGS20B) and tyrosinase for *C. pseudoglaebosa* (LIIIS36B) (Figure 3). Thus, since the enzyme extracts obtained in this assay did not decolorize the dye tested when applied directly, it is possible that the ability for decolorization of these yeasts could be due to the presence of constitutive enzymes or could be due to the result of a complex metabolism that depends on living yeast cells and may involve various enzymes and metabolic processes. Therefore, further studies should be conducted to understand and clarify the mechanisms behind the decolorization process.

2.4. CONCLUSIONS

Tested strains, especially *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) both isolated as in consortium, were effective in removing above 50% color, both by adsorption or biodegradation, from commercial synthetic dyes and simulated textile effluents, depending on the different classes of dye, at higher concentrations than commonly used in the textile industry. Reactive dyes were the class that in general seemed to be removed more efficiently by true decolorization. These selected yeasts may be used as a potential biotechnological tool to treat wastewater resulting from the textile industries instead of classical chemical treatments, which could be an advantage and a promising solution for this environmental worldwide problem, contributing to the circular economy and eco-sustainability. Nevertheless, the mechanisms behind the decolorization process by these yeasts need to be clarified.

SUPPLEMENTARY MATERIAL

Diagrams of the preparation of the simulated effluents

1.1. Simulated textile effluents

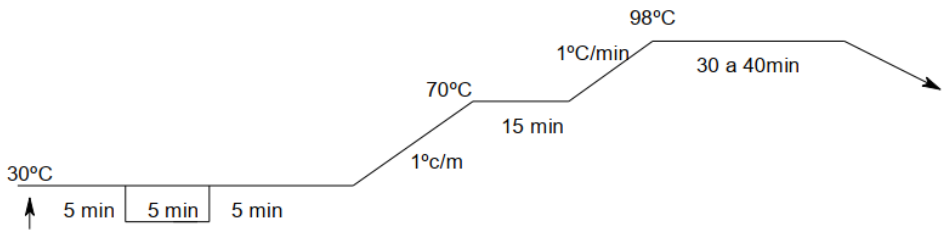
1.1.1. Everacid

1) Dyeing:

a) 1 g/L equalizer

0.5-1.5 g/L acid donor agent

Desired concentration of dye (%)



Equalizer

Acid donor agent

pH 6-6.5

b) Wash with water at 50 ºC – 10 min.

c) Cold wash – 10 min.

2) Fixation:

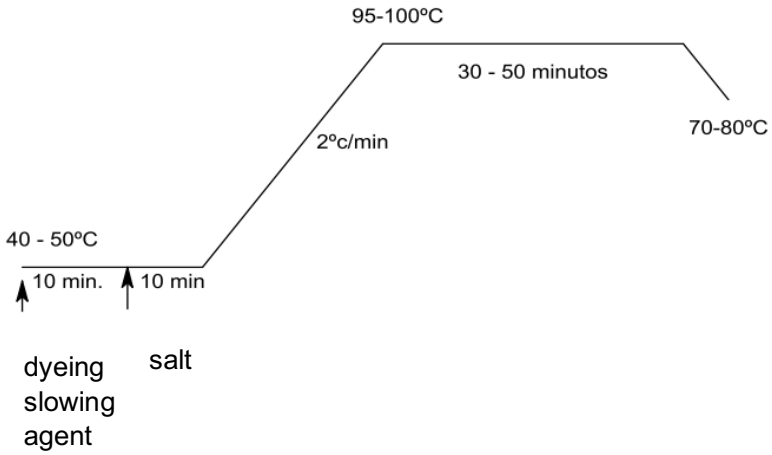
- 2-3% Fixer solution
- 1 g/L acid buffer
- pH - 4.5-5
- 20 min at 70 ºC

3) Softening:

- 2% softening agent
- 1 g/L acid buffer
- pH - 4.5-5
- 20 min at 40 °C

1.1.2 Everdirect Supra

1) Desired concentration of dye (%)
   Desired concentration of salt (g/L)
   0.2 – 0.5 g/L dyeing slowing agent



1.1) Cold wash – 10 min

1.2) Cold wash – 10 min

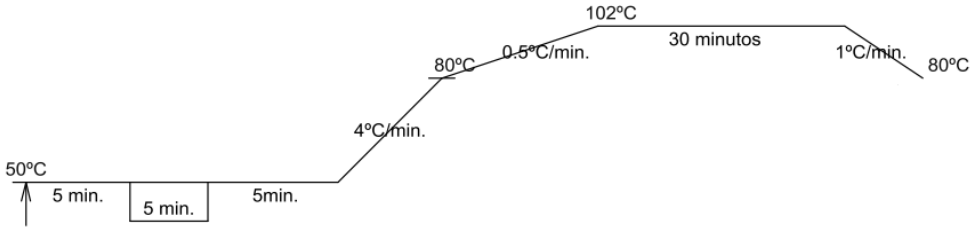
1.3) <u>Fixation:</u>

- 1 – 2% Fixation agent
- 1 g/L acid buffer
- pH – 4.5 – 5
- 10 min at 60 ºC

1.1.3 Basic

1.) Dyeing

- 1 – 2 g/L dyeing slowing agent
- 1.5 g/L acid buffer
- 1 g/L dispersant agent
- 5% of sodium sulfate
- Desired concentration of dye (%)



slowing agent Dyes

sodium sulfate

dispersant agent

acid buffer

pH 4.5 - 5

1) Reducing wash:

- 1 g/L detergent
- 1 g/L sodium carbonate
- 1.5 g/L of sodium hydrosulphite

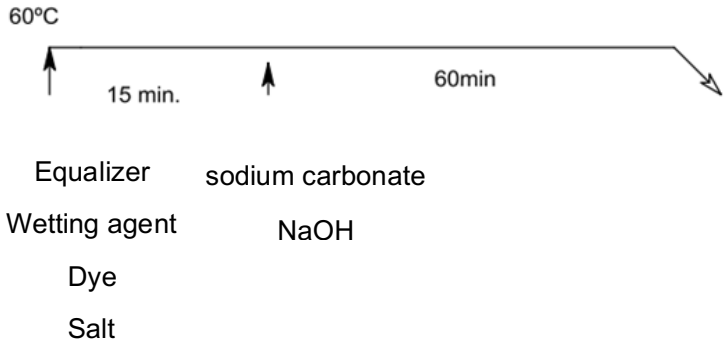
- 15 min at 60 °C

- Wash at 50 ºC – 10 min
- Wash at 50 ºC – 10 min

1.1.4 Everzol

1)

- 1 g/L equalizer
- 0.2 g/L wetting agent
- Desired concentration of dye (%)
- Desired concentration of salt (g/L)
- 5 g/L of sodium carbonate
- Desired concentration of NaOH 38 ºBé (mL/L)



1.1) Cold wash – 10 min

1.2) Neutralization 50 ºC with 0.5 g/L – 1 g/L product – 10 min

1.3) Soaping

- 1 g/L soaping agent
- 15 min at 100 °C

1.4) Cold wash

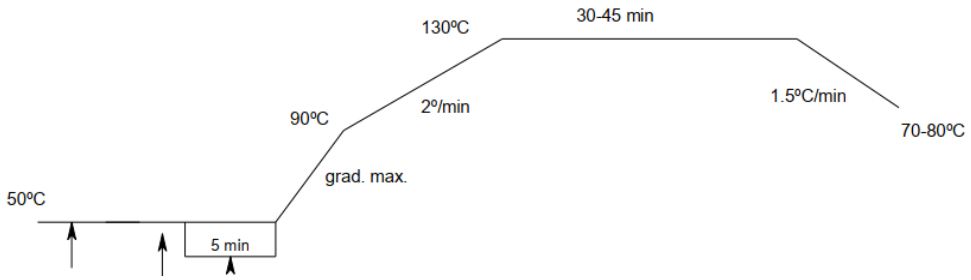
1.1.5 Disperse

1) Pre-wash

- 1.5 g/L detergent
- 1 g/L solubilizer solution
- 20 min at 75 ºC

2) Dyeing

- 1 g/L dispersant/equalizer agent
- 1 g/L dispersant
- 1 -1.5 g/L acid buffer
- (pH – 4.5)
- Desired concentration of dye (%)



Dispersant Dye

Dispersant agent

Acid buffer

3) Reducing wash

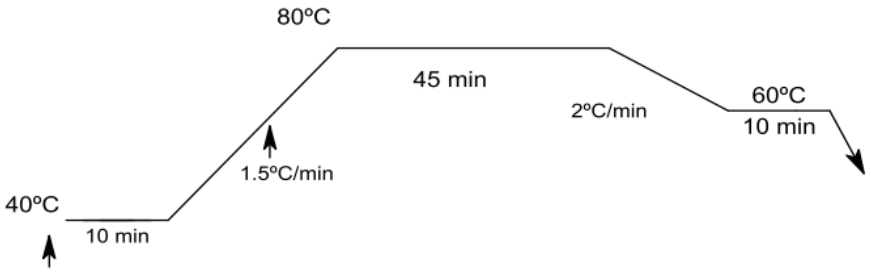
- 4 g/L sodium hydrosulphite
- mL/L sodium hydroxide 38 ºBé
- 1 g/L Toxal NP/5
- 15 min at 80 ºC

- wash/ neutralize
- softening

1.1.6 Samovat

1) Dyeing

- 1 g/L sequestering agent
- 1 g/L dispersant agent
- 0.5 g/L equalizer agent
- Desired concentration of dye (%)
- Desired concentration of of sodium hydrosulphite (g/L)
- Desired concentration of NaOH 38 ºBé (mL/L)



Sequestering

Dispersant

Equalizer

Dye

NaOH 38 ºBé

sodium hydrosulphite

1) Wash
2) Oxidation

- mL/L H_2O_2 200 vol.
- pH: 5-5.5 (with acetic acid)
- 15 min at 55-60 ºC

3) Soaping

- 1 g/L dispersant agent
- g/L sodium carbonate
- 15 min at 100 °C

- Wash/dry

CHAPTER 3

REMOVING COLOR WHILE LOWERING TOXICITY: THE CASE FOR DECOLORIZATION OF TEXTILE DYES AND SIMULATED EFFLUENTS WITH YEASTS

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3.1. INTRODUCTION

Environmental pollution is currently on the global agenda. Modern industrialization is responsible for the formation of a wide range of environmental pollutants. Textile industry, in particular, produces high volumes of effluents that contain a complex mixture of synthetic dyes (Dellamatrice *et al.,* 2017). Textile dyes are very difficult to degrade, and complex combinations of chemicals are needed for dyeing processes (Croce *et al.,* 2017; Dellamatrice *et al.,* 2017). Specifically, azo dyes are one of the most widespread class of synthetic dyes, being applied in various areas, from the textile to paper and additives industries (Al-Tohamy *et al.,* 2020; Ali *et al.,* 2021). These dyes are characterized by the presence of one or more azo bonds (-N=N-) with aromatic rings, which through different substitutions on the aromatic nucleus give them a range of diverse structures (Ali, 2010). Thus, azo dyes have a versatile and recalcitrant nature that makes them very difficult to eliminate (Ali, 2010). These wide range of colored compounds present in wastewaters may be responsible for toxic, mutagenic and carcinogenic effects, being persistent in water and having also a huge impact on photosynthesis of aquatic ecosystems (Mahmoud, 2016; Kristanti *et al.,* 2016; Paz *et al.,* 2017). As most of the dyes are soluble in water, they are easily absorbed through the skin and by inhalation, increasing the risks of cancer and other diseases that could cause irreversible damages to liver, kidneys and nervous system (Sudha *et al.,* 2014).

Classic wastewater treatment methods are considered to have low efficacy, are usually expensive and may generate toxic by-products (Saratale *et al.,* 2011; Mahmoud, 2016; Kurade *et al.,* 2017). In contrast, biological treatments are a promising method to potentiate the decolorization and degradation of dyes in textile wastewaters, possibly leading to mineralization of organic pollutants (Imran *et al.,* 2015; Paz *et al.* 2017). Over several years, numerous species of organisms have been tested for the decolorization and degradation of several dyes (Saratale *et al.* 2011; Solís *et al.* 2012; Paz *et al.* 2017). Bacteria are the most studied, together with filamentous fungi, but algae and yeasts might play an important role at treating textile effluents (Imran *et al.* 2015; Martorell *et al.,* 2017). Yeasts have an added value in the process of dye decolorization as they are easy to grow and maintain and are capable of removing color both through adsorption and through the production of a wide variety of enzymes such as laccase, azoreductase, manganese peroxidase (MnP), lignin peroxidase (LiP), NADH-DCIP reductase, among others (Solís *et al.,* 2012; Zaharia and Suteu, 2012; Kurade *et al.,* 2015; Guo *et al.* 2020; Ali *et al.* 2021). Different yeast strains such as *Galactomyces* spp. (Kurade *et al.* 2017; Guo *et al.,* 2019), *Saccharomyces cerevisiae* (Mahmoud, 2016), *Trichonosporon* spp. (Martorell *et al.,* 2012), *Pichia pastoris* (Saravanan *et al.,* 2021) and several strains of *Candida* spp. i.e. *C. boidinii*, *C. rugosa*, *C. tropicalis*, *C. pseudoglaebosa* and *C. parapsilosis* as well as *Yarrowia lipolytica* have shown to decolorize

reactive dyes (Yang *et al.,* 2013; Martorell *et al.,* 2017; Wang *et al.,* 2021; Chapter 2 of this thesis). Dyes such as Reactive Black 5, Direct Violet, Reactive Brilliant Red K2BP, Reactive Yellow 84 and Reactive Yellow 141were also successfully degraded by yeasts (Jadhav *et al.,* 2008; Kurade *et al.,* 2012; Singh, 2015).

Nevertheless, biological degradation of synthetic dyes may lead to the formation of various metabolites, such as aromatic amines, potentially exhibiting toxicity to the environment if discharged into the ecosystems (Ali *et al.,* 2021). To access its toxicity, tests using organisms from different trophic levels should be used (Raghukumar *et al.,* 2008; Khan *et al.,* 2013). Bacteria and algae could be used to represent primary producers whereas primary and secondary consumers may be represented by aquatic invertebrates (e.g., *Daphnia magna*) and aquatic vertebrates (e.g., zebrafish), respectively. Higher plants such as lettuce seeds (*Lactuca sativa*) are also used for the determination of toxicity in environmental samples (Priac *et al.,* 2017).

In this study, three selected yeast strains, previously isolated from a wastewater treatment plant, were assessed for their capacity of degradation of some reactive, direct, and basic dyes, individually and in simulated effluents. For the best of our knowledge, this is the first time that the toxicity of the resulting treated streams from this wide range of dyes and simulated effluents, containing the end-products of decolorization resulting from these yeasts metabolism, was determined to evaluate their safety when discharged into the environment.

3.2. MATERIAL AND METHODS

3.2.1. Biological material

Three yeast strains previously isolated from wastewater treatment plants: *Candida parapsilosis* (HOMOGS20B - now onward 20B), *Yarrowia lipolytica* (HOMOGST27AB - now onward 27AB), and *Candida pseudoglaebosa* (LIIIS36B - now onward 36B), were used to perform decolorization of dyes and simulated effluents in test. These yeasts were selected according to the results from the previous Chapter.

3.2.2. Culture media and Dyes

The Normal Decolorization Media (NDM) and Normal Solid Decolorization Medium (NSDM), were prepared as stated in Chapter 2 (2.2.) and used for culture maintenance. Cultures were inoculated at 3% v/v at 30 ºC for 48 hours and tested under sterile conditions. Minimum Decolorization Media (MDM) – NDM without yeast extract – was used in the decolorization assays for HPLC analysis to eliminate the interference of yeast extract components on the analysis. Glucose (20 g/L) was used as culture medium for the toxicity tests to eliminate possible interferences of the NDM in the assays.

Solutions of commercial synthetic dyes (kindly provided by AQUITEX, SA): Everzol group – Red ED-3B (Evz-Red), Yellow LX (Evz-Yellow), Navy ED (Evz-Navy); Supra Everdirect Blue FFRL (SEd-Blue) and Basic Blue GRL-F200% Itocryl (B-Blue), were prepared in deionized water at 4 g/L. The respective simulated effluents (Evz-Red$_{ef}$, Evz-Yellow$_{ef}$, Evz-Navy$_{ef}$, SEd-Blue$_{ef}$ and B-Blue$_{ef}$ - acronym used hereinafter in this chapter) were prepared at 3% (m/v) by the company AQUITEX, SA (Porto, Portugal) by simulating the industrial processes of dying at a lab scale, as described in Chapter 2 (2.2. and 2.2.3.). At the end of the decolorization experiments, the dye was no longer detected.

3.2.3. Decolorization assays

Fresh overnight pre-inoculums of the yeast strains were prepared in NDM and incubated at 30 °C. Erlenmeyer flasks with MDM (for HPLC assays) or with only glucose (20 g/L) as a growth medium, containing each dye at 25 mg/L or dyed simulated effluents (2.5 g/L) were inoculated with 3% of each yeast pre-inoculum and incubated at 25 °C/100 rpm/48 hours (in duplicate). Biotic controls without dyes/effluents were established at the same conditions. The supernatant with the resulting decolorized products was recovered by centrifugation (5000 rpm/10 min) and frozen at -20 °C until used for further analysis. Dye decolorization was monitored using Specord S600 (Analytik Jena, Germany) spectrophotometer using the supernatants obtained from each decolorization assay. The percentage of the decolorization was calculated using Equation 1 (Chapter 2- 2.2.5.).

3.2.4. Analysis of the decolorization streams

Analysis of the products resulting from yeasts decolorization in MDM was carried out based on the method of Mata *et al.* (2015) with some modifications. Briefly, analysis was conducted on HPLC-DAD (Water Series 600, Mildford MA. USA) with a Kromasil® C18 column, 250 x 4.6 mm i.d. 5 µm particle size and 100 Å pore size with a guard column (Kromasil® 3.0 x 4.6 mm). The mobile phase was composed of sodium phosphate buffer (0.7 mL/min – 25 mM, pH 5.5) and acetonitrile (Merck, pure grade) running on a 30-min linear gradient from 100:0 to 50:50 (v/v), followed by a 5-min linear gradient up to 15:85 (v/v) and ending with a step back to 100:0 for further 5-min. All dye standard solutions were prepared in ultra-pure distilled water and run for their respective peak identification. Each sample, biotic control and standard solution were properly filtered (0.22 µm filter) and analyzed in triplicate.

3.2.5. Toxicity tests

3.2.5.1. TOXI-ChromoTest™

TOXI-ChromoTest™ kit (Version 4.0, EBPI, Canada) was used for the determination of the supernatant toxicity, following the manufactures directions. This test is a rapid, sensitive, and cost-effective bacterial-based bioassay kit for the determination of toxicity in environmental samples based on the ability of toxic substances in test to inhibit the de novo synthesis of an inducible β-galactosidase enzyme in a highly permeable mutant of *Escherichia coli*. According to the kit directions, lyophilized bacteria was rehydrated, for 15 minutes at room temperature, in a cocktail containing a specific inducer of β-galactosidase enzyme and essential factors required for a successful recovery from their stressed condition. Then, the bacteria (100 µL) were directly exposed in a 96-well microplate filled with the controls (4 µg/mL of mercury chloride, diluent solution, simulated effluents, glucose, and yeasts growth) and test samples in glucose culture medium (diluted at 50%), during an incubation period of 90 minutes at 37 ºC. After that, 100 µL of blue chromogen was added to the wells and incubated again at 37 ºC for 30 min. Each sample was tested in duplicate. Results were established by blue color development that indicated nontoxicity, whereas no color development indicated toxicity of the substances in test and were interpreted qualitatively by naked eye and quantitatively with a microplate reader (Thermo Scientific Multiskan Go, Finland) (600 nm). Samples below 50% are considered to have “low toxicity”, and above 50% are considered “moderate toxicity”. If no blue color is developed samples are considered to have “high toxicity” (100%). Therefore, the darker the blue color developed the lower the % of toxicity. Samples in glucose culture medium (diluted at 50%) were tested. Results were interpreted qualitatively by naked eye and quantitatively with a microplate reader (Thermo Scientific Multiskan Go, Finland) (600 nm) with the following equation:

$$Toxicity\ (\%) = 100 - \left(\frac{Abs\ sample}{Abs\ control}\right) x\ 100$$

(Equation 2)

According to Equation 2, samples below 50% are considered to have “low toxicity”, and above 50% are considered “moderate toxicity”. If no blue color is developed, samples are considered to have “high toxicity” (100%). Therefore, the darker the blue color developed the lower the % of toxicity.

3.2.5.2. DAPHTOXKIT F

DAPHTOXKIT F (MicroBioTest Inc., Belgium) was performed to determine the acute toxicity of the samples. This test is a fast and sensitive assay for acute toxicity screening, based on

immobility or mortality of freshwater crustacean *Daphnia magna* when in contact with the toxicants. The kit protocol was followed according to the manufacturer's directions and the OECD guideline 202 (OECD 2004). Briefly, *Daphnia magna* ephippia (dormant eggs) from a commercial kit, were hatched under continuous illumination of 6000 LUX during 72 hours at 20-22 ºC, in a proper standard aerated freshwater. Then, 2 hours before doing the tests, neonates were fed with spirulina powder and after that, they were transferred to the test wells filled with 10 mL of the samples and controls to be tested. Due to the highly sensitivity of these organisms to the culture medium (NDM and MDM), glucose culture medium was used (20 g/L) to eliminate the influence of the toxicity of these solutions, and the assay was prepared using 50% of the decolorization streams. Samples of the end products obtained only with glucose (diluted at 50%) were tested. With a micropipette, 20 actively swimming neonates were transferred to the first well (negative control row) and then exactly 5 *Daphnia* neonates from that were transferred into the 4 wells of the corresponding row (replicas). The multiwell plate was covered with parafilm and incubated at 20 ± 2 ºC in darkness. Results were recorded as alive, immobilized, or dead neonates, at 48 hours after incubation.

3.2.5.3. Phytotoxicity test – Lactuca sativa

Toxicity in organisms from a higher trophic level was determined through the germination test of *Lactuca sativa* seeds according to the Priac *et al.* (2017) method. Seeds were washed with bleach prior to use and were rinsed with sterile distilled water for preventing mold formation. In triplicate, empty Petri dishes were prepared with 2 filter paper disks that were wetted with 3 mL of the respective test solution, ensuring no bubble formation. Then, with a tweezer, 15 seeds were well distributed on each plate. Closed plates were incubated at room temperature (20 °C) in a dry and dark place for 7 days. The supernatant obtained in glucose-containing medium (20 g/L) was tested after being diluted at 50%, as well as the original dyed simulated effluents. Supernatant resulting from biotic controls, glucose, and dH_2O was performed as test controls. Results were obtained by counting the number of germinated seeds and measurement of their root length (carefully with a ruler) from the node (thicker region of transition between the root and the stem) to the root apex.

3.2.5.4. Mutagenicity assay – Ames Test

MOLTOX® *Salmonella* Mutagenicity Assay Kit (TRINOVA BIOCHEM, Germany) was used to test the potential mutagenicity of obtained decolorization streams. This kit replicates the Ames assay for mutagenicity research. According to the kit guidelines, the *Salmonella typhimurium* strain TA98 used is a mutated strain for the histidine operon (His-). This mutation made them incapable of synthesizing histidine, requiring exogenous histidine. In addition, *S. typhimurium* strain TA98 has also altered cell walls that increase the cell's permeability to certain high molecule-weight materials, such as dye molecules. Thus, exposure to mutagens may result in

genetic reversions in the histidine operon resulting in the restoration of the wild type which can synthesize histidine and the growth of the bacterial colonies. Simulated effluents (Evz-Navy$_{ef}$, Evz-Yellow$_{ef}$, Evz-Red$_{ef}$, SEd-Blue$_{ef}$, and B-Blue$_{ef}$) and samples of the streams after decolorization with the selected yeasts, using glucose (20 g/L) in the culture medium (36B_Evz-Navy$_{ef}$, 36B_Evz-Yellow$_{ef}$, 36B_Evz-Red$_{ef}$, 36B_SEd-Blue$_{ef}$, 36B_B-Blue$_{ef}$, 20B_Evz-Navy$_{ef}$, 20B_B-Blue$_{ef}$, 27AB_B-Blue$_{ef}$), were tested. Abiotic controls of glucose-containing-medium and supernatant of biotic controls obtained after yeasts grown with glucose were also tested. Results were obtained by counting the colonies that were able to grow on the kit-specific agar plates.

3.2.6. Statistical analysis

Results obtained in the experiments were expressed in terms of means (average) and standard deviation (S.D.). Normality distribution was first verified, and one-way ANOVA with Tukey post hoc was performed, with a level of significance of 0.05, using IBM® SPSS® Statistics 26 (SPSS Inc., IBM Corporation, NY, USA).

3.3. RESULTS AND DISCUSSION

3.3.1. Decolorization capacity of selected yeasts and analysis of the decolorization streams

The capacity of selected yeasts *C. parapsilosis* (HOMOGS20B), *Y. lipolytica* (HOMOGST27AB), and *C. pseudoglaebosa* (LIIIS36B) to decolorize the dyes and dyed simulated effluents was carried out in MDM. These yeasts were selected from a wastewater treatment plant so they presented advantages since they are already accustomed to these environments and are easy to maintain and perform the decolorization, which could be used as a new alternative to other microorganisms and enzymes. The percentage of decolorization of the dyes (Evz-Navy, Evz-Yellow, Evz-Red, SEd-Blue, and B-Blue) and respective simulated effluents (Evz-Navy$_{ef}$, Evz-Yellow$_{ef}$, Evz-Red$_{ef}$, SEd-Blue$_{ef}$ and B-Blue$_{ef}$) are shown in Figure 3.1.

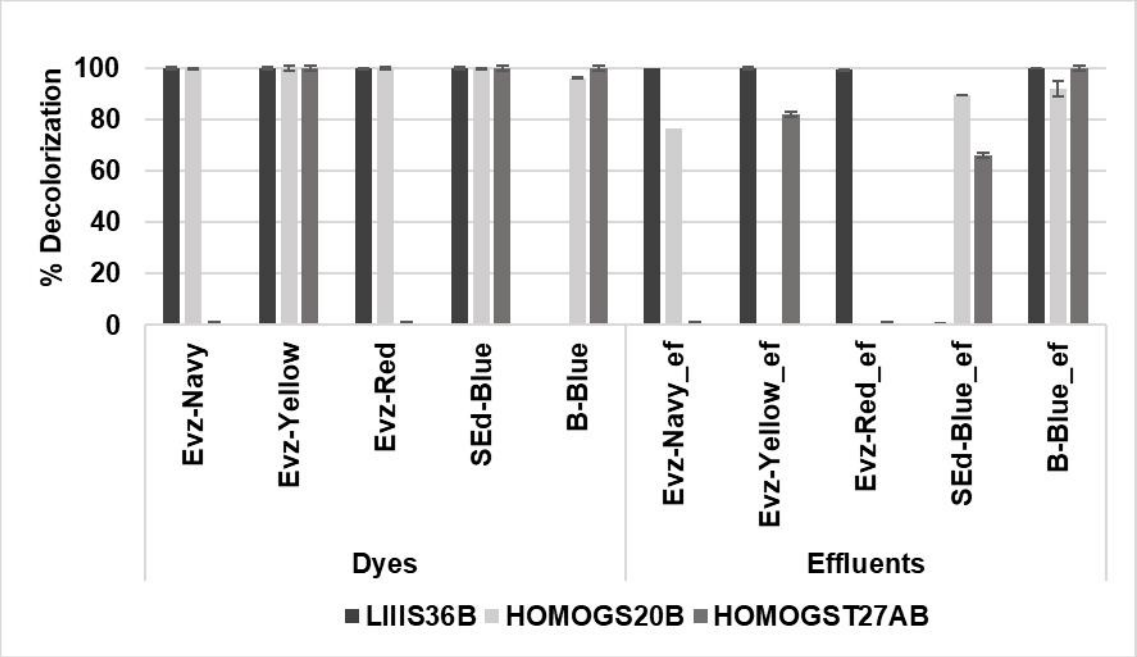

Figure 3.1 – Decolorization of dyes and dyed simulated effluents by yeasts *C. pseudoglaebosa* (LIIIS36B), *C. parapsilosis* (HOMOGS20B), and *Y. lipolytica* (HOMOGST27AB) in MDM.

In general, in this study, dyes were decolorized at a high percentage (> 90%) for almost all yeasts in study. However, B-Blue dye was not decolorized by *C. pseudoglaebosa* (LIIIS36B) and the same occurred for Evz-Navy and Evz-Red dyes by *Y. lipolytica* (HOMOGST27AB). The decolorization was less efficient in the simulated effluents: color from SEd-Blue$_{ef}$ was not removed by *C. pseudoglaebosa* (LIIIS36B), whereas Evz-Yellow$_{ef}$ and Evz-Red$_{ef}$ were not decolorized by *C. parapsilosis* (HOMOGS20B) and color from Evz-Navy$_{ef}$ and Evz-Red$_{ef}$ was not removed by *Y. lipolytica* (HOMOGST27AB). Among the three candidates, *C. pseudoglaebosa* (LIIIS36B) was the most effective, completely decolorizing four of the five simulated effluents. These could be due to the different characteristics and chemical structures of each dye and simulated effluent and the capacity/adaptation that each yeast strain had to degrade the target compounds in the study. In fact, dye decolorization effectiveness is deeply influenced by the complexity of the dye structure and by the specifications of the enzymes that each microorganism possesses as well as the abiotic conditions (Solís *et al.,* 2012). The fact that lower color removal occurred in the simulated effluents could be due to the other constituents of the simulated effluents that are generally used in the textile industry, which were not present in the dye solutions but that can impair the strain efficiency. Components such as dispersants, equalizers, fixation and wetting agents, detergents, sodium carbonate, sodium sulfate, and salt, are needed to improve and increase dyeing textile quality processes and could be responsible for these differences (Pinheiro *et al.,* 2004; Guo *et al.,* 2020; Wang *et al.,* 2021). The presence of these compounds contributes to the increase of recalcitrance of the effluents through inhibiting organisms' growth and development (Ben Mansour *et al.,* 2012; Guo *et al.,* 2020; Danouche *et al.,* 2021).

The yeast's ability to decolorize dyes and simulated effluents without another external carbon source rather than the dyes and simulated effluents was also tested. These tests were performed to minimize the interferences that other medium components could have in toxicity tests. However, since these yeasts could not decolorize dyes and effluents without a complementary carbon source (data not shown), glucose (20 g/L) was added to the culture medium. Even though yeasts were not able to totally decolorize any of the dyes tested, the decolorization of the simulated effluents occurred, even if at a lower rate than when using the media containing nutritional components (Figure 3.2).

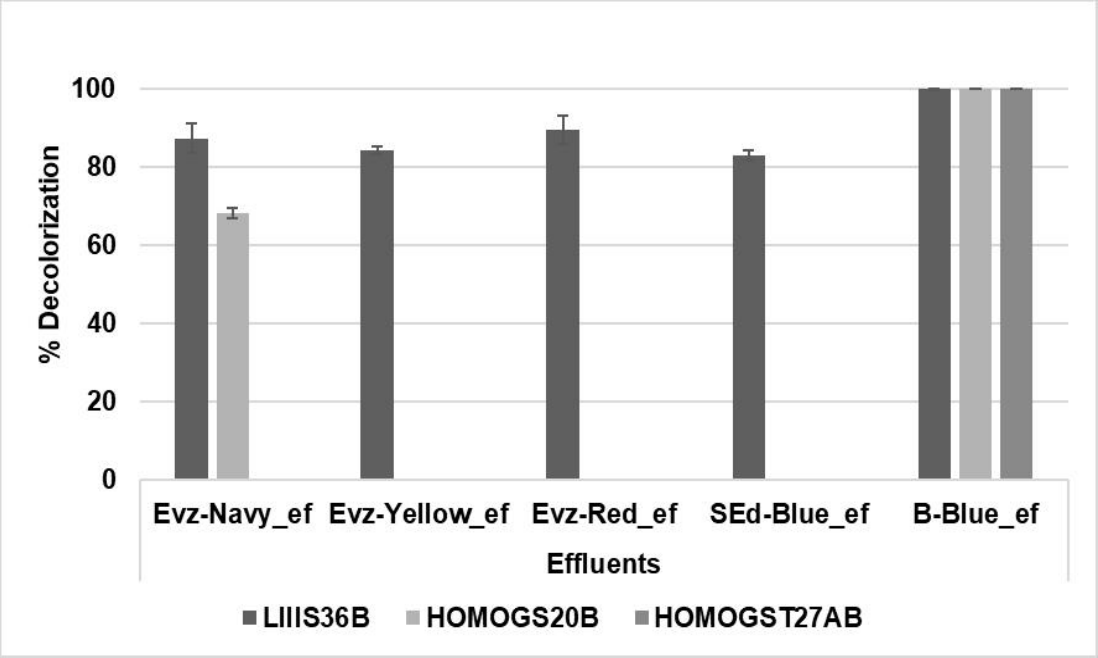

Figure 3.2 – Decolorization of effluents by yeasts in glucose-containing medium (20 g/L).

Only *C. pseudoglaebosa* (LIIIS36B) was able to remove color efficiently from all simulated effluents tested (> 80%). *C. parapsilosis* (HOMOGS20B) only decolorized Evz-Navy$_{ef}$ and B-Blue$_{ef}$; and *Y. lipolytica* (HOMOGST27AB) could only remove the color from B-Blue$_{ef}$.

3.3.1.1. Decolorization stream compounds

Dye solutions and simulated effluents, both before and after the decolorization processes, were analyzed by HPLC. An example of an HPLC chromatogram is shown in Figure 3.3. Peaks resulting from yeasts' metabolism were detected in control biotic assays (Figure 3.3-a) and only the peaks resulting solely from dye or effluent degradation were considered. The example of chromatograms from HPLC intends to demonstrate the peaks of the decolorization stream compounds presented in the samples of colored effluent treated with the yeasts (Figure 3.3-b), by comparison with the peaks presented in the sample without dye (Figure 3.3-a) and with the chromatogram where only the peak of the original dye is seen (Figure 3.3-c). Thus, in the sample Figure 3.3-b it was possible to notice that the peak of the original dye was no longer present, which could indicate that it could have been degraded by the yeast, causing the formation of the peaks of degradation stream compounds.

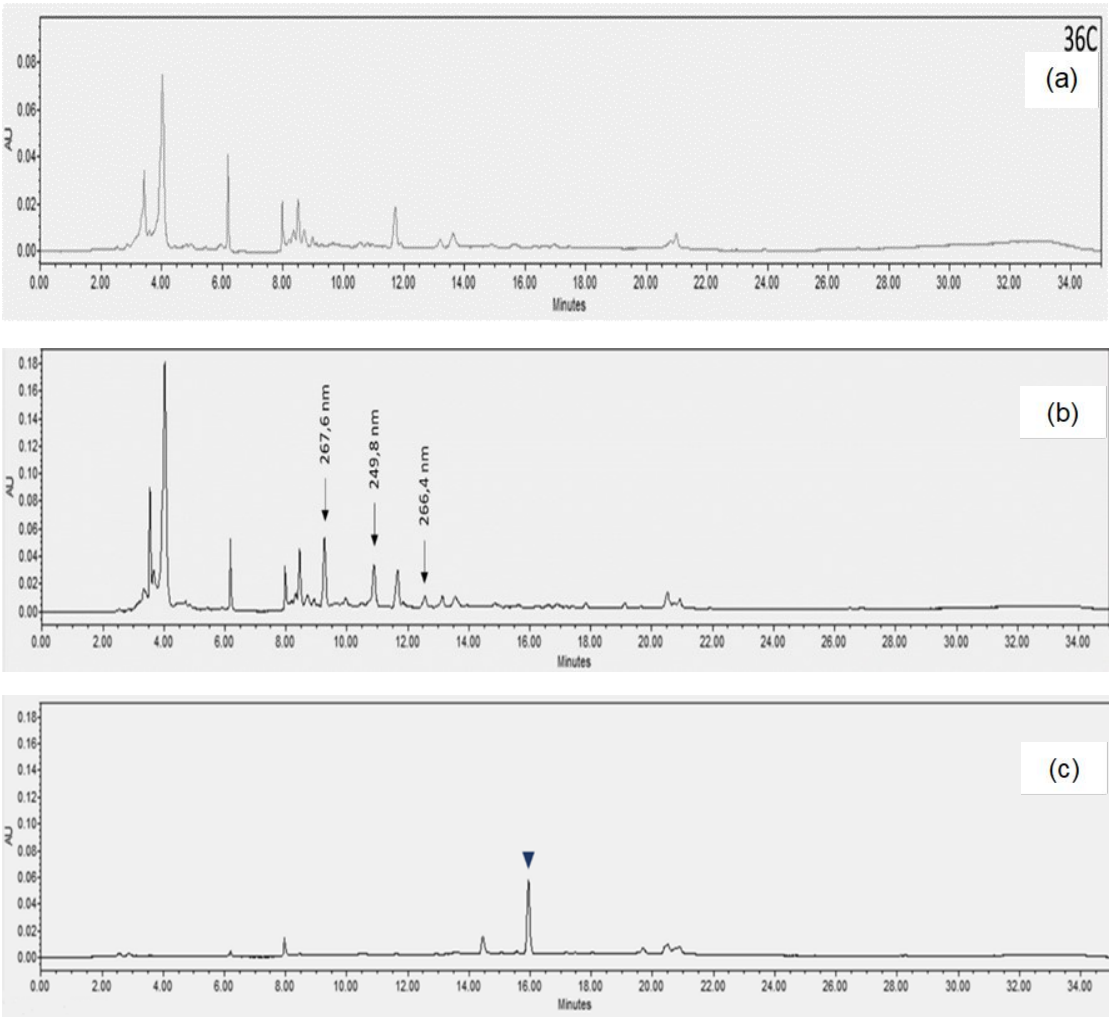

Figure 3.3 – Example of an HPLC chromatogram. Supernatant of culture medium after growth of *C. pseudoglaebosa* (LIIIS36B) (a) without dye and (b) with dye Navy Everzol ED, (c) standard dye Navy Everzol ED (634 nm). ↓ - Decolorization stream peaks; ▼- Dye Navy Everzol ED peak.

The decolorization performed by yeasts could lead to the formation of compounds with different chemical structures of the original compound (Danouche *et al.,* 2021). Table 3.1 shows some decolorization compounds formed by the yeasts after decolorization, distributed in a range between 208.5 and 290.2 nm of absorbance.

Table 3.1 – Peaks (as absorbance in nm) found in HPLC after decolorization of dyes and simulated effluents by yeasts *C. parapsilosis* (HOMOGS20B), *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) using MDM. Identified peaks correspond only to the formation of new compounds. Peaks present before the decolorization process are not indicated.

		Decolorization Compounds										
Yeasts	Samples	Abs (nm)										
LIIIS36B	Evz-Navy						249.8		266.4	267.6		
	Evz-Yellow							252.2				290.2
	Evz-Red						247.0			267.6		
	SEd-Blue											
HOMOGS20B	Evz-Navy						249.8		267.0			
	Evz-Yellow											290.2
	Evz-Red											
	SEd-Blue											
	B-Blue	208.5	216.7		232.1		241.5	257.9	267.6		284.2	284.2
HOMOGST27AB	Evz-Yellow											290.2
	B-Blue										284.2	
LIIIS36B	Evz-Navy$_{ef}$					245.0	249.8	252.0	265.2	266.4		
	Evz-Yellow$_{ef}$											
	Evz-Red$_{ef}$											
	BSEd-Blue$_{ef}$											
HOMOGS20B	Evz-Navy$_{ef}$			228.5			249.8	252.2				
	B-Blue$_{ef}$	210.9										
HOMOGST27AB	Evz-Navy$_{ef}$						248.6	252.2				
	Evz-Yellow$_{ef}$										284.2	
	B-Blue$_{ef}$	210.9										

Since dyes spectra have their characteristic absorption peaks in the visible region ranging between 400 and 700 nm (Pinheiro *et al.,* 2004), the detected peaks correspond only to compounds from degraded dyes and no extra peaks were detected in this region. Nonetheless, 36B_SEd-Blue, 20B_Evz-Red, 20B_SEd-Blue, 36B_Evz-Yellow$_{ef}$, 36B_Evz-Red$_{ef}$, and 36B_BSEd-Blue$_{ef}$ did not show any compounds resulting from the dye degradation, which could be due to complete degradation of the molecules.

New compounds formed due to decolorization can be more or less toxic to the environment depending on how the degradation process is performed by the degrading organisms (Danouche *et al.,* 2021). Azo dyes are known to be the most commonly used group of synthetic dyes worldwide (Saratale *et al.,* 2011). The chemical structure of azo dyes is characterized by

the presence of one or more azo bonds (–N=N–), typically conjugated to aromatic rings, often containing different substituents. This structure allows the displacement of the electrons, in transitions in the ultraviolet-visible (UV-Vis), absorbing the light in the zone of the visible (Pandey *et al.,* 2007; Saratale *et al.,* 2011). In general, azo dyes could be biologically degraded through the reductive cleavage of azo bounds which results in dye decolorization forming colorless solutions. The formed resulting metabolites are often aromatic amines (Pinheiro *et al.,* 2004; Saratale *et al.,* 2011; Danouche *et al.,* 2021). Compounds ranging from 260 to 300 nm, are known to belong to the aromatic amines group (Pinheiro *et al.* 2004). Aromatic amines are formed through the degradation of azo dyes by microorganisms that can also generate products with biotoxicity (Danouche *et al.,* 2021). Most of the metabolites found in the present study absorbed in the range of 266.4-290.2, which could mean that these degradation products could be aromatic amines. However, when comparing the peaks found in this study with main regions of UV-visible spectra from bibliographic references (Pinheiro *et al.,* 2004), none of the degradation products could be identified further. Products of decolorization ranging 240-260 nm were well distributed through the dye and simulated effluents, especially in blue color dyes and effluents. Nevertheless, in some of the decolorized dyes and effluents, no peaks were found, thus the original dye or simulated effluent could possibly have been fully degraded by yeasts.

3.3.2. Toxicity tests

The toxicity of the yeast-degraded resulting streams was evaluated. Tests were performed with commercial kits that use organisms from different trophic levels to ensure the representativeness of the ecosystems. Toxicity tests were only performed with simulated effluents because of operational issues at the time of the experiment. Moreover, real effluents are widely variable, and the results could be influenced by non-controllable factors. Using simulated effluent allowed a more controlled experiment. Only the decolorization streams from simulated effluents supplemented with glucose were tested to minimize the interferences that other medium components may have. Thus, 36B_Evz-Navy$_{ef}$, 36B_Evz-Yellow$_{ef}$, 36B_Evz-Red$_{ef}$, 36B_SEd-Blue$_{ef}$, 36B_B-Blue$_{ef}$, 20B_Evz-Navy$_{ef}$, 20B_B-Blue$_{ef}$, 27AB_B-Blue$_{ef}$ were selected for toxicity tests.

3.3.2.1. TOXI-ChromoTest™

Quantitative results from the TOXI-ChromoTest™ are presented in Figure 3.4 and show that the simulated effluents before treatment with yeasts exhibited high toxicity as the non-treated effluent had the capacity to inhibit the de novo synthesis of the highly permeable mutant *E. coli* β-galactosidase. In contrast, after yeast's treatment, the simulated effluent streams developed blue color in every sample showing their lower toxicity according to the kit instructions. In the controls, a lighter blue color was developed, which means that the toxicity

was considered moderate (above 50%, yet below 60%). Moderate toxicity of glucose may not be very relevant for the assay since it is consumed by the metabolism of the yeasts (Aracagök and Cihangir, 2013). Most of the treated samples showed low toxicity (below 40%), except for 27AB_B-Blue$_{ef}$ (49%), which means that the reduction of toxicity after decolorization by yeasts ranged from 51 to 66%.

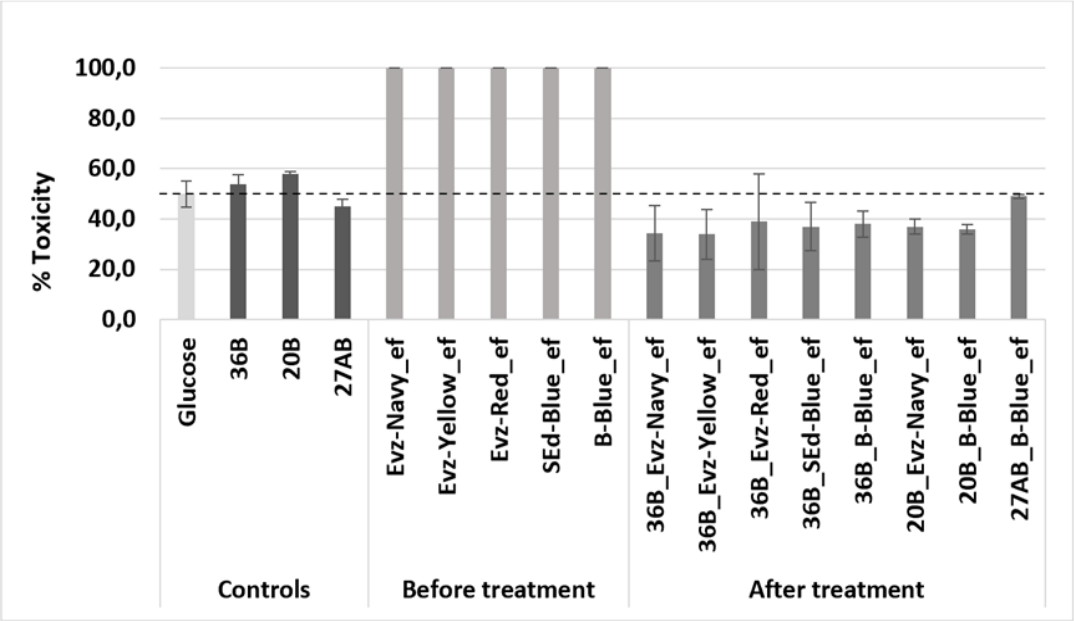

Figure 3.4 - Toxicity of effluents before and after decolorization, and respective controls (glucose, *C. pseudoglaebosa* LIIISB36B (36B), *C. parapsilosis* HOMOGS20B (20B), *Y. lipolytica* HOMOGST27AB (27AB)) assessed using TOXI-ChromoTest™. The dashed line indicates 50% of toxicity.

Overall, toxicity decreased after treatment with yeasts when compared to the original effluents that are classified as highly toxic by this test. Several authors showed that dye toxicity decreased after treatment with some yeasts: Tan *et al.* (2019) and Song *et al.* (2017) indicated that the degradation and the detoxification of Acid Red B azo dye was efficient through the use of a halotolerant yeast *C. tropicalis* SYF-1 (from high - 80% to low toxicity – 35%) and *Pichia occidentalis* (from 80% to 5% toxicity), respectively. Jadhav *et al.* (2010) stated that a microbial consortium of *Pseudomonas* sp. succeeded in decolorizing a real textile effluent as well as the Reactive Orange 16 dye and the bio-treated samples were revealed to be non-toxic.

3.3.2.2. DAPHTOXKIT F

The toxicity of the simulated effluents before and after decolorization treatment by yeasts performed with DAPHTOXKIT F is presented in Table 3.2.

Table 3.2 – Toxicity of simulated effluents before and after decolorization processes in glucose, and respective controls (glucose, LIIISB36B (36B), HOMOGS20B (20B), HOMOGST27AB (27AB)), using DAPHTOXKIT F.

	Controls				Before treatment					After treatment							
	Glucose	36B	20B	27AB	Evz-Navy$_{ef}$	Evz-Yellow$_{ef}$	Evz-Red$_{ef}$	SEd-Blue$_{ef}$	B-Blue$_{ef}$	36B_Evz-Navy$_{ef}$	36B_Evz-Yellow$_{ef}$	36B_Evz-Red$_{ef}$	36B_SEd-Blue$_{ef}$	36B_B-Blue$_{ef}$	20B_Evz-Navy$_{ef}$	20B_B-Blue$_{ef}$	27AB_B-Blue$_{ef}$
Survival	×	×	×	×	✓	✓	✓	✓	×	×	✓	✓	×	×	✓	×	×
pH	7.8	2.8	2.8	3.5	6.7	6.5	6.9	7.0	6.2	4.9	6.8	6.9	3.2	3.3	5.6	3.4	3.4

✓- survivor *Daphnia* (0% mortality); × - non survivor *Daphnia* (100% mortality). Shaded cells show pH of solutions below 5.

According to this test, before treatment, only B-Blue$_{ef}$ was toxic for *Daphnia*. After decolorization, streams 36B_Evz-Navy$_{ef}$, 36B_SEd-Blue$_{ef}$, 36B_B-Blue$_{ef}$, 20B_B-Blue$_{ef}$ and 27AB_B-Blue$_{ef}$ were toxic to these organisms. Interestingly, the pH of these treated effluents that were toxic for *Daphnia*, was lower than 5 and that may have contributed for *Daphnia* mortality. An exception was the glucose medium which has a pH of 7.8, and was fatal for *Daphnia* in this test, meaning that the glucose solution was somehow indeed toxic for these organisms, besides pH. In fact, some authors stated that these organisms are sensitive to low pH (Ghazy *et al.,* 2011). The acidic pH of the streams may be due to the fermentation metabolism of the yeasts and the end-products generated. This could be corroborated by the results of the controls performed only with each yeast in glucose, that had a very low pH (below pH=3). Actually, there seems to be a relation between the pH values and these organisms' survival, since in all samples with a pH value near to neutral value, the neonates were able to survive after treatment. Ghazy *et al.* (2011) reported that the suitable range of pH for *Daphnia* must be above 4.55 and below 10.13. In the samples that had pH values below 5, the neonates did not survive. B-Blue$_{ef}$ was toxic by itself before treatment and was the only simulated effluent that was toxic after treatment with the three yeasts in study (36B_B-Blue$_{ef}$, 20B_B-Blue$_{ef}$ and 27AB_B-Blue$_{ef}$), meaning that the decolorization process did not reduce its toxicity. Toxicity found could be due to the adjuvant compounds such as surfactants, detergents, solvents, salts, on the simulated effluent or even from the dye degradation compounds that were formed by the decolorization capacity of the yeasts in study which is different for every dye molecule. Curiously, 36B_Evz-Yellow$_{ef}$, 36B_Evz-Red$_{ef}$ and 20B_Evz-Navy$_{ef}$ streams, despite being treated with the same yeasts, their pH was nearly neutral which could mean that the dye degradation (and adjuvants compounds) somehow compensated the pH from the yeasts metabolism and the streams were not toxic for *Daphnia*. An exception of the pH issue was the glucose medium which had a pH of 7.8 and was toxic for *Daphnia* too. Yeasts use preferably glucose as carbon source to grow and metabolize the effluents when it is available (Aracagök

and Cihangir, 2013). Therefore, the toxic effect of glucose on the decolorization streams was not significant since glucose should have been completely consumed during the decolorization process. So, we could assume that glucose did not affect the toxicity of the streams after treatment.

Assays using daphnids are an excellent method for evaluation of acute aquatic toxicity of dyes since these organisms are very sensitive (Abe *et al.,*2019). *Daphnia magna* was used by Croce *et al.* (2017) as a model for evaluate acute toxicity of 42 commercial dyes from different classes and its studies revealed that 9 formulations were in fact toxic below 100 mg/L. Disperse Red 1 was also toxic for *Daphnia magna* according to Vacchi *et al.* (2016). Some studies already mentioned that decolorization of some selected azo dyes by a bacterial consortium consisting of *Providencia rettgeri* strain HSL1 and *Pseudomonas* sp. SUK1 proved to be effective, and the dye-degraded metabolites were not toxic to *Daphnia magna* (Lade *et al.,*2015). Also, Franciscon *et al*. (2009) decolorized and biodegraded four different azo dyes using the bacteria *Klebsiella* sp. strain VN-31 to non-toxic metabolites for *Daphnia magna*. Similar results were stated by Elisangela *et al.* (2009) using *Staphylococcus arlettae*.

3.3.2.3. Phytotoxicity test – Lactuca sativa seeds

The phytotoxicity assay was performed to evaluate the impact that the products of decolorization resulting from treatment of the simulated effluents by yeasts metabolism had on plants. Reuse of the treated water in field irrigation could also be a possibility if biodegradation by yeasts is able to decrease phytotoxicity of dyed effluents (Ayed *et al.,*2011). Germination of *Lactuca sativa* seeds before and after yeast decolorization is shown in Figure 3.5.

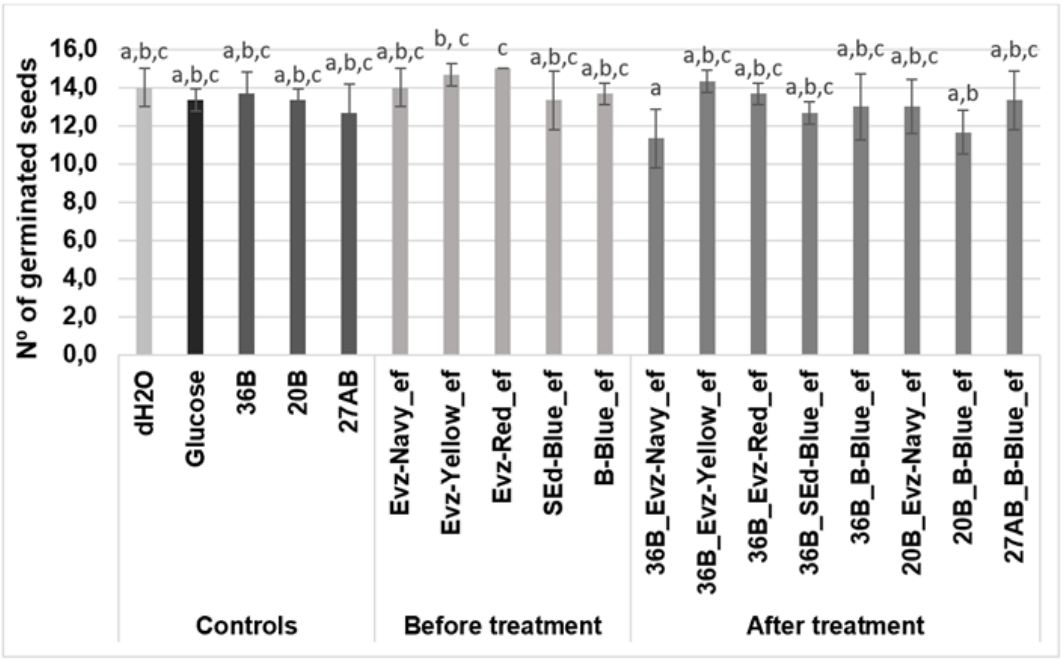

Figure 3.5 – Number of germinated seeds with effluents before and after treatment, and respective controls (glucose, *C. pseudoglaebosa* LIIISB36B (36B), *C. parapsilosis* HOMOGS20B (20B), *Y. lipolytica* HOMOGST27AB (27AB)). Different superscript letters for significant differences ($p < 0.05$).

The number of germinated seeds was high and very similar for almost all the samples and controls. Significant differences were only found for 36B_Evz-Navy$_{ef}$ and Evz-Yellow$_{ef}$ and Evz-Red$_{ef}$, and between 20B_B-Blue$_{ef}$, and Evz-Red$_{ef}$. Nevertheless, simulated effluents were not toxic to the germination of lettuce seeds.

Yet, root length (mm) after seed germination with controls and samples before and after yeast treatment is shown in Figure 3.6.

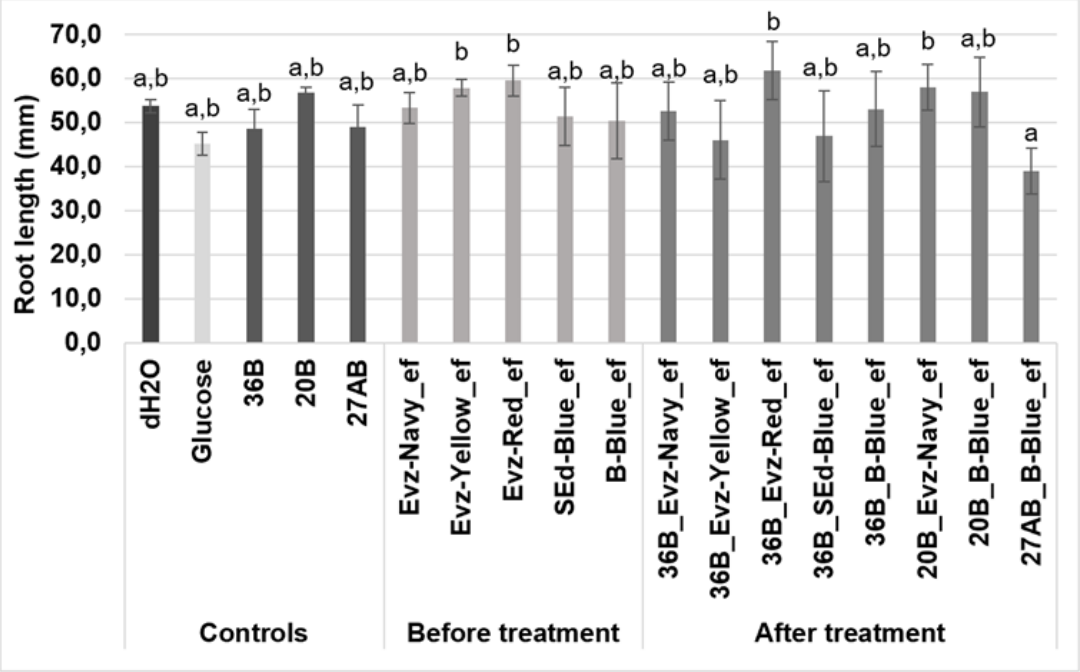

Figure 3.6 – Root length (mm) after seed germination with effluents before and after treatment in glucose, and respective controls (dH_2O, glucose, *C. pseudoglaebosa* LIIISB36B (36B), *C. parapsilosis* HOMOGS20B (20B), *Y. lipolytica* HOMOGST27AB (27AB)). Different superscript letters for significant differences ($p < 0.05$).

The root length of germinated seeds varied between 40 to 60 mm. Comparing with the control deionized water, there seemed to be no evident variation. Glucose medium was the only control that led to smaller root length but with no significant difference. The decolorization streams after yeast treatment led to bigger root length in the case of 36B_Evz-Red_{ef}, 20B_Evz-$Navy_{ef}$ and 20B_B-$Blue_{ef}$. However, 27AB_B-$Blue_{ef}$ had the lowest root length, significantly different from Evz-$Yellow_{ef}$, Evz-Red_{ef}, 36B_Evz-Red_{ef} and 20B_Evz-$Navy_{ef}$.

Lettuce seeds (*Lactuca sativa*) is one of the most common plant species recommended by several international environmental protection agencies as bioindicator in phytotoxicity tests for soil and water samples (Priac *et al.,* 2017). In this study there was no toxicity for samples before and after treatment. Similar results occurred in the studies of Almeida *et al.* (2019) that referred that toxicity of an azo dye Acid Blue 161 to *Lactuca sativa* seeds after being degraded with a combination of electrochemical oxidation and the yeast *S. cerevisiae* was only slightly lower than before treatment.

3.3.2.4. Mutagenicity test – Ames Test

There was no duplication of the number of spontaneous revertant of *S. typhimurium* TA 98 strain in MGA plates in none of the samples tested (data not shown). This fact indicated that simulated effluents, before and after treatment, were not mutagenic.

To the best of our knowledge, this is the first study that comprises a battery of toxicity tests and a mutagenicity test for the evaluation of these dyes and simulated effluents before and after the treatment with different yeasts. Some studies regarding azo dyes biodegradation tested the mutagenicity of biodegradation' metabolites and original compounds, with diverse results. Bafana *et al.* (2009) studied the cytotoxicity and mutagenicity of Direct black 38, a benzidine-based azo dye, and the metabolites resulting from the treatment with *Enterococcus gallinarum* and found that mutagenicity decreased after treatment. The research of Dawkar *et al.* (2010) showed the non-mutagenic and non-carcinogenic nature of the degraded products of the sulfonated azo dye Red HE7B by *Bacillus* sp. VUS. Rodrigues de Almeida *et al.* (2019) evaluated the degradation and the decrease in the mutagenicity of Acid Blue 161 and Procion Red MX-5B by a system using *Aspergillus terreus* and *Saccharomyces cerevisiae* with an electrochemical oxidation process as a pretreatment method.

Overall, results obtained from toxicity and mutagenic tests in our research showed that the biodegradation of dyed effluents by yeast strains resulted into the formation of end molecules with very low to no toxicity and no mutagenic effect for the different trophic levels.

3.4. CONCLUSIONS

In the present investigation, several dyes commonly used in textile industries, and respective simulated effluents were degraded by three selected yeasts and their color was effectively removed. *C. pseudoglaebosa* (LIIIS36B) was the most effective yeast since could completely decolorize four of the five simulated effluents. Products resulting from the simulated effluents degradation by yeasts were detected and were further subjected to toxicity tests. Interestingly, treated samples did not show toxicity to bacteria and lettuce seeds but were inconclusive for *Daphnia*. In fact, the toxicity decreased after treatment with yeasts, showing that the treatment did not produce very toxic end-products. Furthermore, no mutagenic effect was detected. At the end, the biological treatment of textiles wastewaters presented in this study showed high potential to be applied in the textile industry, ensuring the environmentally safe discharge of resulting wastewaters.

CHAPTER 4

ASSESSMENT OF DRYING CONDITIONS FOR STORAGE OF A YEAST-BASED DECOLORIZATION SOLUTION FOR APPLICATION IN TEXTILE INDUSTRIAL WASTEWATER TREATMENT PLANTS

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4.1. INTRODUCTION

The textile industry has a high environmental impact due to the highly pollutant liquid effluents generated (Sen *et al.,* 2016; Dellamatrice *et al.,* 2017). These dyes are easily absorbed, causing cancer and other diseases due to their toxic, mutagenic, and carcinogenic effects (Sudha *et al.,* 2014). Dyed effluents can also cause several environmental problems in photosynthetic activity, creating anoxic conditions in water bodies that are lethal to aquatic organisms (Kunz *et al.,* 2002; Danouche *et al.,* 2021). Traditional treatment methods have reduced efficiency, high costs and can form toxic wastes to be treated separately, increasing substantially the cost and complexity of these methods (Saratale *et al.,* 2011; Mahmoud, 2016; Kurade *et al.,* 2017). Biological methods are generally environmentally friendly since they can lead to the complete mineralization of organic pollutants or adsorption by living or dead biomass at a lower cost (Paz *et al.,* 2017; Danouche *et al.,* 2021). Microorganisms involved in dye biodegradation are diverse and have been studied in recent years (Das and Mishra, 2017). Yeasts like *Galactomyces geotrichum* (Kalyani *et al.,*2009), *Saccharomyces cerevisiae* (Aksu and Dönmez, 2003; Mahmoud, 2016), and *Candida* spp. (Yang *et al.,*2013; Martorell *et al.,*2017), have been recently studied for dye degradation. These microorganisms are easy to grow and maintain in culture and produce various enzymes. However, these biological solutions have limitations, such as application and viability over long periods of time. Formulations that can be easily stored for a long time, maintaining their decolorization capacity, are in demand.

To facilitate production processes, freeze and spray-drying methods are widely used to preserve microbial cultures for application in modern industries such as lactic and wine industries (Połomska *et al.,*2012). Freeze-drying is often thought to be the most convenient and successful method of preserving microorganisms (Abadias *et al.,*2001; Połomska *et al.,*2012). This process removes water in a frozen sample by sublimation under reduced pressure with minimum chemical alteration of the samples since it is a low-temperature process (Barbosa *et al.,*2015). This is an advantage over other processes, especially for biological materials and thermal-sensible products. In addition, freeze-dried products are efficiently rehydrated (Castro *et al.,*1997; Barbosa *et al.,*2015). However, freeze-drying is an expensive and time-consuming process compared to spray-drying. Spray-drying transforms a sample solution directly into a dried powder with lower costs, which justify being frequently chosen for industrial processes such as in wine, pharmaceutical and food industries (Aponte *et al.,*2016; Dadkhodazade *et al.,*2021). The sample solution is sprayed into a chamber with hot dry air and evaporates rapidly into a spray-dried powder. The high temperatures used in this process may lead to a decrease or loss of some chemical properties, and sugar molecules

can cause stickiness causing operating difficulties. Nonetheless, spray drying is an inexpensive, rapid, simple, and continuous process (Barbosa *et al.,*2015).

Microorganisms could be challenging to dry due to the high temperatures used in spray-drying and low temperatures in freeze-drying processes that can cause several damages to the cell wall and cell membranes, which are considered to be the main site that leads to lethal cell damages (Jesus and Maciel Filho, 2014; Tang *et al.,* 2020). To overcome some of the problems associated with the drying processes, cryoprotecting agents such as skim milk powder or maltodextrin have extensively been used as a suspending agent before drying processes, with positive results (Tang *et al.,*2020). They minimize stickiness during the drying process and crystallization of molecules during storage (Barbosa *et al.,*2015). They are also used to protect microorganisms' cells, improving survival throughout the drying processes (Castro *et al.,* 1997; Połomska *et al.,* 2012; Barbosa *et al.,* 2015).

Another possible form of microbial immobilization involves encapsulation in a matrix of sodium alginate and calcium chloride. The addition of the capsules to a sodium citrate bath allows the capsules to retain their core liquid, facilitating exchanges between liquid and the entrapped microorganisms. The main advantages of encapsulation are the protection of the microorganisms against the external environment and consequently greater resistance during the treatment process (Qi *et al.,* 2005; Sousa *et al.,* 2015; Holkem *et al.,* 2016).

In the present study, a dried yeast-based product with dye-decolorization capacity was developed. The main objective was to evaluate the viability of yeast strains cells over time after being subjected to drying processes and storage conditions. The yeasts' ability to decolorize a simulated textile effluent overtime after the freeze-drying process to assess stability was also evaluated. In addition, yeast immobilization within alginate-calcium capsules and its viability and decolorization capacity was also tested. An inference of the formulation amount required for industrial proposes and associated costs were estimated.

4.2. MATERIAL AND METHODS

4.2.1. Biological material

Three yeast strains previously isolated from wastewater treatment plants in the north of Portugal were selected for freeze-drying assays according to Chapter 2 – 2.2.1.: *Candida parapsilosis* (HOMOGS20B), *Yarrowia lipolytica* (HOMOGST27AB) and *Candida pseudoglaebosa* (LIIIS36B). *Yarrowia lipolytica* (HOMOGST27AB) was selected among the three for the spray-drying assay because of safety and operational limitations related to the lab-scale equipment.

4.2.2. Culture media

The Normal Decolorization Media (NDM) and Normal Solid Decolorization Medium (NSDM) were used for culture maintenance and cell viability assays and were prepared according with the described in Chapter 2 (2.2.1.).

4.2.3. Simulated textile Navy Everzol ED effluent

The simulated effluent Navy Everzol ED was prepared by mimicking the industrial process in a lab-scale fabric dying device, according with the described in Chapter 2 (2.2.3.).

4.2.4. Yeasts' growth and preparation for drying

Overnight pre-inoculum (30 °C/24 h) of each yeast strain was cultivated with a 2% inoculum in NDM and incubated at 25 °C/48 h/100 rpm (in duplicate). Cultures were centrifuged (Hettich Universal 320R, Tuttlingen, Germany) at 5000 rpm for 10 min, and pellets were washed with peptone water (Sigma-Aldrich, St. Louis, MO USA). Cells were then mixed in sterilized (110 °C /15 min) skim milk powder (SKM) (Oxoid, Hampshire, England) or sterilized maltodextrin (MDX) (Sigma-Aldrich, St. Louis, MO USA) solutions at 10% (w/v). Solutions for freeze-drying were frozen at -80 °C until use.

4.2.4.1. Spray-drying

Y. lipolytica (HOMOGST27AB) samples (in 400 ml of 10% of SKM) were dried in a lab-scale spray-dryer (BÜCHI Mini Spray Dryer B-191, Flawil, Swiss) with an inlet air temperature adjusted to 110 °C. The outlet temperature (55 °C) was controlled by flow rate (pump = 10%) and inlet air temperature parameters (optimized conditions). The dried powder (approximately 3 g) was collected in a single cyclone air separator system. A pilot-scale spray-drying with *Y. lipolytica* (HOMOGST27AB) suspended in 1 L of 10% SKM was performed to ensure enough yeast powder (approximately 70 g) to test the decolorization capacity. Due to equipment limitations, drying conditions were inlet temperature of 150 °C and outlet temperature of 98 °C.

4.2.4.2. Freeze-drying

Each sample, previously frozen (-80 ºC), was desiccated under vacuum (0.05 mbar) for 3 days in a freeze-drier (Telstar LyoQuest -55, Tokyo, Japan) at room temperature, and the condenser was cooled at -55 °C.

4.2.5. Storage conditions after freeze-drying

Each freeze-dried sample was stored in individually sealed sterilized plastic bags inside glass flasks filled with dried silica gel (Pronalab, Vista Hermosa, Mexico) to control humidity. Flasks were kept at two different temperatures (4 °C and room temperature) in the absence of light.

4.2.6. Viability of yeasts cells after freeze-drying

The viability of yeast cells was tested before and after freeze-drying throughout time (t = 0 d; 15 d; 60 d; 90 d; 210 d). Briefly, 2 g of freeze-dried powder was dissolved in 20 mL of peptone water (1 g/L), and decimal suitable dilutions were plated in duplicate by the drop count technique (20 µL) into Petri dishes with NSDM. After incubation at 25 °C for 48 h, colonies were counted, and the log CFU/g was calculated.

4.2.7. Decolorization capacity after freeze-drying

4.2.7.1. With yeasts' recovery step

Decolorization capacity of freeze-dried yeasts was evaluated to test if the color removing capacity of the yeasts was still held. A yeast pre-inoculum was performed with 25 mg of dried powder in 10 mL of NDM and incubated at 25 °C for 24 h. Then, each inoculum was added to 5 g/L of Navy Everzol ED simulated effluent (kindly provided by AQUITEX, SA) in the proportion of 1:5 and incubated at 25 °C/100 rpm/72 h.

4.2.7.2. Without yeasts' recovery step

Decolorization capacity of the freeze-dried yeasts' powder added directly to the simulated effluent was also evaluated. This test was performed to prove the simplicity of application of the obtained formulation in effluent decolorization. Dried yeast powder (25 mg) of *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) at 1%, 2%, and 5% (with SKM), were added directly to the simulated effluent Navy Everzol ED (5 g/L) and incubated at 25 °C/100 rpm/72 h.

4.2.8. Immobilization in Alginate capsules

To prepare alginate-calcium capsules loaded with *Y. lipolytica* (HOMOGST27A), 2% pre-inoculum of yeast was added in flasks with 200 mL of NDM medium and incubated at 25 ºC, 100 rpm for 48 hours. Then, was centrifuged for 15 minutes at 5000 rpm and the pellet

obtained was washed with peptone water and then was resuspended in sodium alginate (40 g/L), forming capsules by extrusion in a calcium chloride solution (20 g/L). The capsules formed remained in a calcium chloride solution for about 30 minutes to harden. The formed capsules were about 3 mm and after encapsulation the yeasts showed a growth of 10^8 CFU/g. To understand its viability and decolorization capacity, decolorization tests were carried out with the capsules in flasks with 25 mL of simulated effluent of Navy Everzol ED (diluted 6x) and Navy Everzol ED dye (100 mg/L) and various percentages of capsules were tested: 1% (250 mg), 2% (500 mg) and 5% (1.25 g). Then, the concentration of capsules in the process was increased to 20% (5 g) and 40% (10 g) of capsules. In order to make the matrix more open and the connections weaker, and to make the effluent more intimately in contact with the yeast, new tests were performed with a considerably lower concentration of sodium alginate (15 g/L). The capsule formation method was maintained, but after hardening in calcium chloride (20 g/L), the capsules were washed in deionized water and then placed in a sodium citrate solution (55 mmol/L) for another 30 minutes. The incubation was carried out again in a flask for 48 hours, with 25 mL of effluent and 20% (5 g) and 40% (10 g) of alginate capsules.

Also, a laboratory system with some similarities to a bioreactor, where a preparative column (40 cm) was packed with the selected capsules and submitted to aeration creating conditions, was tested. For this experiment, a preparative HPLC column and an air diffuser were used to aerate the system. The capsules were placed on the column to about half of their volume (corresponding to ± 60 mL sodium alginate) and 40 mL of simulated effluent of Navy Everzol ED was added. The aeration system was continuously turned on and decolorization was observed over time.
Regarding their stability over time, the capsules were stored at 25 ºC and 4 ºC in a dark and dry place. The stability was evaluated for two months in terms of growth viability.

4.2.9. Statistical analysis

Results obtained in the experiments were expressed in terms of means (average) and standard deviation (S.D.). Normality distribution was first verified, and a t-student test was performed, with a level of significance of 0.05 ($p < 0.05$), using IBM® SPSS® Statistics 26 (SPSS Inc., IBM Corporation, NY, USA).

4.3. RESULTS AND DISCUSSION

4.3.1. Freeze-drying

Yeast growth viability before and after freeze-drying (t = 0 days) with different cell protectors was assessed. Freeze-drying was carried out individually for the three yeast strains using SKM and MDX as cell protectors at 10%. Results are shown in Figure 4.1.

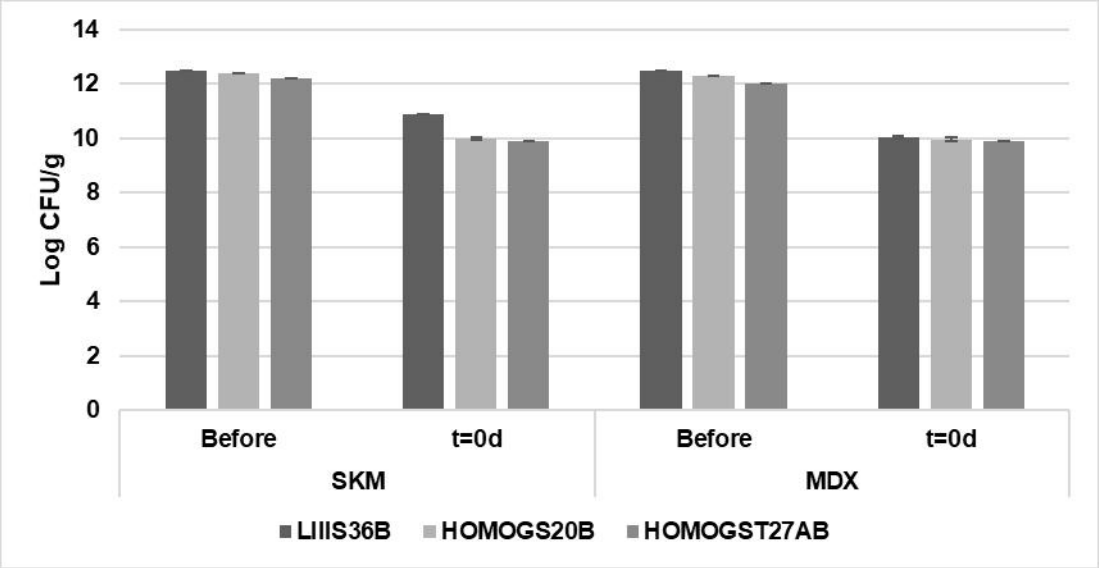

Figure 4.1 – Viability of yeast cells (in log CFU/g) before and after (t = 0 days) freeze-drying process using SKM and MDX as cell protectors.

The viability of yeast cells (Figure 4.1) was very similar for the two protector agents, both before (12 log CFU/g) and after lyophilization (t = 0 days) (10 log CFU/g) with similar results for both yeast strains. However, there was a reduction of approximately 2 logs in all cases, except for the yeast *C. pseudoglaebosa* (LIIIS36B) with SKM, with a decrease of only 1 log (from 12 log CFU/g for 11 log CFU/g). There were no significant differences between SKM or MDX in protecting the cell's viability during the freeze-drying process. This is in accordance with Tang *et al.* (2020) who studied the viability of *Lactobacillus acidophilus* FTDC 3081 after being freeze-dried with 10% SKM and 10% MDX.

Microorganisms tend not to survive well when exposed to drying processes. Exposure to extreme temperatures and osmotic pressures can cause damage to the cell integrity that could be lethal (Tang *et al.,*2020). Adding cell cryopreservatives like milk, sugars, polyols, and amino acids to maintain and protect cells from damages during drying processes is well documented (Abadias *et al.,*2001). These substances are essential in preserving freeze-dried cells' viability since, besides the cell damage protection, they provide support material of dry residue with a defined physical structure that facilitates rehydration. Several studies show that the addition of SKM and MDX could reduce cell death during drying processes (Abadias *et al.,*2001; Tang

*et al.,*2020). SKM powder has been widely used to ensure that the lyophilized preparation maintains a uniform porous structure, allowing easy sample drying and rehydration after drying processes (Połomska *et al.,*2012). The incorporation of MDX is known to improve physical stability. Other sugars, such as trehalose and maltose, are used too and are proven to enhance resistance to thermal treatments (Lodato *et al.,*1999). Although some authors suggested that trehalose and other sugars and mixtures between other compounds had better results in protecting cells from damage, we chose to study SKM powder and MDX mainly because of their economic viability and commercial availability in the market.

4.3.2. Viability of freeze-dried yeasts' cells throughout storage time

Yeasts cells viability was tested over time during nearly seven months of storage (210 days). Freeze-dried cells using SKM and MDX as protector agents were stored at 4 °C and room temperature in a dark and dry place. Results are presented in Figure 4.2 for SKM and Figure 4.3 for MDX.

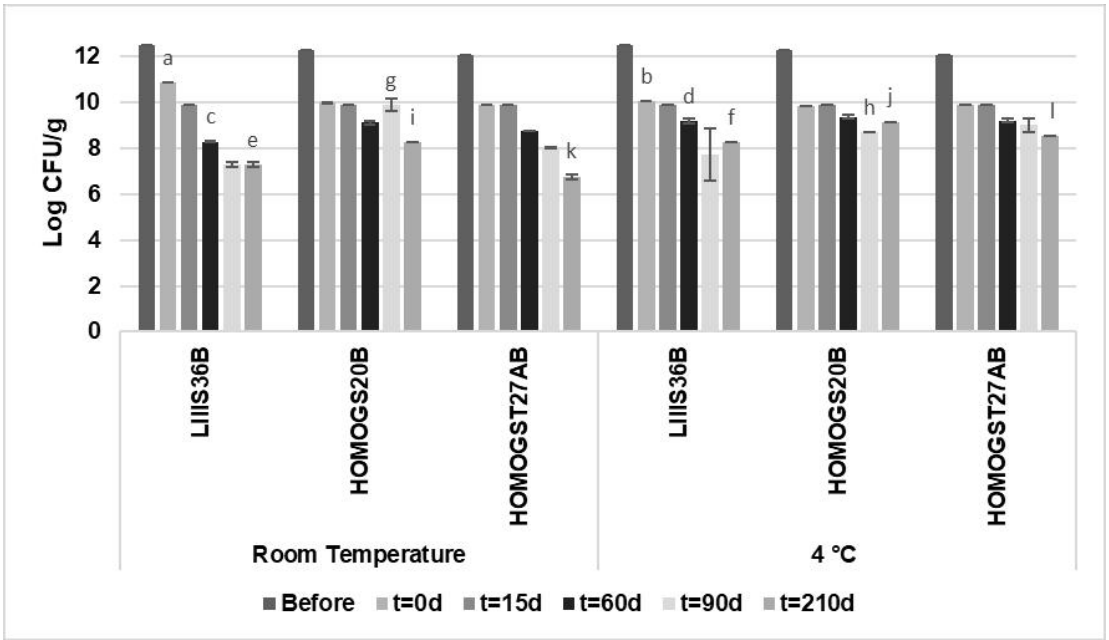

Figure 4.2 – Viability of yeast cells over time at room temperature and 4 °C with SKM as a cell protector agent in freeze-drying. Different superscript letters for significant differences ($p < 0.05$).

When using SKM as a cell protector, all yeasts suffered a decrease in viability of about 2 logs CFU/g after freeze-drying (from 12 logs CFU/g to about 10 logs CFU/g). Over time, a decrease in viability occurred both at room temperature and at 4 °C for all yeasts. The reduction was of about 2 to 3 logs, with the viability of around 8 logs CFU/g at 210 days of storage. Comparing the two storage conditions, yeasts kept at room temperature showed a higher decrease (about 3 logs) in cell viability than at 4 °C, which only decreased by about 2 logs. This was expected since cooling temperatures for storage are commonly known to be better in preserving cell

viability and were in line with the studies of Tang *et al.* (2020). Nevertheless, the viability of freeze-dried yeast cells was still high at room temperature, above 6 log CFU/g after 210 days, which could also be an effective method for drying and storing yeast cells.

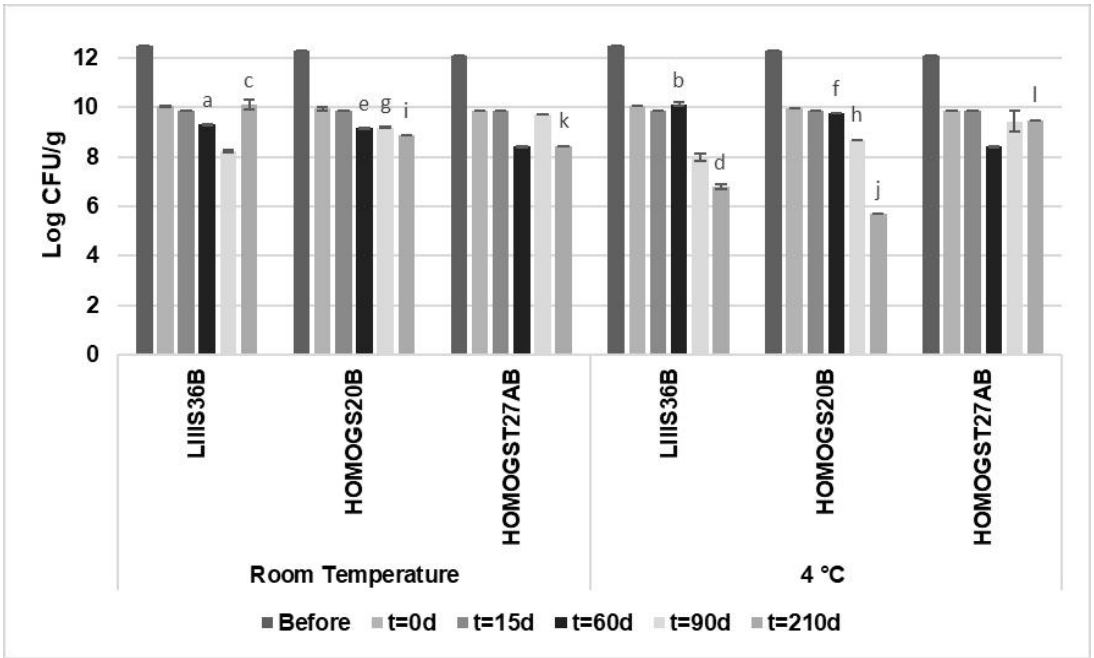

Figure 4.3 - Viability of yeast cells over time at room temperature and 4 °C with MDX as a cell protector agent in freeze-drying. Different superscript letters for significant differences ($p < 0.05$).

Figure 4.3 shows the results of viability when using MDX as a cell protector in freeze-drying. There was a decrease in viability for all yeasts of about 2 logs after freeze-drying (from 12 logs CFU/g to 10 log CFU/g). Over time, viability decreased again by about 2 logs (from 10 log CFU/g to 8 log CFU/g) for almost all yeasts at room temperature. At 4 °C, strains *C. pseudoglaebosa* (LIIIS36B) and *C. parapsilosis* (HOMOGS20B) showed a more pronounced decrease in viability (from 10 logs CFU/g to slightly below 6 logs CFU/g), which went against the previously obtained results for SKM. These results indicate significant differences between MDX storage conditions as cell protector agents in freeze-drying. Comparing the two protecting agents, there were significant differences between them and between storage conditions for all yeasts. SKM seems to have more steady results for all yeasts and storage conditions. MDX also protected the cells, but at 4 °C did not seem effective in the long run (t = 90 days and t = 210 days), especially for *C. pseudoglaebosa* (LIIIS36B) and *C. parapsilosis* (HOMOGS20B). These findings were in contrast with Tang *et al.* (2020) that states that MDX was a better protective agent than SKM for *L. acidophilus* FTDC 3081. Nevertheless, the viability of freeze-dried yeasts' cells was still high, at about 6 logs CFU/g, representing a decrease from 12 logs to 6 logs CFU/g, which means that both protecting agents are efficient.

4.3.3. Decolorization ability after freeze-drying

4.3.3.1. With yeasts' recovery

Besides the viability and stability of the freeze-dried yeasts' cells, its decolorization ability was also evaluated to understand whether the freeze-drying process influenced the yeasts' decolorization ability of a simulated effluent, chosen according to industry representativeness. A pre-inoculum of every freeze-dried yeast strain was prepared in NDM and then added to a simulated effluent of dark blue color (Navy Everzol ED). The reduction of color was observed after 24, 48 and 72 hours of incubation. Results are shown in Table 4.1 and Table 4.2 for storage at 4 °C and room temperature, respectively.

Table 4.1 – Percentage of decolorization of simulated effluent Navy Everzol ED over time for freeze-dried yeasts, stored at 4 °C. Samples observed at t = 24 hours; t = 48 hours and t = 72 hours of incubation; The darkest blue color represents the original color of the dye, which indicates 0% decolorization; the lighter blue color, indicates that decolorization has occurred - 100% represents total decolorization.

			t=60 days			t=90 days			t=210 days		
			t_{24h}	t_{48h}	t_{72h}	t_{24h}	t_{48h}	t_{72h}	t_{24h}	t_{48h}	t_{72h}
4 °C	LIIIS36B	SKM	80%	100%	100%	80%	100%	100%	0%	0%	0%
		MDX	100%	100%	100%	100%	100%	100%	0%	20%	50%
	HOMOGST27AB	SKM	0%	50%	50%	0%	60%	60%	0%	0%	0%
		MDX	20%	100%	100%	40%	60%	60%	0%	0%	0%
	HOMOGS20B	SKM	0%	50%	50%	0%	80%	100%	0%	20%	50%
		MDX	20%	70%	70%	20%	80%	100%	0%	20%	50%

C. pseudoglaebosa (LIIIS36B) maintained the capacity to remove color from the effluent completely in 48 hours for both SKM and MDX at 60 and 90 days of storage. Moreover, with MDX, color was removed effectively after only 24 hours of incubation. At 210 days of storage, this yeast strain dried with MDX could only decolorize 50% of the effluent and could not remove any color when dried with SKM at 72 hours of incubation. After the long storage time, yeast cells may have taken longer to recover or were not able to recover properly, impairing the decolorization ability. Regarding yeast strain *Y. lipolytica* (HOMOGST27AB) with SKM, the color was removed at 50% at 60 days of storage and 60% at 90 days of storage. As for cells preserved with MDX, effluent color was effectively removed at 60 days of storage (100%), and at 90 days of storage, only 60% of color was removed at 72 hours. At 210 days of storage, the decolorization capacity was lost completely. Freeze-dried *C. parapsilosis* (HOMOGS20B) cells with SKM as cell protector were able to remove effluent color at 50% at 48 hours after 60 days of storage and at 100% at 72 hours after 90 days of storage. With MDX, decolorization only

reached 70% at 48 hours after 60 days and was effective (100%) at 72 hours after 90 days of storage. After 210 days, yeast could only remove 50% of effluent color with both cell protectors. Strains *Y. lipolytica* (HOMOGST27AB) and *C. parapsilosis* (HOMOGS20B) had a slow growing capacity when compared with *C. pseudoglaebosa* (LIIIS36B), which could explain some of the results obtained, namely, the decolorization capacity that in some cases only occurred after 48 hours. The storage time could affect these yeasts' decolorization capacity since results showed that at 210 days the decolorization activity was very low.

Overall, stored at 4 °C, strain *C. pseudoglaebosa* (LIIIS36B) was more efficient in removing color from the simulated effluent, especially with MDX as a cell protector, for at least 90 days of storage. The freeze-dried yeasts' strains, both with SKM and MDX, were able to grow in effluent Navy Everzol ED and were effective in removing color after being stored for at least 90 days.

Table 4.2 - Percentage of decolorization of simulated effluent Navy Everzol ED over time for freeze-dried yeasts, stored at room temperature. Samples observed at t = 24 hours; t = 48 hours and t = 72 hours of incubation; The darkest blue color represents the original color of the dye, which indicates 0% decolorization; the lighter blue color, indicates that decolorization has occurred - 100% represents total decolorization.

			t=60 days			t=90 days			t=210 days		
			t_{24h}	t_{48h}	t_{72h}	t_{24h}	t_{48h}	t_{72h}	t_{24h}	t_{48h}	t_{72h}
T_{room}	LIIIS36B	SKM	100%	100%	100%	100%	100%	100%	0%	0%	20%
		MDX	100%	100%	100%	100%	100%	100%	0%	20%	40%
	HOMOGST27AB	SKM	60%	100%	100%	60%	80%	80%	0%	0%	0%
		MDX	40%	100%	100%	40%	80%	80%	0%	0%	0%
	HOMOGS20B	SKM	0%	0%	70%	0%	60%	80%	0%	50%	70%
		MDX	50%	50%	100%	50%	60%	80%	0%	0%	0%

Freeze-dried yeast strain *C. pseudoglaebosa* (LIIIS36B) effectively removed the color from the effluent (100%) in 24 hours, both at 60 and 90 days of storage with SKM and MDX. However, after 210 days of storage, only 20% and 40% of color were removed for SKM and MDX, respectively, which corroborates that the storage time influences the ability of decolorization. Strain *Y. lipolytica* (HOMOGST27AB) could remove color effectively (100%) with both SKM and MDX, at 60 days of storage after 48 hours. After 90 days, this strain with both cell protectors decolorized the simulated effluent at 80%. However, at 210 days of storage, none could remove color when stored at room temperature. *C. parapsilosis* (HOMOGS20B), after 60 days of storage with SKM, could remove 70% of color, 80% after 90 days and 70% after 210 days of storage. With MDX, yeast strain *C. parapsilosis* (HOMOGS20B) could remove color effectively (100%) in 72 hours after storage for 60 days and that decreased to 80% after 90 days. At 210 days, this yeast strain could not remove the color of the simulated effluent.

The same behavior of decolorization capacity was observed for *C. parapsilosis* (HOMOGS20B) and *Y. lipolytica* (HOMOGST27AB) which could be explained by the low growth capacity of these two yeasts when compared with *C. pseudoglaebosa* (LIIIS36B).

Strain *C. pseudoglaebosa* (LIIIS36B) was the most effective strain in removing color from the simulated effluent, for both cell protectors, and was effective after 90 days of storage. Nevertheless, the strains were able to decolorize and grow with both cell protectors for the simulated effluent Navy Everzol ED in study.

4.3.3.2. Without yeasts' recovery

An assay was performed to verify if freeze-dried yeasts powder was efficient in decolorizing if directly applied to the simulated effluent (without a previous recovery). Table 4.3 provides the results obtained from this experiment.

Table 4.3 – Decolorization of simulated effluent Navy Everzol ED (5 g/L) for freeze-dried yeasts *C. pseudoglaebosa* (LIIIS36B), *Y. lipolytica* (HOMOGST27AB), and *C. parapsilosis* (HOMOGS20B) with SKM, at 1%, 2%, and 5%, applied directly without yeasts' recovery. The darkest blue color represents the original color of the dye, which indicates 0% decolorization; the lighter blue color, indicates that decolorization has occurred - 100% represents total decolorization.

	1%			2%			5%		
	t_{24h}	t_{48h}	t_{72h}	t_{24h}	t_{48h}	t_{72h}	t_{24h}	t_{48h}	t_{72h}
LIIIS36B	0%	0%	0%	0%	50%	50%	20%	100%	100%
HOMOGST27AB	0%	0%	0%	20%	100%	100%	20%	100%	100%
HOMOGS20B	0%	0%	0%	0%	0%	0%	0%	0%	0%

Freeze-dried yeast strain *C. pseudoglaebosa* (LIIIS36B) was able to completely decolorize (100%) the simulated effluent at 5% in 48 hours, but only removed 50% of color at a 2% concentration and was not able to decolorize at 1% even after 72 hours. Freeze-dried yeast strain *Y. lipolytica* (HOMOGST27AB) with SKM was also able to totally decolorize the simulated effluent at 5% and 2% of powder in 48 hours but was not able to remove color at 1%. Yeast *C. parapsilosis* (HOMOGS20B) could not decolorize at any concentration tested.

Results show that the direct application of a freeze-dried powder in a textile effluent could be a convenient and straightforward solution for dye decolorization. In addition, this yeast-based solution had considerable stability in simple storage conditions. To the best of our knowledge, there are no studies referring to dye decolorization with freeze-dried microorganisms. The stabilized dehydrated yeast-based solution presented in this study would be quite promising, if cost-efficient, to be applied in the textile industry to decolorize colored effluents as a starter culture to colonize wastewater treatment plants for textile industries before releasing effluents into the municipal collectors.

4.3.4. Spray-drying

Spray-drying assays were only conducted with *Y. lipolytica* (HOMOGST27AB) because of equipment limitations and safety issues, with SKM and MDX as cell agent protectors. Drying process conditions were optimized to minimize losses from the process itself. The maximum yield obtained was 45.5% with SKM and 40.9% with MDX. At a lab-scale spray-dryer, where sample quantities are usually very small and the losses are high, such yield values are common (Barbosa *et al.,*2015). For that reason, we could not test its viability over time as not enough material was available. Temperatures were also optimized (T_{inlet} = 110 °C and T_{oulet} = 55 °C) to increase yield and not compromise yeasts' cell viability due to their thermal sensitivity. The viability of *Y. lipolytica* (HOMOGST27AB) cells before and after (t = 0 days) spray-dryer with SKM and MDX is shown in Figure 4.4.

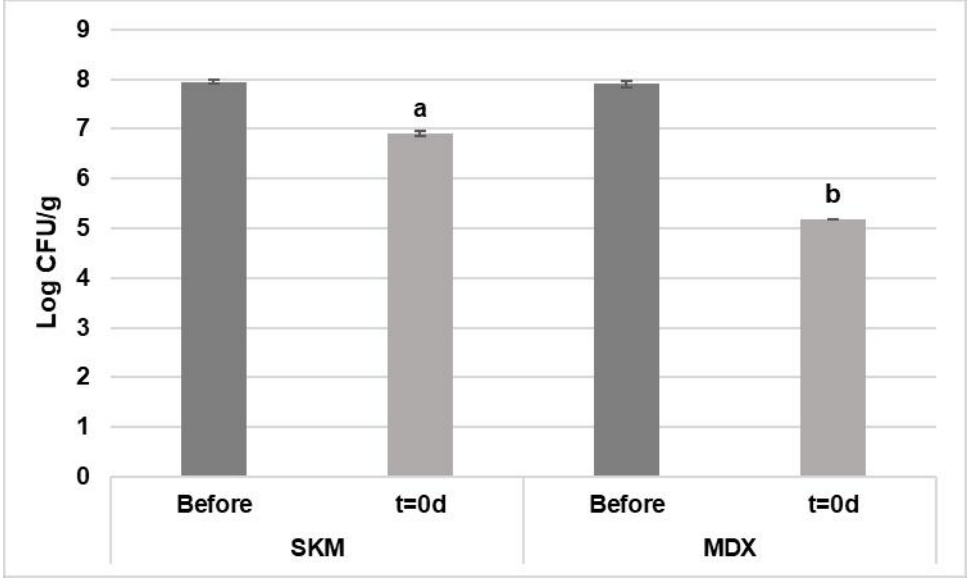

Figure 4.4 – Viability of *Y. lipolytica* (HOMOGST27AB) cells before and after (t = 0 days) spray-drying, with SKM and MDX as cell-protecting agents. Different superscript letters for significant differences ($p < 0.05$).

Cells of *Y. lipolytica* (HOMOGST27AB) survived the spray-drying tested conditions and grew with both cell protectors in the study (Figure 4.4). Even so, when analyzing viability with SKM before and after (t = 0 days) the drying process, cell viability decreased 1 log (from 8 logs CFU/g to 7 log CFU/g) instead of the 3 logs that decreased with MDX (from 8 logs CFU/g to 5 log CFU/g). There were significant differences between the protecting agents indicating that SKM was more efficient in protecting cells than MDX after spray-drying in the conditions tested. Although to the best of our knowledge, there are no studies that have tested spray-dried yeasts with these two protector agents under similar conditions, Tang *et al.* (2020) tested

SKM and MDX at 5, 10, and 20% with *Lactobacillus acidophilus* FTDC 3081 and concluded that MDX was more effective than SKM.

Comparing these results with those obtained from freeze-drying (Figure 4.1), in the last, viability decreased by 2 logs both in SKM and MDX, while in spray-dry the reduction was only 1 log for SKM and 3 logs for MDX. Therefore, SKM seems to be more efficient in protecting yeast cells when compared with MDX. This fact was not in accordance with the studies of Tang *et al.* (2020) that stated that MDX was more efficient than SKM at 10% with *Lactobacillus acidophilus* FTDC 3081.

Therefore, considering the promising results obtained, a pilot-scale (10x scale-up) spray-dryer process was carried out with inlet and outlet temperatures higher than on lab scale, due to equipment limitations. *Y. lipolytica* (HOMOGST27AB), previously recovered and suspended in SKM at 10% was sprayed, and the resulting process yield was about 70%, which was a considerable increase over the lab scale. However, due to the very high temperatures, yeasts could not survive after the pilot-scale spray-dry. Several studies have shown that the viability of microorganisms depends mainly on the inlet and outlet temperatures during the drying process (Jesus and Maciel Filho, 2014). Maintaining the same conditions of optimized lab-scale spray-drying in a pilot-scale spray-dryer with SKM would probably be an adequate solution for preserving and maintaining yeasts' viability and decolorization capacity. Moreover, this method has the advantage of being a more economical and less time-consuming technique, suitable for application in industrial facilities for effluent decolorization, if the decolorization capacity is maintained.

4.4. IMMOBILIZATION WITH ALGINATE CAPSULES

Another strategy to immobilize and stabilized yeasts involves encapsulating them in a matrix of sodium alginate and calcium chloride. The main advantages of encapsulation are the protection of the yeast against the external environment and consequently greater resistance of the formulation during the effluent treatment process.

Following the approach used for the other drying methods, the yeast *Y. lipolytica* (HOMOGST27A) was used to test the prove of concept for the production of alginate capsules. Alginate capsules loaded with yeast were successfully produced (Figure 4.5) with a yeast growth rate of 10^8 CFU/g. The alginate capsules were then tested for their decolorization capacity with the effluent Navy Everzol ED, using 1%, 2%, 5%, 20% and 40% of capsules with 25 mL of effluent, during 48 hours of incubation.

Although subtle, after incubation, only the flasks with 40% capsules showed a slight decolorization, while the 20% did not (Figure 4.5 – top row). Regarding dye adsorption, the test capsules with 20% concentration showed significant dye adsorption. As for the test with 40% concentration, the capsules showed less adsorption, which could mean that the little decolorization that took place was possibly related with the yeasts activity and was not due to adsorption (Figure 4.5 – bottom row).

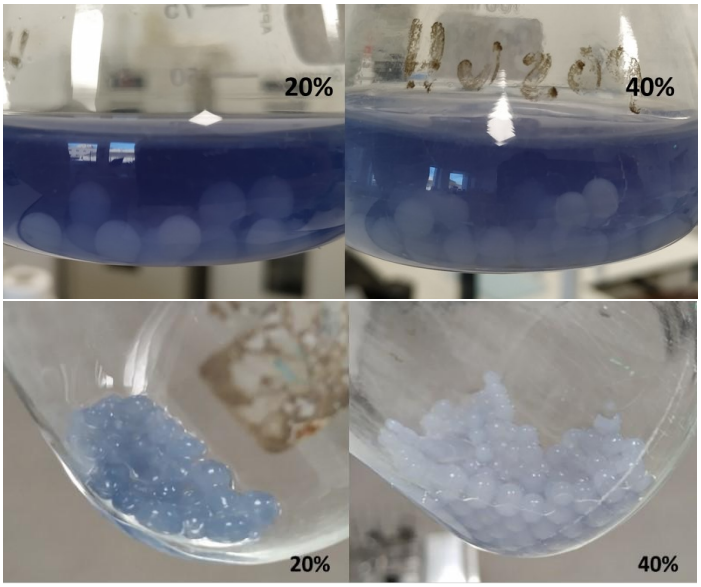

Figure 4.5 – Decolorization test (top row) and color adsorption (bottom row) of simulated effluent of Navy Everzol ED (diluted 6x) with yeast *Y. lipolytica* (HOMOGST27A) capsules in flask at concentrations of 20% and 40%.

One of the possible reasons for the ineffectiveness of decolorization could be the insufficient passage of the effluent through the matrix of the capsules, that could not reach the yeasts to be metabolized. This issue could be due to the very closed calcium-alginate matrix. In order to make these connections weaker and the matrix more open, new tests were performed in the same way but with a considerably lower concentration of sodium alginate (15 g/L) and placed in a sodium citrate solution (55 mmol/L) for 30 minutes. Sodium citrate allows the capsules to retain their liquid core, facilitating exchanges between effluent and yeast. However, the decolorization was not effective even under these conditions.

The possibility to pack a preparative column with the capsules in a preparative HPLC column with a continuous air diffuser was set up and would simulate a bioreactor for bioremediation (Figure 4.6). For these experiments, the capsules were placed in the column at about half of their volume (corresponding to ± 60 mL sodium alginate) and then 40 mL of simulated effluent of Navy Everzol ED was added.

This system packed with initial alginate capsules was able to decolorize the effluent in 14 hours (Figure 4.6). Using this system and replacing by new capsules, it was able to decolorize the dye in about 5 hours. Possibly the ratio between the quantity of capsules and effluent and the aerated environment may have contributed to the efficiency of the decolorization.

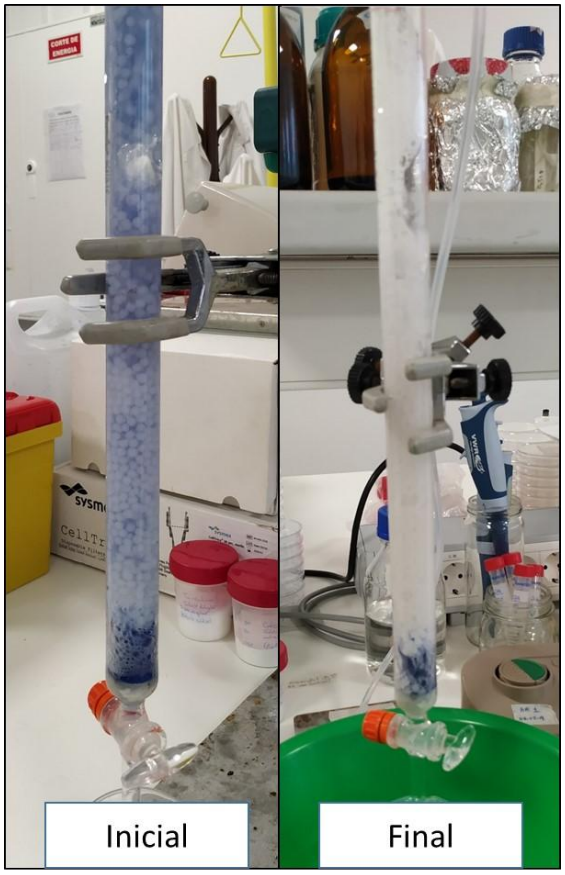

Figure 4.6 - Decolorization of simulated effluent of Navy Everzol ED (6x diluted) in a preparative column using yeast *Y. lipolytica* (HOMOGST27A) capsules.

Regarding the stability of the capsules prepared with sodium citrate throughout the time, the capsules were stored at 25 ºC and 4 ºC in a plastic container (100 mL) in a dark and dry place. The stability and viability of the yeast growth was evaluated after two months (Figure 4.7).

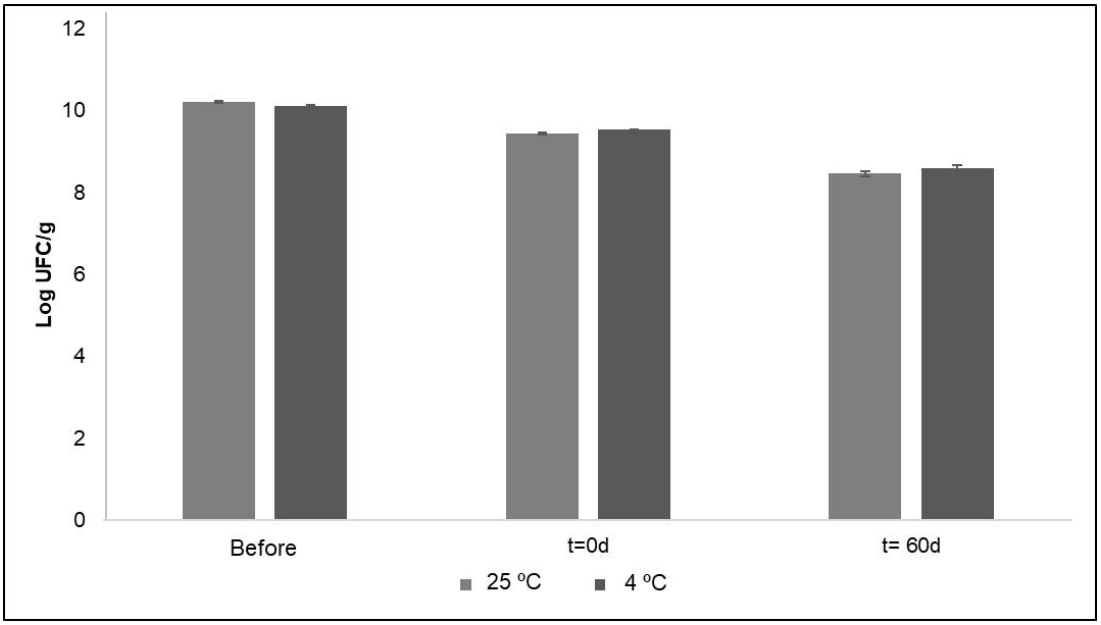

Figure 4.7 - Viability over time of encapsulated yeast *Y. lipolytica* (HOMOGST27A) stored at 25°C and 4°C.

After encapsulation, the yeast *Y. lipolytica* (HOMOGST27A) decreased 1 log in its viability and after two months of storage it presented a decrease of another logarithmic cycle. Only after two months of storage were evaluated, however the growth at the end of this time is very relevant, so although tested for experimental restrictions, the stability of this formulation is longer.

Final formulation

Considering the results obtained, the selected formulation of yeast immobilized by encapsulation consisted of 3 mm in diameter of capsules, with a matrix of calcium-alginate and yeast. According to this formulation, and taking into account the method used, for each 100 mL of capsules (formulated with 1.5 g of sodium alginate, 1 g of calcium chloride and 0.8 g of sodium citrate) used to pack the column, approximately 175 mL of NDM medium would be needed to grow 2.5 g of yeast.

4.5. ESTIMATION OF THE AMOUNT OF FORMULATION REQUIRED AND ASSOCIATED COSTS

The two formulations developed are quite different and therefore require different modes of application and consequently different amounts of final product. The decolorization method with encapsulated yeast requires a greater amount of final formulation applied to the effluent than the decolorization with freeze-dried yeast. However, capsules can be reused several times and therefore can be advantageous over freeze-dried yeast. In order to compare formulations tested in the laboratory, an estimate of cost was made considering the amounts of reagents and yeast needed for each of the formulations, as well as the associated costs (Table 4.4).

Table 4.4 - Composition of developed formulations and cost of reagents for **1 kg** (lyophilized yeasts) or **1L** (encapsulated yeasts) of formulation.

Formulation	Reagents	Cost (€/kg) [a]	Total Cost (€)	
Freeze-dried Yeast	*Skim Milk*	2	2	6.5
	Culture medium	100	4.5	
Encapsulated Yeast	Sodium Alginate	3.2	0.05	0.47
	Calcium Chloride	0.1	0.001	
	Sodium Citrate	0.82	0.007	
	Lactose	60	0.2	
	Culture medium	100	0.44	

[a]Source of reagents costs:

https://m.made-in-china.com/?gclid=EAIaIQobChMIqNvair3T6AIV04jVCh0a9QmdEAAYASAAEgKj2vD_BwE (2020)

Table 4.5 - Estimate of the amount of formulation and yeast needed to decolorize **1 m^3** of simulated effluent Navy Everzol ED (diluted 6x) and costs associated with the reagents needed for its preparation.

	Freeze-dried Yeast	**Encapsulated Yeast**
Yield (volume of effluent treated per kg of yeast)	132 L of treated effluent	264 L of treated effluent
Required yeast (wet weight) for 1 kg or 1 L of formulation	250 g	25 g
Mass or Volume of formulation required to decolorize 1 m^3 of effluent	30 kg	1200 L
Estimated cost to treat 1 m^3 of effluent	195 €	368 €
Number of decolorization cycles	1	8
Estimated cost per decolorization cycle	195 €	46 €

The yield values presented in this table considered that the calcium-alginate capsules used are capable of carrying out 8 decolorization cycles.

In Table 4.5, it is possible to verify that, for a tank with 1 m^3 of effluent, a greater initial investment is necessary in terms of the formulation of encapsulated yeasts, since it would be necessary to produce 1200 L. However, these yeast capsules can perform up to 8 decolorization cycles, that means, they could decolorize up to 8 m^3 of effluent. Thus, the estimated cost for each decolorization cycle would be just 46 €. In the case of freeze-dried yeasts, they only carry out one decolorization cycle, and the value per cycle is higher (195 €). Thus, considering the number of decolorization cycles for each formulation, the cost of treatment with freeze-dried yeast is ~0.2 € per liter of effluent and with encapsulated yeast is ~0.05 €, per liter of effluent. The estimated costs only consider the necessary reagents and culture medium (values obtained in laboratory reagents that would reduce a lot with industrialization), and it will also be necessary to consider all the production costs associated with each process. The simulated effluent used was only composed of chemicals and with a high dye load. In a textile wastewater treatment plant, the effluent will have a lower dye load and also would have other microorganisms associated with the biological treatment process that may help with the decolorization.

4.6. CONCLUSIONS

Both cell protectors, SKM and MDX, effectively protected yeasts' cells from damage that could be caused by both drying processes, although the use of SKM in freeze-dyer allowed lower reduction than MDX. The viability and decolorization capacity of the freeze-dried yeasts cells were maintained for at least 90 days, both at 4 °C and room temperature, especially for *C. pseudoglaebosa* (LIIIS36B). The viability of *Y. lipolytica* (HOMOGST27AB) was also maintained after spray-drying in lab-scale conditions. Thus, these drying processes could be used for cell immobilization of a standardized yeast-based solution, in particular *C. pseudoglaebosa* (LIIIS36B), which could be used for decolorizing industrial textile wastewaters since it combines stability, efficiency, and easiness of production and application in real industrial facilities. Also, yeast encapsulation process considering a matrix of calcium-alginate could be an interesting method with a reasonable cost, for decolorizing textile dyes in industrial textile wastewater facilities.

CHAPTER 5

BIOAUGMENTATION OF AEROBIC GRANULAR SLUDGE WITH DYE-DECOLORIZING YEAST FOR TEXTILE INDUSTRIAL WASTEWATER

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5.1. INTRODUCTION

Water is the most valuable natural resource for life on earth. Industrial wastewaters generated by the textile industries are one of the most important sources of global water pollution. These industries generate high amounts of dyed polluted effluents that are discharged into water bodies leading to ecological and adverse human health effects (Dellamatrice *et al.,*2017). These wastewaters are often charged with azo dyes that have intense color, together with surfactants, detergents, salts, solvents, and other compounds that are difficult to degrade because of their recalcitrancy. The amount of these compounds in textile wastewater is difficult to generalize because they differ in composition, depending on many different factors such as the type of process, chemicals, dyes, fabrics, and machinery used. However, dye effluents are usually high in color, pH, suspended solids (SS), chemical oxygen demand (COD), biochemical oxygen demand (BOD), metals, temperature, and salts (Yaseen and Scholz, 2019). Azo dyes are composed of one or more azo groups (R-N=N-R') that could be microbiologically reduced to amines, leading to dye decolorization under anaerobic/aerobic conditions (Saratale *et al.,*2011). Alternating anaerobic/aerobic conditions can be more effective for the treatment of dye-containing effluents (Sarvajith *et al.,*2018).

Activated sludge systems are commonly used for biological wastewater treatment but need large surface areas for system implementation that, in some regions, are a limited resource (Adav *et al.,*2008). Aerobic granular sludge (AGS) is an innovative technology for wastewater treatment with many advantages compared with conventional activated sludge systems. The lower initial investment cost, either in terms of energy consumption and less space needed due to the higher compactness of the granules formed with excellent settling properties, is one of the main advantages of AGS (Adav *et al.,*2008). The high tolerance to chemical toxicity, good effluent clarification, and high biomass retention contribute to the increasing interest in AGS technology, since the amount of wastewater that can be treated in the bioreactor increases (Adav *et al.,*2008; Amorim *et al.,*2018; Oliveira *et al.,*2020b; Ely *et al.,*2022). Moreover, the simultaneous removal of carbon and nutrients with high shock load resistance, all in a single reactor, contributes to the ecological benefits of this technology (Bassin *et al.,*2011; Ely *et al.,*2022). AGS comprises suspended microorganisms that bend together to form spherical aggregates that form clusters (de Kreuk *et al.,*2005). These structures are formed by an extracellular polymeric substance (EPS) matrix produced by the microorganisms involved, not needing an external carrier (Beun *et al.,*1999; Oliveira *et al.,*2020). EPS is composed of proteins, polysaccharides, humic compounds, and nucleic acids (Flemming, 2016). EPS leads to the creation of a layered structure that protects microorganisms from direct contact with the external environment and from the toxicity of the wastewater contaminants (Flemming, 2016). Additionally, some studies showed that AGS technology is

efficient in degrading toxic contaminants such as fluorophenol and endocrine disruptors (i.e.) among others (Amorim *et al.,*2016; Ely *et al.,*2022).

Despite all the benefits that AGS technology promises, the microorganisms present in the bioreactors may not be able to degrade specific pollutants. Single strains or microbial consortia have been used to bioaugment AGS systems for degrading contaminants such as herbicides and organofluorines, among others (Duque *et al.,*2011; Quan *et al.,*2015; Oliveira *et al.,*2021b). A few studies regarding the use of bacterial strains with dye-decolorizing capacity using AGS in SBR with synthetic wastewater were investigated with good results. Maqbool *et al.* (2020) tested *Pseudomonas aeruginosa* strain ZM130 for remediation of a reactive black-5 dyed wastewater with more than 80% of color removal; and Franca *et al.* (2020) tested a mix of bacterial strains in an SBR fed with a synthetic textile wastewater containing the dye Acid Red 14 with 91% of color removal. Also, results from the studies of Lourenço *et al.* (2015) demonstrated the potential application of non-specific bioaugmented AGS technology for dyed wastewater, with color removal of 75-80%. In addition, some yeasts are also being used for bioaugmentation with promising results. Louhasakul *et al.* (2019) tested *Yarrowia lipolytica* for bioaugmentation and biovalorization of oily industrial wastes, and Wen *et al.* (2022) used a high-efficiency salt-tolerant yeast, *Meyerozyma guilliermondii* W2, to biofortify a pilot-scale wastewater treatment plant to treat high-salinity organic wastewater. However, to the best of our knowledge, there are no previous reports on the use of yeast strains to carry out bioaugmentation in AGS systems.

In the present study, AGS granulation was promoted simultaneously with bioaugmentation with a dye-decolorizing yeast tolerant to salinity. The objective of this study was to access the ability of the yeast-bioaugmented AGS to remove an azo dye commonly used in the textile industry in saline environments. The reactor performance for main nutrient removal processes and the composition of the microbial community were evaluated during reactor operation.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals and materials

Textile dye Navy Everzol ED (NE), kindly provided by the company AQUITEX, SA (Porto, Portugal), was prepared by dissolving the dried powder in distilled water at the desired concentration. Stock, standard and working solutions of dye were filtered on syringe nylon membrane filters (0.22 µm pore-size) to sterilize. Acetonitrile, potassium chloride, and potassium phosphate monobasic (phosphate buffer) for HPLC mobile phases were purchased from Merck (Darmstadt, Germany). HPLC grade solvents were filtered with 0.45 µm Glass microfiber filters (Whatman™). Ultrapure water was supplied by a Milli-Q Gradient A-10 (Millipore) system (18.2 MΩcm, organic carbon ≤ 4 µg/L). SBR influent media were prepared with analytical-grade chemicals (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany). Sodium acetate was purchased from Merck (Darmstadt, Germany). All other chemicals used in this study were analytical grade (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany).

5.2.2. Cultivation conditions

A yeast strain, *Yarrowia lipolytica* (HOMOGST27AB), previously isolated, with decolorizing capacity for Navy Everzol ED dye, was selected for reactor bioaugmentation according to the results of the previous screening in Chapter 2. The inoculum at 2% was prepared in Normal Decolorization Medium (NDM) and incubated in an orbital shaker at 25 ºC/100 rpm, to a final concentration of $1.6x10^6$ CFU/mL. After centrifugation and washing with SBR medium (de Kreuk *et al.,*2005), the recovered pellet was reconstituted in 10 mL of SBR medium and introduced into the reactor through a syringe during the aeration phase on day 3 of the reactor operation. Pure cultures of *Y. lipolytica* were maintained in Normal Solid Decolorization Medium (NSDM).

5.2.3. AGS sequencing batch reactor (SBR) set-up

A lab-scale SBR with 110 cm of height, 6.5 cm of internal diameter, and 2.325 L of working volume was inoculated with the activated sludge from the aeration tank of an industrial wastewater treatment plant from a local brewing industry (Porto, Portugal) and bioaugmented with *Y. lipolytica* (HOMOGST27AB) inoculum prepared as described above. The bioreactor was operated in 3 hours cycles during the bioaugmented granulation process and then in 6 hours cycles (after day 65), at room temperature (25 ± 2 ºC). The experiment was carried out without pH and oxygen control. The system worked into four stages: 60 minutes of inlet anaerobic feeding, aeration (112 and 292 minutes for the 3 and 6 h-cycles, respectively), gradual decrease from 30 to 3 minutes of settling, and 5 minutes of effluent withdrawal. The inlet feeding was introduced from the bottom of the bioreactor; bottom aeration was sparged

at an airflow rate of 4 L/min and the withdrawn liquid in each cycle was approximately 40%. Automatic timers were used to control pumps during cycles. The SBR influent wastewater was composed of two solutions, as described by De Kreuk *et al.* (2005). Briefly, solution A was composed of $C_2H_3NaO_2$ 63 mM, $MgSO_4.7H_2O$ 3.6 mM, and KCl 4.7 mM, and solution B was composed of NH_4Cl 35.4 mM, Na_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM, and 10 mL/L of trace element solution (de Kreuk *et al.,*2005). NaCl (12 g/L) was added to solution B throughout the experimental period to mimic the salinity of a textile industrial wastewater (Kokabian *et al.,*2013). In each cycle, a total of 89 mL of solution A and B together with 772 mL of tap water was introduced from the bottom of the reactor to fulfill the working volume. The dye NE was introduced weekly (1 cycle/week) into the reactor from the top in phase II at 25 mg/L, phase III at 15 mg/L, and phase IV at 7.5 mg/L. Phase I (granulation) and phase V occurred without dye (Table 5.1).

Table 5.1. - Operating conditions tested in the SBR.

Phase	Length of operation (days)	Days of operation	Cycle time (h)	Anaerobic feeding (min)	Aeration (min)	Inlet acetate concentration (mM)	Inlet Dye concentration (mg/L)
I	0-89	90	3 (0-64d)	60	85-112	5.9	-
			6 (65-89d)	60	292		
II[a]	90-145	55	6	60	292	5.9	25
III[a]	146-243	97	6	60	292	5.9	15
IV[a]	244-271	27	6	60	292	5.9	7.5
V	272-289	17	6	60	292	5.9	-

[a] Dye Navy Everzol ED applied 1 cycle/week.

5.2.4. Analytical methods

Samples from the bioreactor were regularly collected (out of the dye addition days) from the inlet, influent, and effluent and filtered through nylon membrane syringe filters (0.45 µm pore-size) (Chromafil® PET filters, Macherey-Nagel, Germany) to remove biomass.

Quantification of nitrite (NO_2^--N), nitrate (NO_3^--N), phosphate (PO_4^{3-}-P), and ammonium (NH_4^+-N), was performed with photometric test kits (Spectroquant®, Merck Millipore, USA), according to the manufacturer's instructions. Total organic carbon (TOC) was measured using a Total Organic Carbon Analyzer (Shimadzu, Japan). Total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge retention time (SRT), and sludge volume index after 5 minutes of settling (SVI_5), were determined according to Standard Methods (APHA, 1998). The AGS bed volume was determined at the end of the settling period using a graduated scale placed on the reactor column.

Quantification of the Navy Everzol ED dye was carried out by chromatography based on the method of Mata *et al.* (2015) with some modifications: analysis was conducted on HPLC Agilent 1260 Infinity II (California, USA) with a Kromasil® C18 column, 250 x 4.6 mm i.d. 5 µm particle size and 100 Å pore size with a guard column (Kromasil® 3.0 x 4.6 mm). The mobile phase used was composed of sodium phosphate buffer (0.7 mL/min – 25 mM, pH 5.5) and acetonitrile running on a 30 minutes linear gradient from 100:0 to 50:50 (v/v), followed by a 5 minutes linear gradient up to 15:85 (v/v) and ending with a step back to 100:0 for another 5 min. Each sample was analyzed in duplicate and stored at -20 ºC until analysis.

The adsorption capacity of the aerobic granular sludge was estimated using equation 3:

$$\text{Adsorption capacity} = \frac{V\ (Ci - Cf)}{W}$$

(Equation 3)

where V is the working volume of the SBR, W is the dry weight of granules inside SBR, and C_i and C_f are the initial and the final dye concentrations in each cycle, respectively.

5.2.5. Recovery of Y. lipolytica from AGS and identification

To ascertain if the dye-decolorizing yeast was incorporated into the granules, isolation of *Y. lipolytica* from the reactor AGS was carried out by plating crushed granules onto agar (NSDM). Colonies that had the size, color, and morphology that corresponded to *Y. lipolytica* were selected. DNA extraction was performed using PowerSoil® DNA Isolation Kit (Mo Bio, USA), according to manufacturer instructions. The internal transcribed spacer ITS was amplified with universal primers ITS-4 (5' TCCTCCGCTTATTGATATGC-3') and ITS-5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). PCR was performed using 25 pmol of each primer, 25 µL of NZYTaq 2x Green Master Mix, 1 µL of DNA, and 22 µL of nuclease-free H_2O. Amplification occurred for 35 cycles, with 3 minutes denaturation at 95 ºC, followed by 30 s annealing at 50 ºC for ITS and 2 minutes extension at 72 ºC and with a final extension at 72 ºC for 10 min. The quality of DNA was evaluated by electrophoresis on 0.8% agarose gel. Sequencing was performed at Eurofins genomics (Leipzig, Germany), using ITS-5 primer. To confirm the phylogenetic affiliation, similarity searches were performed using the BLAST program from the National Centre of Biotechnology Information website: http://www.ncbi.nlm.nih.gov/ (accessed on 9 July 2021).

5.2.6. Microbial community analysis of AGS

AGS samples from the bioreactor were regularly collected through the operation during the aeration period of the cycle to obtain representative samples of the microbial population. Under aseptic conditions and using a sterile potter and pestle, the biomass sample was crushed, and the resulting suspension was stored at -20 ºC until use. Some samples were selected representing the different phases of the bioreactor (days 0, 86, 142, 177, 240, and 266) for further analysis.

DNA extraction was performed using PowerSoil® DNA Isolation Kit (Mo Bio, USA), according to manufacturer instructions. DNA extracted was quantified using a Qubit fluorometer (Invitrogen, USA) according to the manufacturer's protocol. DNA bioreactor samples were used to perform next-generation sequencing (NGS) analysis through Eurofins (Konstanz, Germany). This platform was used for DNA amplification, preparation of library sequencing, and bioinformatic analysis. Two primers, covering the V3-V4 hypervariable region, (357F – TACGGGAGGCAGCAG 800R – CCAGGGTATCTAATCC) were used, and paired-end sequencing based on 16S rRNA gene was conducted (Illumina MiSeq platform). Microbiome analysis comprehends demultiplexing, clipping of primer sequences, merging, quality filtering and microbiome profiling. To assign taxonomic information of the OTUs, DC-MEGABLAST alignments of cluster representative sequences to the sequence database were performed. Reference sequences with a minimum of 70% and 80% of identity and representative sequence, respectively, were selected and then OTUs and taxonomic assignments were

performed with the QIIME software package (version 1.9.1, http://qiime.org/, accessed on 27 September 2022). The raw sequence data were deposited in Sequence Read Archive (SRA) from NCBI database, under the accession number BioProject ID PRJNA885052 (http://www.ncbi.nlm.nih.gov/, accessed on 27 September 2022).

5.2.7. EPS Extraction

An experimental assay was performed at the end of the reactor operation to verify if the dye was linked to the EPS present in the granules or to the biomass. EPS was extracted by adding sodium carbonate (Na_2CO_3) and heating at 80 ºC continuously mixing according to Felz *et al.* (2016).

5.2.8. Image analysis

AGS representative samples were collected during the aeration phase throughout bioreactor operation, and images under a magnifying glass coupled to a digital camera (C-5060WZ, Olympus, Japan) were randomly captured to observe the AGS morphology.

5.2.9. AGS ability for dye removal in batch conditions

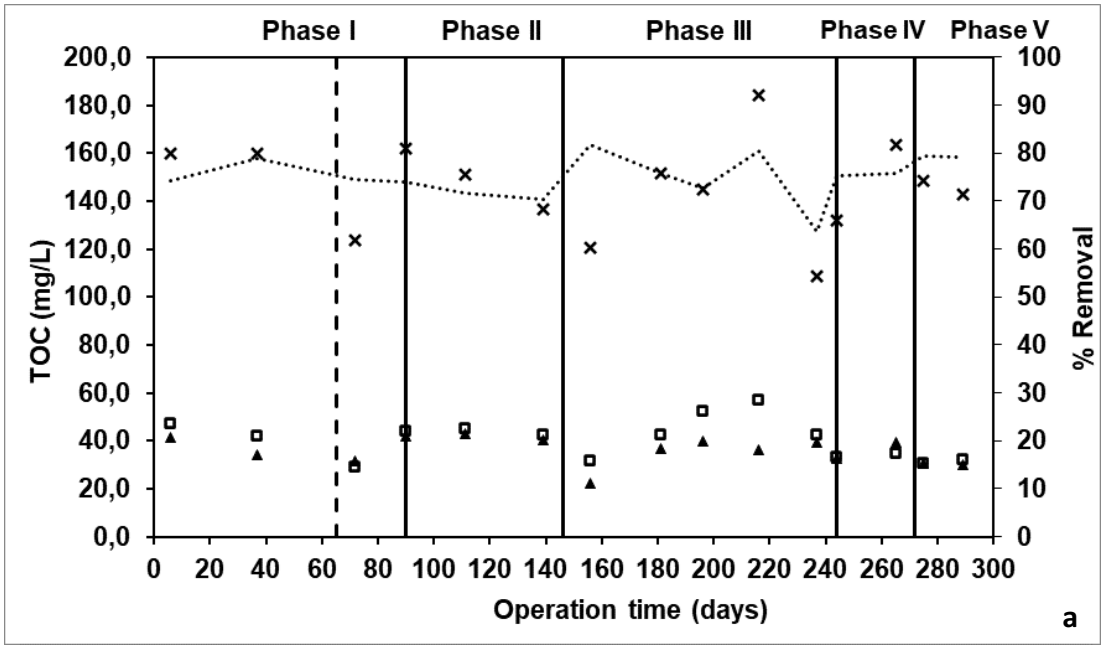
AGS ability for removing Navy Everzol ED dye was evaluated in culture flasks. The experiments were conducted in 100 mL sterile flasks containing 50 mL of SBR influent medium with [NE] = 7.5 mg/L and 5 mL of AGS from the bioreactor and heat-inactivated AGS (adsorption control). Cultures were incubated in an orbital shaker at 25 ºC at 100 rpm. Experiments were performed in duplicate under sterile conditions and protected from light. Abiotic control was also established.

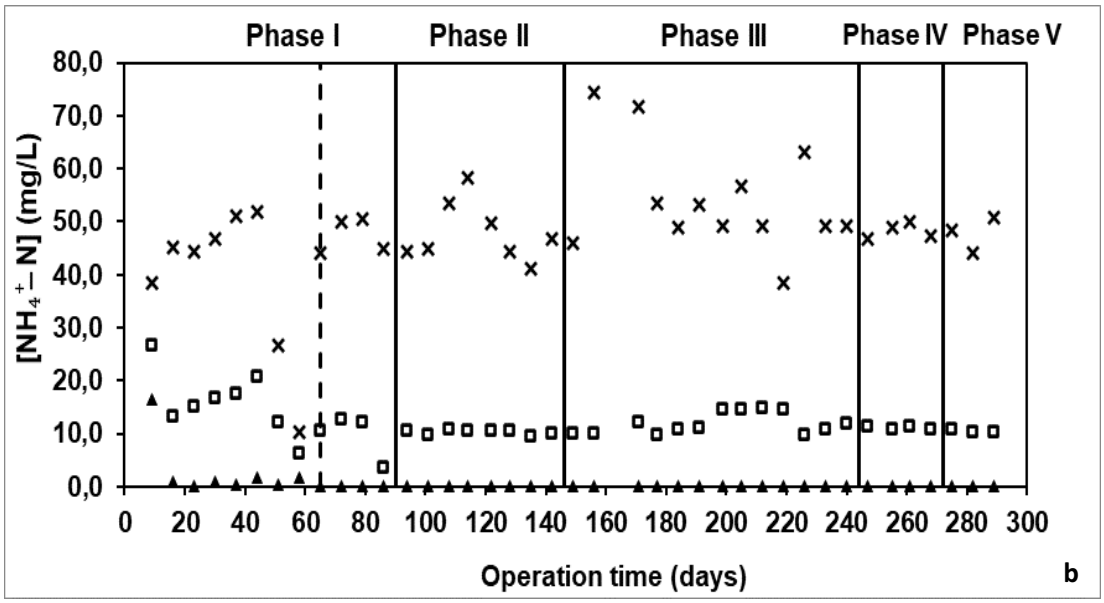
5.3. RESULTS AND DISCUSSION

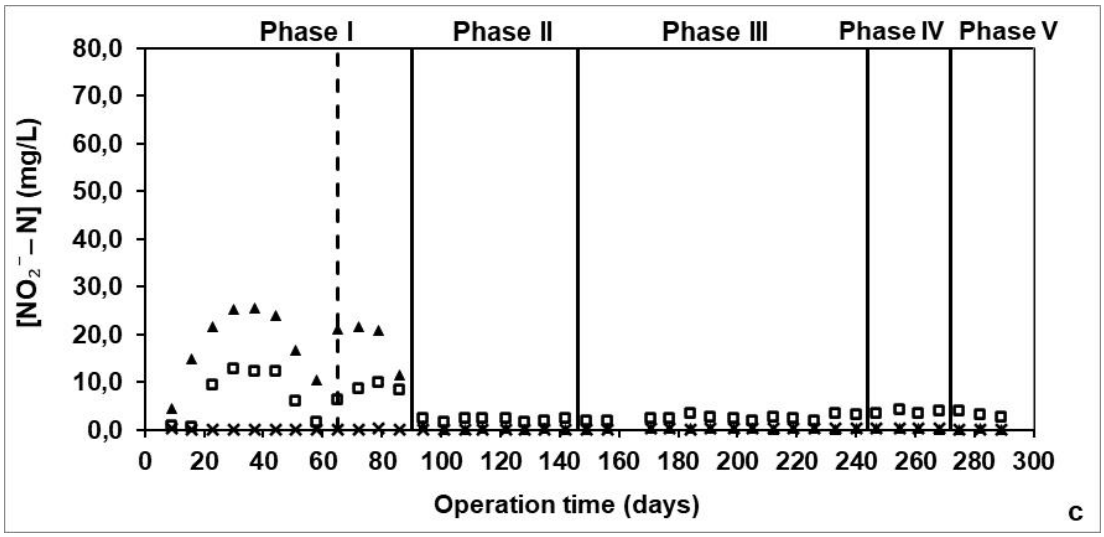
A bioreactor was inoculated with activated sludge from an industrial wastewater treatment plant and a dye-decolorizing yeast *Yarrowia lipolytica* (Phase I) to form bioaugmented granular sludge, under salty conditions (12 g/L). In the subsequent phases (II to IV), a textile dye (Navy Everzol ED) was inserted at different concentrations (25, 15, and 7.5 mg/L) to evaluate whether the decolorization of the dye would occur. Dye feeding was ceased in phase V. The reactor performance in the removal of carbon and nutrients, granular biomass properties, and microbial community were evaluated.

5.3.1. Reactor performance – removal of C and N

Figure 5.1 shows the profile of the carbon, ammonium, nitrate, and nitrite during AGS-SBR operation. The reactor performance was evaluated throughout the operation.




Phase I
Phase II
Phase III
Phase IV
Phase V
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
[NH4+ – N] (mg/L)
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
Operation time (days)
b


Phase I
Phase II
Phase III
Phase IV
Phase V
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
[NO2- – N] (mg/L)
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
Operation time (days)
c

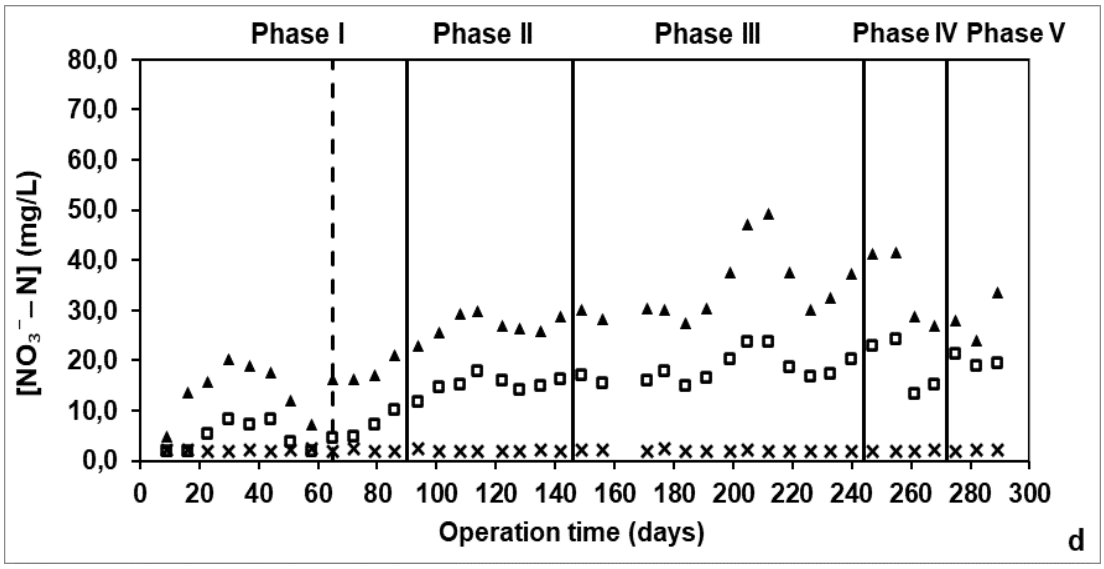


Figure 5.1 – TOC (a), NH_4^+-N (b), NO_2^--N (c), and NO_3^--N (d) concentrations during AGS-SBR operation. Concentrations in reactor influent (×), after anaerobic feeding (□) and effluent (▲), are shown. The doted lines indicate the removal percentage. The dashed vertical line indicates the beginning of 6h-cycles.

The bioreactor exhibited efficient carbon removal, with values of about 40 mg/L in the effluent (Figure 5.1-a), stable throughout the reactor operation (~75% removal). Although the carbon supplied was practically all consumed in the anaerobic phase, some amount of carbon was still present in the effluent (about 40 mg/L). This could be due to the presence of non-biodegradable organic matter. In the anaerobic phase of the bioreactor, microorganisms such PAOs (Polyphosphate-accumulating organisms) and GAOs (Glycogen-accumulating organisms) are of extreme importance for carbon removal. The selection of PAOs and GAOs is favored due to the absence of oxygen as a terminal electron acceptor. These organisms commonly present in AGS-SBR are intracellular accumulators of storage polymers from volatile fatty acids and use acetate from the influent medium as carbon and energy source for the aerobic phase (Lopez-Vazquez *et al.,*2009). In our bioreactor, removal of phosphate was not efficient (data not shown). Several authors mentioned that the presence of salt (NaCl) is difficult for the overall performance of the bioreactor, especially the effective removal of phosphate (Zhao *et al.,*2016; Oliveira *et al.,*2021a). High salinity may interfere directly or indirectly with cell division, inhibiting some microorganisms' growth and affecting the performance of the AGS-SBR (Zhao *et al.,*2016). Nevertheless, the high content of salt did not affect the efficiency of ammonium removal or the nitrification process. Figure 5.1-b shows that the ammonium provided to the SBR was consumed throughout all phases of the bioreactor operation. Somehow, the AGS structure could have protected the AOBs (Ammonia-oxidizing bacteria) and other nitrifying bacteria from the salt and the dye. All the ammonium (NH_4^-) provided was oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by these bacteria (Figure 5.1-

c, d). The addition of the dye Navy Everzol ED in Phase II (day 94) did not also affect the performance of the SBR. Previous studies with dyes had reported similar results in terms of the stability of carbon removal (Li *et al.,*2015; Sarvajith *et al.,*2018). The AGS-SBR bioaugmentation with *Y. lipolytica* was efficient and did not alter the performance of the SBR. Results from Li *et al.* (2015) showed a similar carbon removal (nearly 80%) in a submerged membrane bioreactor with activated sludge bioaugmented by a yeast *Candida tropicallis* TL-F1 for degradation and detoxification of Acid Red B. Sarvajith *et al.* (2018) stated that removal of carbon, nitrogen, and phosphorus was not impacted by azo dye loading in an AGS reactor operated for 80 days under microaerophilic conditions. Another study reported the efficient removal of chemical oxygen demand and detoxification in a lab-scale activated sludge-based membrane bioreactor bioaugmented with a halotolerant yeast (*Pichia occidentalis* G1) (Song *et al.,*2019). In addition, Assadi *et al.* (2018) stated that the presence of high nitrate concentrations had a very small effect on the decolorization performance of biomass. Furthermore, their results also showed that the reactor could maintain its performance in the presence of the dye compounds.

5.3.2. AGS settling properties

During the experimental operation, the bed volume of the AGS and the solid content of the bioreactor effluent were regularly measured (Figure 5.2). During phase I, the bed volume decreased due to the process of granulation. Phase II started with the addition of 25 mg/L of dye, which seemed to not cause high stress to the AGS since the bed volume did not decrease much. In fact, in Phase III, bed volume reached its maximum value and the solid content released in the effluent was the lowest of that found during SBR operation, which meant that the AGS was operating in good conditions. Bed volume in phases IV and V was maintained but the TSS value increased, probably due to the renovation of granular biomass.

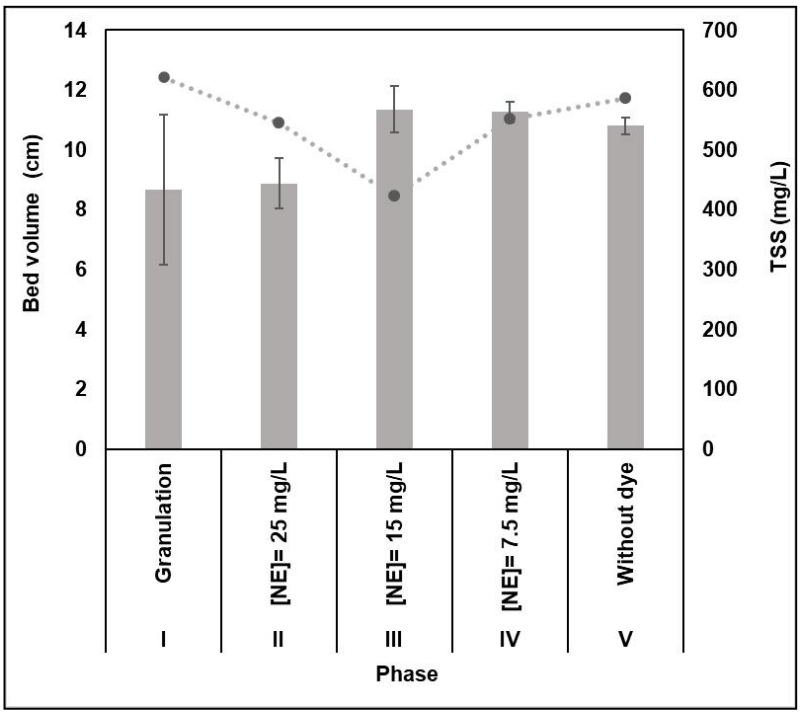


Figure 5.2 - Bed volume and effluent TSS throughout the experimental operation. The average bed volume is represented by columns in each phase and the average of TSS in the effluent is represented by the dotted line.

Table 5.2 summarizes the AGS properties and its physical characteristics during bioreactor operation. MLSS slightly decreases with the addition of the dye in Phase II. In phases III and IV, in which the dye concentration decreased, it was noticed that MLSS tended to increase, reaching its maximum in Phase V, without the dye addition. Accordingly, the same tendency was observed for MLVSS during the bioreactor operation. The ratio between MLVSS and MLSS that represents the biomass activity remained stable during the reactor operation.

Table 5.2 – Aerobic granular sludge properties along bioreactor operation.

Phase	MLSS (g/L)	MLVSS (g/L)	MLVSS/ MLSS	SVI_5 (mL/g_{sst})	SRT (days)
I	9.7	8.82	0.91	20.5	117.4
II	9.0	8.26	0.92	16.7	90.2
III	10.9	9.9	0.91	17.5	97.4
IV	12.3	11.2	0.90	19.3	142.2
V	20.7	18.9	0.91	14.5	231.3

Notes: MLSS, mixed liquor suspended solids; MLVSS, mixed liquor volatile suspended solids; SRT, sludge retention time; SVI_5, sludge volume index after 5 minutes of settling.

SRT decreased in Phase II and then gradually increased along the operation. SVI_5 decreased in Phase II but slightly increased in Phase III and IV, decreasing again in Phase V, indicating more dense and compact granules at the end of operation. Values of SRT and SVI_5 show the increase of AGS compressibility and settleability.

5.3.3. DYE REMOVAL

From Phase II to Phase IV of bioreactor operation, Navy Everzol ED dye was added to one cycle per week at the respective concentration, during the feeding phase, just prior to the start of aeration. Figure 5.3 shows the average of the dye concentration during the reactor cycles of two consecutive days after dye addition. Namely, influent samples collected 5 minutes after the start of the aeration, a sample of the effluent of the same 6 hours cycle, and the subsequent effluents of Day 1 cycles (3 cycles) (mix effluent sample). On Day 2, the influent, effluent, and mix effluent samples were collected.

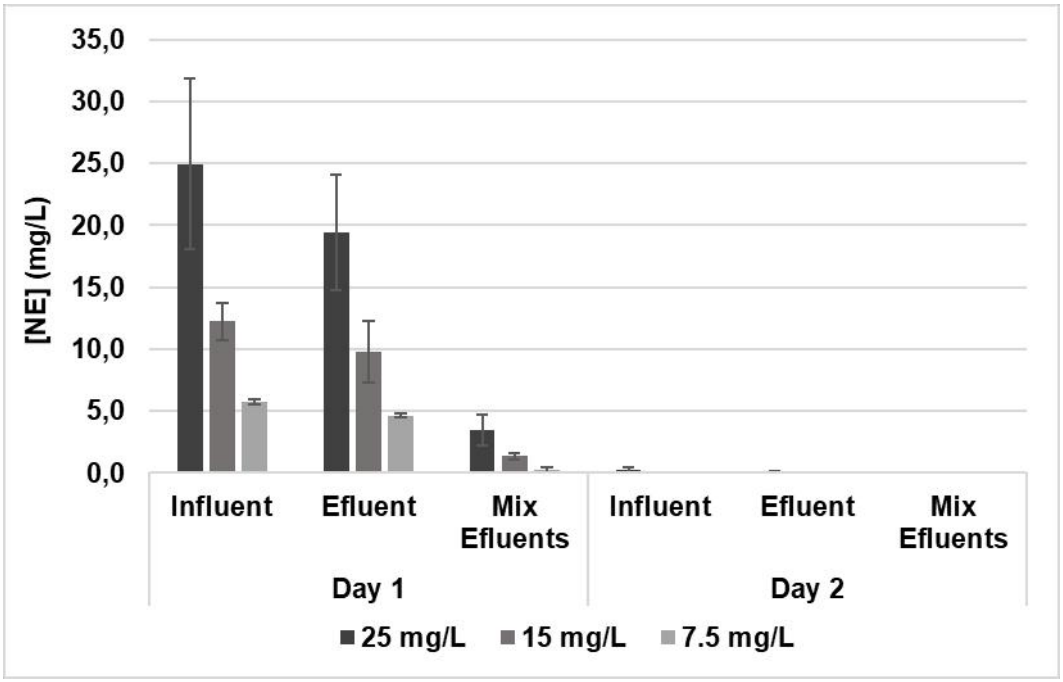


Figure 5.3 - Decrease of dye concentration in the reactor during 2 days of cycles.

Complementary data concerning Navy Everzol ED (NE) concentrations during AGS-SBR operation is shown in Figure 5.4.

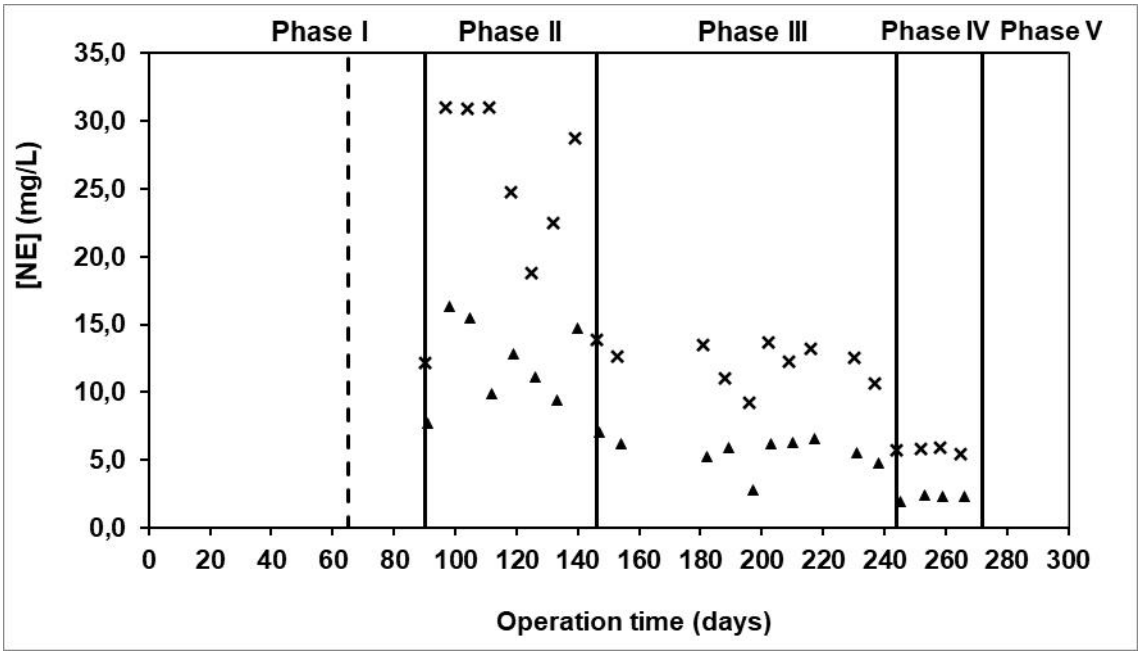


Figure 5.4 - Navy Everzol ED (NE) concentrations during AGS-SBR operation. Concentrations in reactor influent (×) and effluent (▲), are shown. The dashed line indicates the beginning of 6 hours cycles.

Results showed that in Phase II, the dye concentration decreased in the effluent, resulting in the removal of 51.6% of the total dye fed during this period (Table 5.3). In Phase III, the added dye concentration added decreased to 15 mg/L and the removal was 54.1%. In Phase IV, the dye feed decreased to 7.5 g/L, and the dye removal was 60.6%. During each cycle, some dye was retained in the reactor by adsorption of the dye to the aerobic granular biomass (Table 5.3).

Table 5.3 — Summary of the SBR performance for Navy Everzol ED dye (NE) removal and adsorption.

	[NE] mass balance (mg)			
Phase	[NE] in the inlet	[NE] in the effluent	% of Removal	Adsorption (mg/g VSS)
I	-	-	-	-
II	464.4	226.6	51,6 ± 8.9	3.9 ± 1.1
III	284.9	131.7	54.1 ± 7.0	1.86 ± 0.25
IV	53.5	21.1	60.6 ± 4.1	0.98 ± 0.08
V	-	-	-	-

Adsorption capacities of 3.9, 1.86 and 0.98 mg/g VSS were estimated for phases II, III and IV, respectively. No metabolites released were detected in the effluent, corroborating the hypothesis of dye adsorption to the aerobic granules.

There was adsorption of the dye to the granules, which remain colored until the end of reactor operation (no desorption observed). Figure 4 shows the evolution of the granules' color before and after the dye addition. On day 90, before the addition of the dye, granules were yellowish/beige with irregular form, and after the feeding with the dye Navy Everzol ED, the granules began to show some blue dots of dye adsorption all over its surface (Figure 5.5 – b, c, and d).

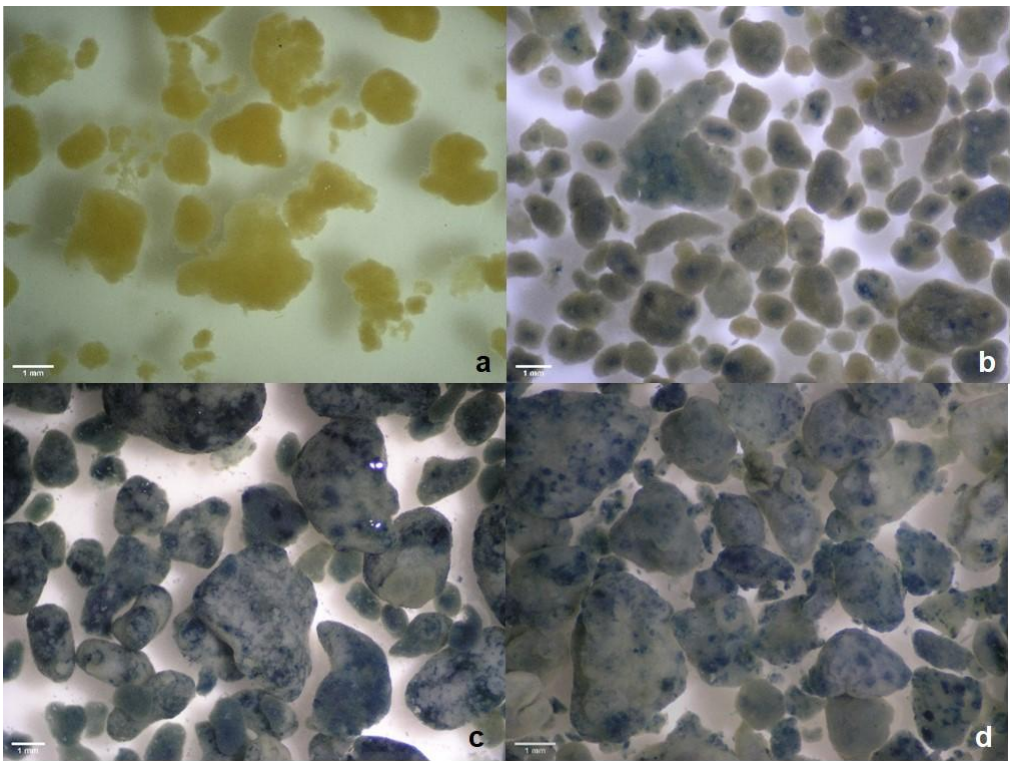


Figure 5.5 – Examples of the evolution of granules' color throughout the reactor operation under a magnifying glass. a - day 90 (before dye); b – day 100; c – day 156; d – day 268.

To evaluate if the dye was linked to the biomass or the EPS from the granules, some assays involving EPS extraction were performed. After the EPS extraction, the supernatant that contained the EPS remained light yellowish and the pellet remained blue, which suggests that the dye did not adsorb to the EPS but to the biomass (Figure 5.6). There are several reports on the capacity of AGS for the biosorption of several contaminants, including dyes (Amorim *et al.,*2007). In a previous study, it was observed that the yeast strain *Y. lipolytica* (HOMOGST27AB) used for the bioaugmentation partially adsorbs the Navy Everzol ED dye (Chapter 2 of this thesis). These observations indicated that the dye could be adsorbed to the biomass, namely to the *Y. lipolytica* (HOMOGST27AB). Some similar studies stated that some microorganisms, inclusively from granular sludge, have the ability to adsorb large molecules of organic pollutants, such as dye molecules (He *et al.,*2021). Meehan *et al.* (2006) tested the

decolorization of Remazol Black-B using a thermotolerant yeast (*Kluyveromyces marxianus* IMB3) and concluded that the decolorization occurred due to biosorption to the yeast cells and not due to a metabolic reaction. Wang *et al.* (2020, 2021) studied a marine bacterium (*Aliiglaciecola lipolytica*) that first adsorbed the azo dye Congo Red onto cells by the secreted EPS (46 %) and then the rest of the decolorization was achieved by the action of an azoreductase and laccase. In addition, immobilized dead cells from *Candida tropicalis* were efficient as a dye biosorbent from textile wastewater (Charumathi and Das, 2012).

Figure 5.6 – Example of the EPS extraction assays. The tube on the left contains the pellet with biomass after the EPS extraction; and the other two tubes show the supernatant with EPS resulting from the first and second successive extractions, in the middle and right tube, respectively.

5.3.4. Ability for dye removal in batch conditions

The ability for dye removal of bioaugmented AGS in batch conditions was investigated. The color of the dye in the supernatant was totally removed both in alive (91%) and heat-inactivated AGS (88%). The granules were blue both in live and heat-inactivated AGS due to the dye adsorption (Figure 5.7 and Figure 5.8). This result confirmed that the AGS biomass was able to remove dye by adsorption and no desorption occurred. It is known that biosorption is common to several yeasts, dead or alive (Aksu, 2005). Aksu and Dönmez (2003) studied and compared the biosorption capacities of several yeasts to remove Remazol Blue reactive dye and concluded that among nine yeasts, *Candida lipolytica* was the one that exhibited the highest dye uptake capacity. In addition, a thermotolerant yeast (*Kluyveromyces marxianus* IMB3) was efficient in adsorbing Remazol Black-B, and immobilized dead cells of *Candida tropicalis* were efficient in removing dyes from textile wastewater (Meehan *et al.,*2006; Charumathi and Das, 2012).

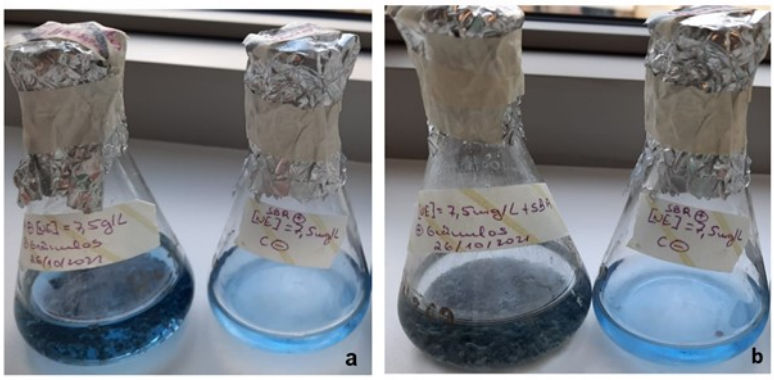


Figure 5.7 – Batch liquid cultures containing aerobic granules in SBR medium with Navy Everzol ED dye ([NE] = 7.5 mg/L). The flask on the right is the abiotic control without granules. a) shows the beginning of the experiment with blue-colored liquid; b) experiment after 24 hours with colorless liquid and colored granules.

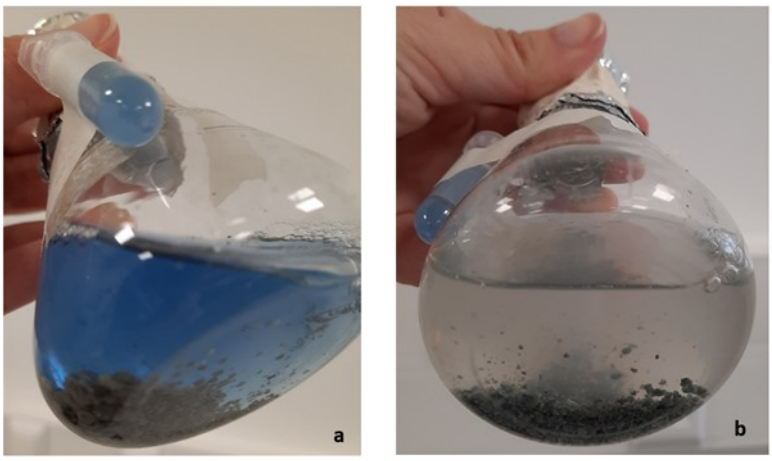


Figure 5.8 – Batch liquid cultures containing heat-inactivated aerobic granules in SBR medium with Navy Everzol ED dye ([NE] = 7.5 mg/L). a) shows the beginning of the experiment with blue colored flask; b) experiment after 24/48 hours with colorless flask and colored granules.

5.3.5. Recovery of the bioaugmented yeast along reactor operation

During reactor operation, several samples of the bioaugmented AGS were collected to evaluate the presence of the dye-decolorizing yeast on the granules. Plating successive dilutions of crushed AGS allowed the recovery of the bioaugmented yeast throughout reactor operation. The identity of the yeast was confirmed by sequencing of the ITS region. These results showed that the yeast *Y. lipolytica* was incorporated into the granules until the end of the reactor operation. To the best of our knowledge, this is the first study reporting the successful formation of aerobic granules bioaugmented with a yeast strain.

In the present study, the dye removal was not complete, even with the dye-decolorizing yeast bioaugmentation. Although the bioaugmentation was successfully achieved, possibly the amount of yeast was not sufficient for the complete dye degradation to occur; it is possible that they faced fierce competition from the other microorganisms present in the reactor. Another explanation could be the time of contact that is needed for the yeast to decolorize the dye. According to previous studies, *Y. lipolytica* (HOMOGST27AB) needs 48-72 hours to decolorize reactive dyes (Chapter 2 of this thesis), so possibly this was a limiting step for the dye decolorization in a 6-hour cycle bioreactor. Another explanation could be the location of the yeast in the granule: in the inner layers, more protected from external toxics, the dye may be inaccessible for the yeasts to degrade (Oliveira *et al.,*2021a).

5.3.6. Microbial community of AGS

A total of 318 OTUs were detected in the AGS microbiome. The bacterial composition of the granules at the class level along with the identification of the respective phylum are presented in Figure 5.9. Bacteria belonging to the Phylum *Proteobacteria* were the most abundant in all samples (47.9%) followed by the *Bacteroidetes* (27.2%); for the activated sludge used to inoculate the bioreactor (day 0), in which it was the dominant Phylum, was the *Ignavibacteriae* (25.4%). This phylum was abundant in the activated sludge inoculum, however, through the granulation *Ignavibacteriae* decreased to a residual number (<1%). *Nitrospirae* and *Acidobacteria* had a similar behavior, being present at 6.1% and 3.4% in the biomass inoculum, respectively, but significantly decreasing in the following phases (<1%). On the other hand, *Acidobacteria* were not present in the initial granules but slightly increased along the subsequent phases. Another phylum that increased during bioreactor operation was *Actinobacteria*, reaching 25.4% in phase IV. Other phyla, such as *Gemmatimonadetes*, showed a relative abundance lower than 2%. Considering class level, overall, *Betaproteobacteria* (19.2%), *Alphaproteobacteria* (18.0%), *Flavobacteriia* (16.9%) and *Actinobacteria* (14.6%) were the most abundant (Figure 5.9). The most abundant class in the seed sludge was *Ignavibacteria* (25.5%) followed by *Betaproteobacteria* (24.3%). In the subsequent samplings (including Phase I-b), the most abundant classes were *Flavobacteriia*, *Alphaproteobacteria*, *Betaproteobacteria*, *Actinobacteria*, and *Gammaproteobacteria*. The abundance of *Actinobacteria* increased from 3.2% in phase I-b to 25.4% in Phase IV. The same tendency was observed for *Cytophagia* (1.8% to 13.3%). The inverse tendency was observed for *Flavobacteriia* that decreased from 30.1% in Phase I-b to 11.2% in Phase IV, *Alphaproteobacteria* (from 27.9% to 15.7%) and *Gammaproteobacteria* (11.4% to 3.5%). The relative abundance of *Betaproteobacteria* varied from 12.5% to 25.0% along the phases. The remaining classes had a relative abundance lower than 2.0%.

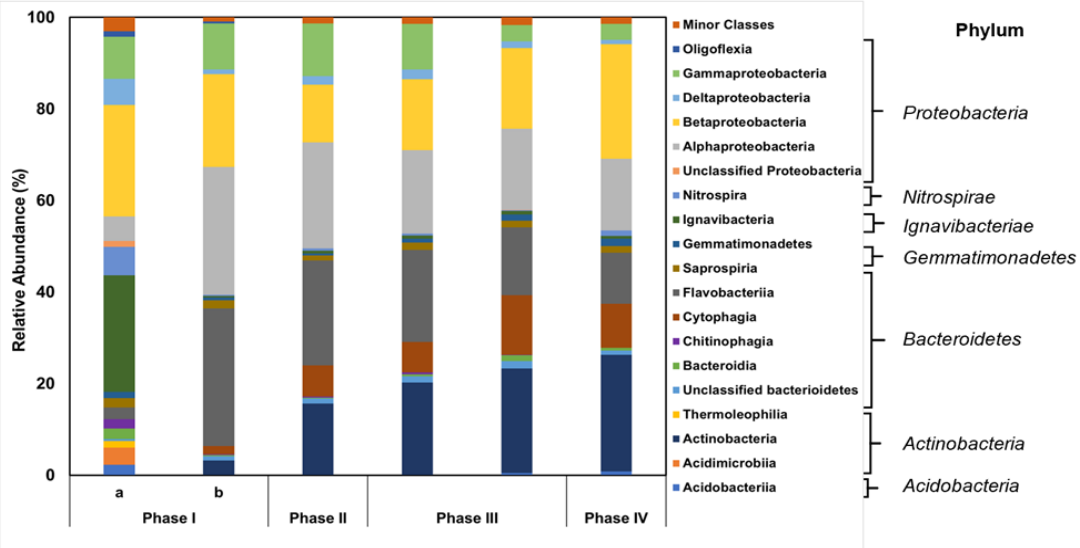
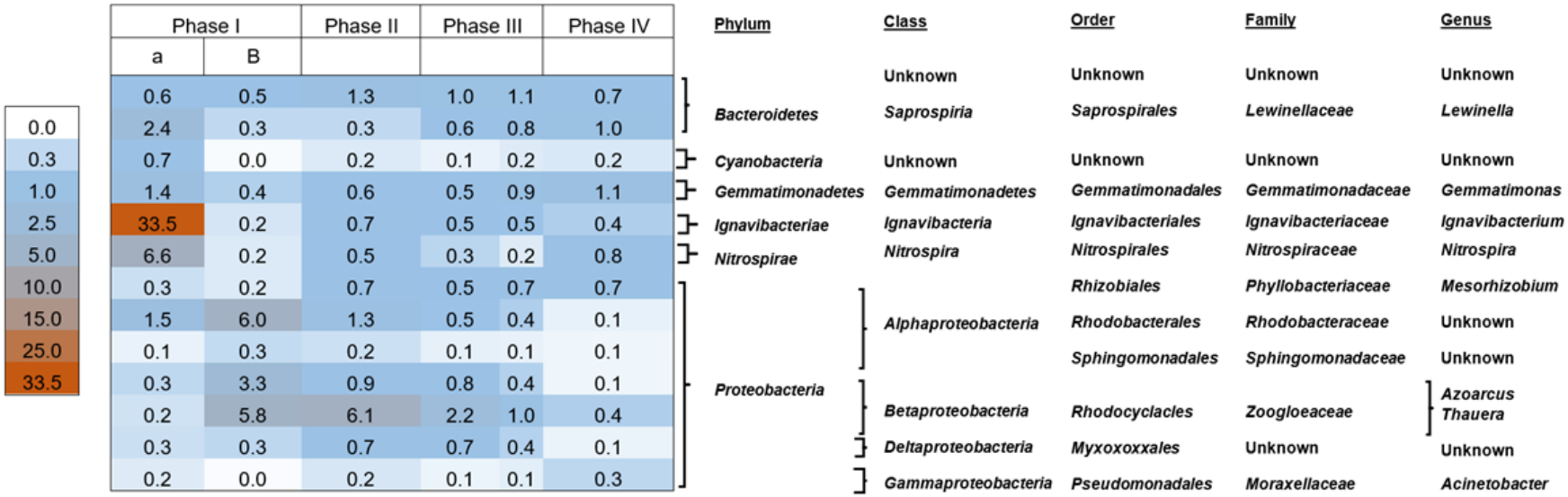


Figure 5.9 - Relative abundance of bacterial groups at class level with their respective phyla. Phase I-IV represents the different phases of the reactor according to Table 1; Phase I-a shows the relative abundance of bacteria of the seed sludge and Phase I-b shows the relative abundance of bacteria in the final of granulation.

Figure 6 represents the heatmap with the most abundant genus of bacteria present in the AGS biomass. When the genus was unknown, the immediately preceding category was represented. *Proteobacteria* was the most abundant phylum of the AGS biomass, comprising seven of the most abundant genera belonging to this phylum (*Mesorhizobium*, Unknown (2), *Azoarcus*, *Thauera*, *Minicystis*, and *Acinetobacter*), though *Ignavibacterium* was the most relative abundant phylum in the seed inoculum.

Organisms from the *Proteobacteria* phylum are generally dominant in AGS and were shown to play a crucial role in the granulation process (Basri *et al.,*2022). These Gram-negative organisms have lipopolysaccharides with cohesive properties on their outer surface that facilitate attachment to the suspended sludge particles and formation of granules (Wu *et al.,*2018). *Proteobacteria* also increase EPS production, promoting the adhesion of floc sludge to become granules (Basri *et al.,*2022). Moreover, the removal of carbon, nitrogen, and phosphorus was shown to increase due to the presence of the organisms from this phylum, playing a significant role in the removal of other pollutants (Xu *et al.,*2022). *Mesorhizobium* and *Thauera* were the most abundant organisms from the *Proteobacteria* phylum found in the AGS-SBR. *Mesorhizobium* is a known nitrogen-fixing bacteria and *Thauera* is an important flocculent aerobic denitrification microorganism that contributes to the degradation of organic matter in wastewater treatment plants and also improves EPS secretion in AGS (Kim *et al.,*2021; Xu *et al.,*2022).



Phase I		Phase II	Phase III		Phase IV	Phylum	Class	Order	Family	Genus
a	B									
0.6	0.5	1.3	1.0	1.1	0.7		Unknown	Unknown	Unknown	Unknown
2.4	0.3	0.3	0.6	0.8	1.0	*Bacteroidetes*	*Saprospiria*	*Saprospirales*	*Lewinellaceae*	*Lewinella*
0.7	0.0	0.2	0.1	0.2	0.2	*Cyanobacteria*	Unknown	Unknown	Unknown	Unknown
1.4	0.4	0.6	0.5	0.9	1.1	*Gemmatimonadetes*	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadaceae*	*Gemmatimonas*
33.5	0.2	0.7	0.5	0.5	0.4	*Ignavibacteriae*	*Ignavibacteria*	*Ignavibacteriales*	*Ignavibacteriaceae*	*Ignavibacterium*
6.6	0.2	0.5	0.3	0.2	0.8	*Nitrospirae*	*Nitrospira*	*Nitrospirales*	*Nitrospiraceae*	*Nitrospira*
0.3	0.2	0.7	0.5	0.7	0.7			*Rhizobiales*	*Phyllobacteriaceae*	*Mesorhizobium*
1.5	6.0	1.3	0.5	0.4	0.1	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	Unknown
0.1	0.3	0.2	0.1	0.1	0.1			*Sphingomonadales*	*Sphingomonadaceae*	Unknown
0.3	3.3	0.9	0.8	0.4	0.1					
0.2	5.8	6.1	2.2	1.0	0.4		*Betaproteobacteria*	*Rhodocyclacles*	*Zoogloeaceae*	*Azoarcus* *Thauera*
0.3	0.3	0.7	0.7	0.4	0.1		*Deltaproteobacteria*	*Myxoxoxxales*	Unknown	Unknown
0.2	0.0	0.2	0.1	0.1	0.3		*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*

Figure 6 - Heatmap representing the most abundant bacterial genus in AGS biomass (% relative abundance). When the genus was unknown, the immediately preceding category was represented.

Bacteroidetes were the second-most abundant Phylum in the AGS-SBR. These Gram-negative, mostly aerobic non-spore-forming bacteria (ex. *Lewinella*) are widely distributed in the environment and are commonly found in wastewater treatment plants (Xu *et al.,*2022). They are one of the most important organisms responsible for the nitrification process of the AGS and the production of EPS and granule formation. On the other hand, an excess of these organisms could damage the stability of granules' structure (Basri *et al.,*2022). In less abundance in the AGS-SBR, we found organisms from *Nitrospirae*, *Ignavibacteria*, *Gemmatimonadetes*, and *Cyanobacteria* phyla. *Nitrospirae* comprises nitrobacteria such as *Nitrospira,* which are common ammonia-oxidizing bacteria that are present in wastewater treatment plants (Xu *et al.,*2022). *Gemmatimonadetes* are known to be a polyphosphate-accumulating microorganism, playing a role in removing phosphate from wastewater and also in the production of EPS (Basri *et al.,*2022). *Ignavibacteria*, the most abundant organisms in the activated sludge provided, are a strictly anaerobic heterotrophic bacterium with the capacity of decompose organic matter in activated sludge (Wang *et al.,*2020a). *Cyanobacteria* are photoautotrophic organisms that are commonly present in wastewater treatment plants and have been reported to be useful for the treatment of wastewater with some chemicals such as antibiotics, pesticides, and detergents since they can metabolize nitrogen, phosphorus, carbon, and sulfur compounds (Kalavathi *et al.,*2001). In the present study, *Cyanobacteria* were present in very low quantity, decreasing from the granulation to the subsequent phases, and therefore possibly not contributing much to the performance of the reactor.

5.4. CONCLUSIONS

In this study, the granulation of activated sludge bioaugmented with the dye-decolorizing yeast *Yarrowia lipolytica* (HOMOGST27AB) in synthetic saline wastewater was successful. AGS-SBR performance in terms of carbon and nitrogen removal from simulated saline wastewater was not affected by the presence of dye Navy Everzol ED. Dye degradation was not achieved by the microbial community present in the AGS-SBR, possibly because the time of contact with the dye was not enough for the decolorization to occur or the proportion of yeast in the AGS was not adequate for dye degradation. Even so, the dye was adsorbed in the granules. Molecular analysis showed the dynamic diversity of AGS-SBR microbial community profile where *Proteobacteria* and also *Bacteroidetes* were predominant. Further studies should be conducted to fully understand the dye adsorption mechanisms by the yeast augmented AGS.

CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE WORK

6.1. GENERAL CONCLUSIONS

The experimental work described in this PhD thesis intends to further the knowledge on the biological decolorization of textile dyes using selected yeasts, and thus to develop potential innovative biotechnological solutions to treat the textile-dyed effluents in order to minimize their environmental impact. The textile industry produces high quantities of effluents with a complex mixture of synthetic dyes that can cause several environmental and health problems. This has led to an increase in the search for new solutions for wastewater treatment.

Initially, a screening to evaluate the performance of three yeast strains (and a combination between them) with a previous indication of decolorization ability was used to select the most efficient yeast at decolorizing a wide range of textile dyes and simulated effluents. Tested strains, especially *Y. lipolytica* (HOMOGST27AB) and *C. pseudoglaebosa* (LIIIS36B) either isolated or in consortium were effective in removing above 50% color, both by adsorption or biodegradation, from commercial synthetic dyes and simulated textile effluents. Reactive dyes were the class that in general seemed to be removed more efficiently by true decolorization (from around 82% to 100%). Yet, the mechanisms behind the decolorization process used by these yeasts could not be unveiled through the methodologies used.

The end-products and the toxicity at different trophic levels of the resulting streams after yeast treatment were investigated to evaluate their safety when discharged into the environment. Although the resulting metabolites could not be identified, the toxicity of the resulting streams decreased after treatment with yeasts, showing that the treatment did not produce toxic end-products. However, treated samples did not show toxicity to bacteria and lettuce seeds but were inconclusive for *Daphnia*. Furthermore, no mutagenic effect was detected.

Thereafter, to stabilize and formulate the decolorizing yeasts, a dried yeast-based product with dye-decolorization capacity was developed. Potential strategies for the preservation and long-term storage of the formulations established were studied: spray and freeze drying, and alginate capsules as an immobilization strategy. The viability of the yeast strains cells after being subjected to drying processes with different cell protectors throughout storage time (at room and refrigerated temperature) was evaluated. Both cell protectors studied, SKM and MDX, effectively protected yeasts' cells from damage that could be caused by both drying

processes, although the use of SKM in freeze-dyer allowed lower reduction than MDX (SKM decreased about 4 logs, whereas MDX decreased about 6 logs, over storage time). The viability and decolorization capacity of the freeze-dried yeasts cells were maintained for at least 90 days, both at 4 °C and room temperature, especially for *C. pseudoglaebosa* (LIIIS36B). The viability of *Y. lipolytica* (HOMOGST27AB) was also maintained after spray-drying in lab-scale conditions. Thus, these drying processes could be used for cell immobilization of a standardized yeast-based solution, in particular *C. pseudoglaebosa* (LIIIS36B), which could be used for decolorizing industrial textile wastewaters since it combines stability, efficiency, and easiness of production and application in real industrial facilities. Also, yeast *Y. lipolytica* (HOMOGST27A) encapsulation within a matrix of calcium-alginate was efficient in dye and simulated effluent decolorization, and its viability over 60 days of storage only decreased by 2 logs. Therefore, this could be another relevant method with a reasonable cost, for decolorizing textile dyes in industrial textile wastewater facilities.

Furthermore, a laboratory-scale reactor with a simulated textile wastewater (containing Navy Everzol ED dye and salinity conditions) and bioaugmented with the decolorizing *yeast Y. lipolytica* (HOMOGST27AB) was tested for the capacity of degrading nutrients and dye color removal. AGS granulation was promoted simultaneously with bioaugmentation with a dye-decolorizing yeast tolerant to salinity. The reactor performance for main nutrient removal processes and the composition of the microbial community were evaluated during reactor operation. The granulation of activated sludge bioaugmented with the dye-decolorizing yeast *Y. lipolytica* (HOMOGST27AB) in synthetic saline wastewater was successfully achieved and its presence was maintained throughout the reactor process. The AGS-SBR performance in terms of carbon and nitrogen removal from simulated saline wastewater was not affected by the presence of dye Navy Everzol ED. However, dye degradation was not achieved by the microbial community present in the AGS-SBR. Even so, the dye was adsorbed in the granules. Finally, the bacterial profile from the bioaugmented AGS was investigated to reveal the communities responsible for the reactor performance and molecular analysis showed a dynamic diversity of AGS-SBR microbial community profile where *Proteobacteria* and also *Bacteroidetes* were predominant.

These yeast-based products may be used as a potential biotechnological tool to treat wastewater resulting from the textile industries instead of classical chemical treatments, however more research is needed regarding operational scenarios for practical application. This could be an advantage and a promising solution for this environmental worldwide problem, contributing to the circular economy and eco-sustainability.

6.2. FUTURE WORK

The research proposed in this PhD thesis encompasses a novelty on its own, concerning the development of innovative biotechnological solutions for the biological decolorization of textile dyes using selected yeasts with dye-decolorizing capacity with the potential to solve problems concerning the presence of dyes in the textile industry effluents considering their environmental impact. Therefore, during the development of this research and from the results obtained, some suggestions for further research are given in this section:

- More studies are necessary to deepen knowledge of the mechanisms behind the decolorization process by the yeasts. Those mechanisms need to be clarified, as it is suggested that not only single enzymes seem to be involved in the decolorization of dyes;

- Identify and explore the enzymatic toolbox present in each yeast to understand its role in dye decolorization as well as its mechanisms and modes of action;

- Identify the metabolites that result from yeast's metabolism after dye decolorization, to understand its nature and its implications for animal and human health as well as for the environment, if released into the aquatic ecosystems;

- Spray-dried yeast should be prepared on a larger scale, under the same lab-scale conditions, to obtain enough quantities to conduct the viability tests over time of storage and the dye decolorization tests, as performed with the freeze-dry.

- Regarding the AGS-SBR bioreactor experiment, it would be interesting to increase the time of contact of the AGS with the dye could contribute to the efficiency of the decolorizing process since it was suggested that this was one of the limiting steps for the low dye decolorizing efficiency;

- Also, increasing the amount of the bioaugmentation yeast in the AGS reactor could be tested to see if it would lead to an increase in the efficiency of dye decolorization since it appears to be in small quantities in the reactor, which may have influenced the results;

- Since lab-scale experiments were executed, it would be interesting that pilot-scale tests would be explored to verify the efficiency of the prototypes developed in a more real industrial context.

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