

AEROBIC DECOLORIZATION OF AZO DYE ORANGE G BY A NEW YEAST ISOLATE *Candida cylindracea* SJL6

DESCOLORAÇÃO AERÓBICA DO AZO CORANTE ALARANJADO G POR UM NOVO ISOLADO DA LEVEDURA *Candida cylindracea* SJL6

Tiago de Araújo Pinho ALCÂNTARA¹; Juliana Mota OLIVEIRA¹;
Norma Suely Evangelista-BARRETO¹; Phellippe Arthur Santos MARBAC¹;
Marcia Luciana CAZETTA^{1,2}

1. Programa de Pós-Graduação em Microbiologia Agrícola, Centro de Ciências Agrárias Ambientais e Biológicas - CCAAB, Cruz das Almas, BA, Brasil; 2. Centro de Ciências Exatas e Tecnológicas - CETEC. Universidade Federal do Recôncavo da Bahia - UFRB, Cruz das Almas, BA, Brasil. malucz@ufrb.edu.br

ABSTRACT: Biodecolorization of the azo dye Orange G was investigated using a new strain of *Candida cylindracea* SJL6, isolated from freshwater samples of the Subaé river in Bahia state, Brazil. Strain SJL6 was identified as *C. cylindracea* on the basis of 26S rDNA region. The various parameters of dye decolorization and cell growth were studied, including the Orange G dye concentration (100 to 500 ppm), temperature (20 to 40 °C), glucose concentration (0 to 5%), and initial pH (3 to 8). Biototoxicity tests were performed using shrimp (*Artemia salina*) to determine the lethal concentration (LC₅₀) and onion bulbs (*Allium cepa*) to determine the cytotoxic and genotoxic effects of both Orange G dye and metabolites formed after decolorization. Up to 90% of decolorization of the Orange G dye at 500 ppm was achieved by *C. cylindracea* SJL6 at 30 °C, pH 3, and 1% glucose. However, the biototoxicity tests showed that there was increased toxicity after decolorization, suggesting partial Orange G dye degradation and production of toxic metabolites.

KEYWORDS: Biodegradation. Fungus. Dyes.

INTRODUCTION

The presence of residues in textile effluents causes significant impacts on the total organic carbon, biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids, salinity, pH, and color, in addition to the recalcitrant nature of azo dyes (FARYAL; HAMEED, 2005; SAVIN; BUTNARU, 2008). The high values of BOD and COD can result in the consumption of dissolved oxygen, causing the death of fish and other aquatic organisms (VON SPERLING, 1996). Furthermore, azo dyes when metabolized by particular enzymes, may generate aromatic amines, which are known for their high toxicity, mutagenicity, and carcinogenicity (SOLÍS et al., 2012).

All this has raised growing concerns and prompted several studies in different fields of knowledge in an attempt to develop effective and low-cost technologies for the removal of dyes from textile effluents. Physical and chemical treatments have been the most widely used; however, they usually require high costs and are not able to completely degrade the molecules, generating significant amounts of chemical sludge, which creates a second pollution problem, waste disposal (ANJANEYULU; CHARY, 2005; SARATALE et al., 2009; BAFANA et al., 2011).

Azo dyes account for 70% of all dyes used in the textile industry (TONY et al., 2009). The success of the wide use of these dyes is related to their easy synthesis, cost-effectiveness, stability, and great structural variety, which enables a wide range of colors and applications. Thus, in addition to the textile industry, these dyes are widely used in the paper, food, leather, cosmetics, and medical industries (CHANG et al., 2004; TELKE et al., 2008).

Bioremediation has been considered an environment friendly and cost-competitive approach that produces less sludge and is able to generate end-products of low toxicity or even achieve total mineralization of compounds (RAI et al., 2005). In this context, many microorganisms have been selected every day for the development of new treatments.

Due to their enzymatic apparatus, fungi have a great potential for decolorization of azo dyes (MORTARELL et al., 2012). Through oxidative and ligninolytic non-specific enzyme systems, these microorganisms are able to mineralize various types of synthetic dyes (YANG et al., 2005). However, several factors hamper the use of filamentous fungi in industrial waste treatment, such as stringent conditions for enzyme production, risk of bacterial contamination under non-sterile conditions of wastewater treatment, and slow growth compared to

that of unicellular organisms, in addition to the production of mycelia, which often makes fungi less adaptable to effluent treatment conditions (ASKU; DÖNMEZ, 2003; YU; MEN, 2005).

The use of yeasts presents several biotechnological advantages, not only because of their rapid growth, but also because of their resistance to unfavorable environments and high efficiency in azo dye decolorization (MORTARELL et al, 2012; YANG et al., 2008; LIU et al., 2011; PAJOT et al., 2011; TAN et al., 2014).

Therefore, the aim of this work was to evaluate various culture parameters for decolorization of the Orange G azo dye by a new strain of *Candida cylindracea* SJL6, as well as to evaluate the toxicity of final products of the degradation process.

MATERIAL AND METHODS

The azo dye used in this study was Orange G (C.I. Name: Acid Orange 10; Sigma–Aldrich, St. Louis, MO, USA). A 10,000 ppm stock solution was prepared by dissolving the dye in distilled water and sterilizing by vacuum filtration (0.22-mm filter, Millipore Corp., Bedford, MA, USA).

Culture medium

The modified Normal Decolorization Medium (NDM) was used for the experiments, which consisted of (%): yeast extract, 0.25; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.013; and glucose, 2 (RAMALHO et al., 2004). After sterilization at 121 °C for 20 min, Orange G was added to the medium at different concentrations (100, 200, 300, 400, and 500 ppm). The pH was adjusted to 5.0 with orthophosphoric acid.

Molecular identification of the yeast

DNA extraction was performed according to Sampaio et al. (2001) and Almeida (2005). Molecular characterization was performed by sequencing of the D1/D2 region of 26S ribosomal DNA using the NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers (O'DONNELL, 1993). For the polymerase chain reaction (PCR), we used the following reagents and concentrations: 10 ng of DNA, 1× enzyme buffer for *Taq* DNA polymerase, 3.7 mM MgCl_2 , 0.6 pmol/μL dNTPs, 0.4 pmol/μL each primer, and 5 U of *Taq* DNA polymerase, with the final volume adjusted to 50 μL with sterile ultra-pure water. DNA of *Saccharomyces cerevisiae* (10 ng) was used as a positive control. Amplification was performed in a

peqSTAR 96× Universal Gradient thermal cycler (PiqLab, Erlangen, Germany) programmed for an initial denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 1 min), and extension (72 °C, 2 min) and a final extension at 72 °C for 5 min. The amplified products were separated on a 0.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The PCR fragments were purified using a GFX PCR kit (Amersham Biosciences, Piscataway, NJ, USA), and then the amplicons were sequenced on an automatic sequencer ABI 310 (Applied Biosystems, Foster City, CA, USA) at the Molecular Genetics Laboratory of Federal University of Recôncavo da Bahia. The second round of sequencing was performed using an ABI PRISM 3100 genetic analyzer (Applied Biosystems) by ACTGene Molecular Analysis LTDA (The Rio Grande do Sul, Brazil). The sequences obtained were compared with those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) at <http://www.ncbi.nlm.nih.gov>.

Decolorization assays

Assays were performed in 250-mL Erlenmeyer flasks containing 50 mL of NDM at varying culture conditions: the dye concentration (100–500 ppm), glucose concentration (0–5%), pH (3–8), and temperature (20–40 °C). After incubation at 150 rpm for 24 h, 2-mL aliquots were taken from the flasks and centrifuged at 5,000 rpm for 10 min. The supernatant obtained was used to analyze both decolorization rate and toxicity, while the biomass was used to determine cellular growth. All assays were performed in triplicate. As the inoculum, 5 mL of a cellular suspension (optical density at 550 nm = 0.8) was used, which was prepared from a 24-h culture grown in NDM.

Analytical method

The decolorization rate was determined by spectrophotometry at 476 nm and calculated using the following equation:

$$\text{Decolorization (\%)} = (A - B)/A \times 100,$$

where A and B represent the initial and final absorbance of the dye, respectively.

Cellular growth was determined by measuring optical density at 550 nm.

Biotoxicity tests

Acute toxicity test using *Artemia salina*

The acute biotoxicity test was performed with the nauplii of *A. salina*, according to Matthews (1995), with some modifications. For hatching, 1 g of *A. salina* cysts was added to 500 mL of artificial

seawater (30 ppt). The cysts were kept at a constant agitation by air pumping for 24 h at room temperature. Then, 10 nauplii of *A. salina* were transferred to test tubes containing 10 mL of artificial seawater and Orange G dye (positive control) or different concentrations (2.08, 4.2, 8.3, 16.6, 25, 33, 50, and 75%) of the supernatant obtained after the decolorization process and incubated at room temperature for 24 h. Artificial seawater was used as a negative control, and all tests were performed in triplicate. The results were obtained by counting the number of alive *A. salina* nauplii in each test tube. The calculation of the lethal concentration (LC₅₀) was performed using the Spearman–Karber Trimmed program (AN, 2006).

Biotoxicity test using onion (*Allium cepa*)

The tests using onion bulbs were performed according to Fiskesjö (1985), modified. Non-sprouted onion bulbs of the same origin, approximately 2 cm in size, with external white cataphylls, were purchased at a local market. Before the test, dry external cataphylls were removed without damaging the root meristem, and the bulbs were washed under running water. To avoid dehydration of primary cells, the bulbs were placed in distilled water and kept in the dark for 48 h. Distilled water was changed daily. After two days, bulbs with the size of 1.5 to 2.0 cm were selected. Five bulbs were used for each treatment. The bulbs were placed in test tubes, with their roots in contact with the supernatant (20 mL) obtained after the decolorization process. The Orange G dye solution

was a positive control, and distilled water was a negative control. After 24 h of exposure, the roots were removed, kept in Carnoy's fixative solution for 24 h, and then stored in ethanol (70%) under refrigeration until slide preparation.

For the slide preparation, roots were removed from ethanol, washed, and hydrolyzed in 1 M HCl at 60 °C for 8 min. Subsequently, a drop of acetic acid (45%) was added to a slide, and the tip was removed from a root, then covered with a coverslip, and crushed. The slide–coverslip assembly was frozen at –80 °C for 5 min, then the coverslip was removed, and the material was stained with 10% Giemsa stain for 2 min. The root material on the blade was observed under an optical microscope at a 400× magnification. About 5,000 cells were analyzed per treatment. The following parameters were observed: (A) a mitotic index (MI) (1,000 cells per bulb); (B) chromosomal aberrations (CAs) in the mitotic cycle (1,000 cells per bulb); and (c) the presence of micronuclei (1,000 cells per bulb). The data were analyzed with the Assisat Software, version 7.7, using analysis of variance, followed by Turkey's test.

RESULTS AND DISCUSSION

Molecular identification

A 616-nucleotide sequence of the D1/D2 region of strain SJL6 showed 99% identity with the sequences of the D1/D2 domain of *C. cylindracea* (Figure 1).

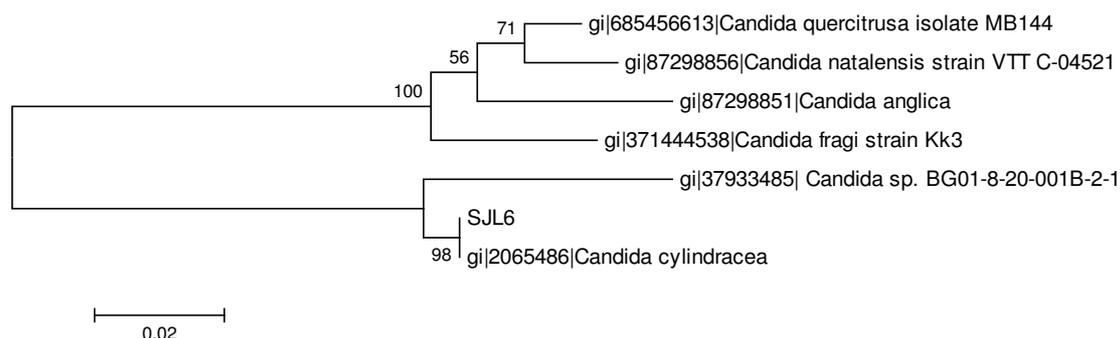


Figure 1. Maximum likelihood phylogenetic tree inferred from the alignment of the D1/D2 region sequences of the yeast rDNA subunit constructed in the MEGA 6 program. The best model selected by the program was the Tamura-Nei + Gamma (TN93 + G) model with partial deletion of missing data and confidence level of 1000 bootstrap repeats.

Effects of various parameters on the Orange G decolorization rate and cellular growth of *C. cylindracea* SJL6

Initial dye concentration

C. cylindracea SJL6 was able to remove more than 90% of the color up to an Orange G concentration of 500 ppm after 24 h, and only a

small biomass reduction occurred with the increasing Orange G concentrations. Thus, it is possible to conclude that *C. cylindracea* SJL6

proved to be quite tolerant to high concentrations of the dye (Figure 2).

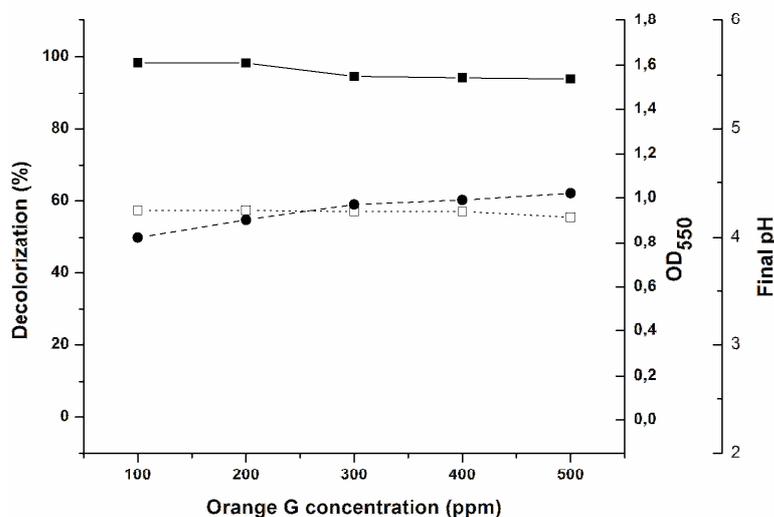


Figure 2. Effect of initial concentrations of Orange G on the decolorization rate (■), cell growth (□), and final pH (●)

Results obtained by other authors have shown an inverse relationship between initial concentrations and dye decolorization. *Pseudozyma rugulosa* Y-48 and *Candida krusei* G-1 decolorized 99% of Reactive Bright Red L-2BP at 200 ppm after 24 h, with the decolorization gradually decreasing as the dye concentrations increased (YU; MEN, 2005). In another study, *Candida tropicalis* TL-F1 was able to achieve a decolorization rate of more than 97% with the Acid Brilliant Scarlet GR dye in the concentration range from 20 to 100 ppm after 24 h; however, there was a rate reduction to less 80% at higher dye concentrations (120 and 140 ppm) (TAN et al., 2014). *Issatchenkia orientalis* JKS6 was able to decolorize up to 99% of the Reactive Black 5 dye at 500 ppm after 36 h; however, the decolorization rate decreased to less than 40% at a 1,000 ppm dye concentration (JAFARI et al., 2014). The decrease in the decolorization rate with an increasing dye concentration suggests a toxic action by dye intermediate metabolites produced after cleavage of the chromophore group (WANG et al., 2009). Textile effluents show variations in residual dye concentrations, and therefore, the ability of microorganisms to degrade dyes in a wide range of concentrations becomes an important factor for effective biodegradation (PARSHETTI et al., 2010; AYED et al., 2011).

Glucose concentration

Figure 3 shows the effects of glucose concentrations on the Orange G dye decolorization.

The results showed that *C. cylindracea* SJL6 was unable to perform decolorization in the absence of an external source of carbon and energy. Although there are reports of yeasts that can use a dye as a sole source of carbon and/or nitrogen for dye degradation, this process is highly associated with the cell growth and primary metabolism (LUCAS et al., 2006; YANG et al., 2008; PAJOT et al., 2011; MORTARELL et al., 2012). Therefore, yeasts do not generally grow without glucose or any other readily metabolizable carbon source (SOLÍS et al., 2012).

In this work, the increase of glucose concentrations showed no significant effect on both decolorization rate and cellular growth, which remained constant at the glucose concentrations from 1% to 5%. These results are very interesting since low carbon source concentrations reduce the costs of developing biodegradation processes. Moreover, high glucose concentrations in the medium can inhibit dye degradation since microorganisms tend to use an external source of energy instead of a dye (QU et al., 2012). However, no such inhibition was observed in this study up to the maximum glucose concentration studied (5%).

For the yeasts *Debaryomyces polymorphus* and *C. tropicalis*, Reactive Black 5 dye degradation was highly dependent on the glucose concentration in the medium. Thus, the color removal increased from 76.4% and 92.7%, respectively, after 60 h to 100% between 18 h and 24 h after rising glucose

concentration from 0.5% to 1% (YANG et al., 2008).

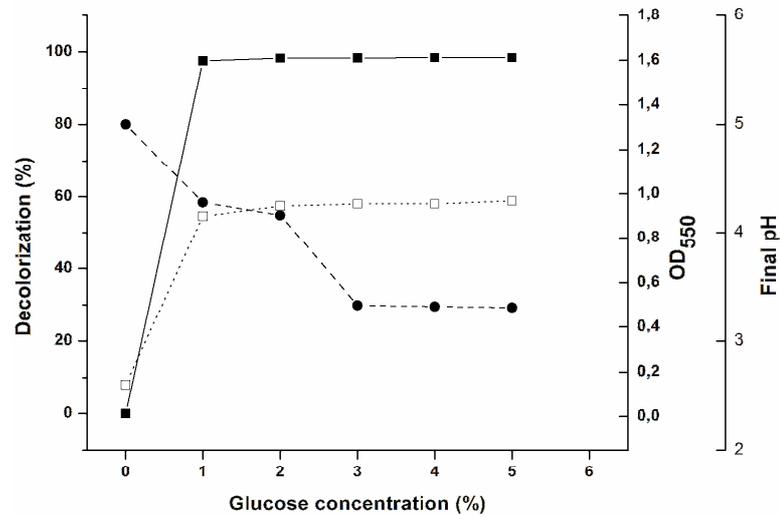


Figure 3. Effect of glucose concentrations on the Orange G dye (200 ppm): decolorization rate (■), cell growth (□), and final pH (●).

The optimal glucose concentration was reported to be between 0.5 and 1% for several yeasts such as *Pichia sp.* TCL upon decolorization of the Acid Red B dye (100 ppm) (QU et al., 2012), *I. orientalis* JKS2 upon decolorization of the Reactive Black 5 dye (JAFARI et al., 2014), and *C. tropicalis* TL-F1 upon decolorization of the Acid Bright Scarlet GR dye (100 ppm) (TAN et al., 2014). In this study, an increased glucose concentration favored the cell growth, and a slight increase in biomass was observed, followed by a decrease in pH. The decrease in pH occurred due to glucose metabolism and positively influenced the dye decolorization since yeasts, in general, adapt better to acid environments.

pH

The decolorization rate was above 98% in the pH range between 3 and 6 (Figure 4) and drastically decreased at pH 7 and 8. The cell growth was not affected by the pH variation, although it was slightly better in the pH range between 4 and 5. These results are interesting for the development of biodecolorization processes using *C. cylindracea* SJL6 since acidic pH inhibits the growth of others microorganisms such as bacteria. It was not possible to correlate the cell growth with the decolorization rate, which suggests that the relationship between pH and decolorization is associated with optimal conditions for the performance and production of enzymes involved in the degradation process.

It is generally known that the maximum fungal growth and the maximum activity of

ligninolytic enzymes involved in azo dye decolorization are observed at a low pH (SHAH; PATEL, 2014). The same has been described for biodegradation of different dyes by yeasts (JADHAV et al., 2008; LIU et al., 2011; TAN et al., 2014). Enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), NADH-dichlorophenol indophenol reductase, and azoreductases are some of the major enzymes involved in dye biodegradation by yeasts (YANG et al., 2005; LUCAS et al., 2006; JADHAV et al., 2008; WAGHMODE et al., 2011).

Temperature

The maximum Orange G decolorization rate and cell growth were observed in the temperature range from 20 °C to 30 °C. However, decolorization ceased completely at temperatures of 35 °C and above, accompanied by a sharp decrease in cell growth (Figure 5). These results can be explained by a loss of cell viability and/or by denaturation of the enzymes responsible for the dye decolorization (WANG et al., 2009).

The temperature effect on decolorization does not follow a pattern and varies according to the characteristics inherent to the microorganisms and/or the enzymes involved in the process. For yeasts, the best results are reported at temperatures in the range from 25 °C to 32 °C (ERTUĞRUL et al., 2008; CHARUMATHI; DAS, 2010; GRASSI et al., 2011; LIU et al., 2011; JAFARI et al., 2014).

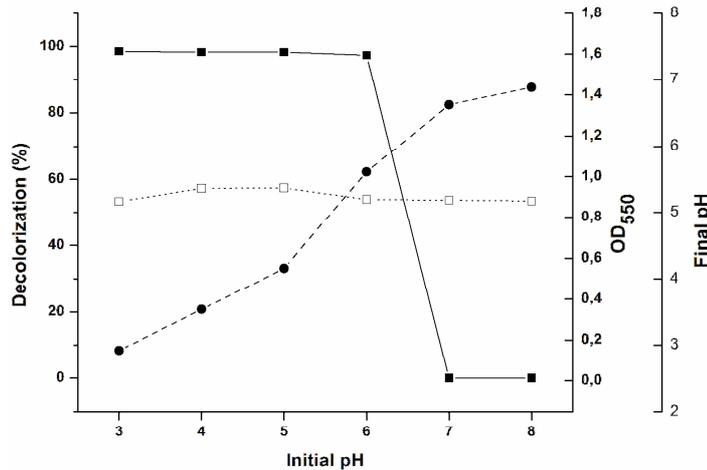


Figure 4. Effect of initial pH on the decolorization rate of Orange G (200 ppm) (■), cell growth (□), and final pH (●).

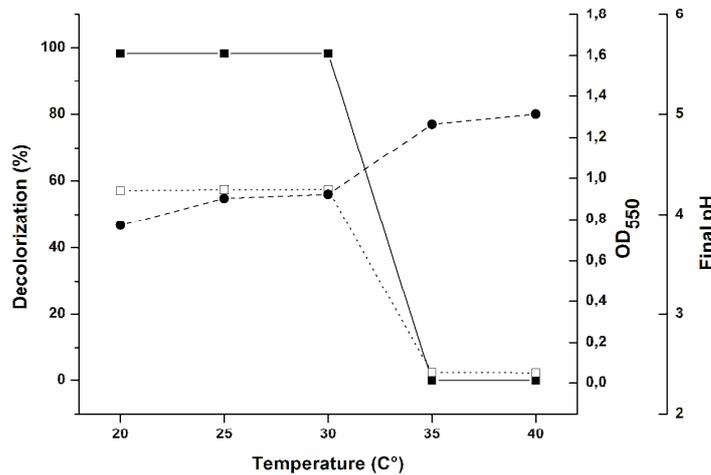


Figure 5. Effect of the temperature on the decolorization rate of Orange G (200 ppm) (■), cell growth (□), and final pH (●).

Biotoxicity tests

The toxicity tests were performed using the nauplii of the brine shrimp *A. salina* as a quick, simple, and inexpensive test, which provides a good indication of the acute toxicity for several compounds and is widely used to determine the toxicity of dyes (PRASAD; RAO, 2013; IQBAL & BHATTI, 2014; PUNZI et al., 2015). The results obtained in this study showed that the decolorization products reached a lethal concentration (LC₅₀) at 17.45%. However, in assays containing only the Orange G dye at different concentrations, no mortality of *A. salina* was observed. These results suggest that the cleavage of the chromophore group produced toxic intermediates (PUVANESWARI et al., 2006). It is common to see the reports about

increased toxicity after dye decolorization processes. Silva et al. (2012) analyzed the discoloration of the Phthalocyanine Reactive Blue 21 dye by peroxidase of turnip and found that the hydrolysis of the chromophore group increased toxicity after enzyme treatment. Almeida and Corso (2014) also observed that the inhibition of the lettuce (*Lactuca sativa*) seed germination increased from 5 to 50%, compared with that caused by the untreated dye, after the Procion Red MX-5B dye was decolorized by *Aspergillus terreus*, indicating incomplete dye degradation and production of toxic intermediates.

To confirm the above results, toxicity tests were performed using onions because they are a good indicator of cytotoxicity, genotoxicity, and

mutagenicity, besides providing a cheap, fast, and sensitive method for toxicity monitoring. According to the results (Table 1), cytotoxicity increased after the Orange G decolorization. Cellular aberrations were observed as anaphase bridges, chromosome fragments, sticky chromosomes in the metaphase, nuclear abnormalities, and the presence of micronuclei, which were evaluated separately. All

MI tests differed statistically. Regarding the CA index and the presence of micronuclei, there were differences between the treatments and control, but not among the treatments. No significant reduction in MI, caused by the exposure of onion bulbs to the Orange G dye, was observed; on the other hand, a higher reduction of MI occurred after dye decolorization (24 h).

Table 1. Total number of cell in interphase and in division: mitotic index (MI), chromosomal aberrations rate (CA) and micronucleus index (MN)

Treatments	Interphase cells	Cells in division	MI (%) **	CA (%) **	MN (%) *
Control	4133	867	17.34 ±0,5 ^a	0.06 ±0.09 ^b	0 ±0 ^b
Orange G	4572	428	8.56 ±0,62 ^b	0.56 ±0.15 ^a	0.2 ±0.19 ^a
Supernatant	4769	231	4.62 ±0,76 ^c	0.46 ±0.21 ^a	0.2 ±0.10 ^a

Control: Distilled water; Significant difference from the negative control *P<0.05 e **P<0.001; (ANOVA – Tukey Test).

The increased toxicity after decolorization in both tests can be attributed to the incomplete dye degradation, indicating a biotransformation process. The metabolites produced after dye decolorization can be more toxic in many cases than the original dye molecule, and their toxicity is commonly attributed to aromatic amines, which are often released after reduction of the azo dye linkage (PINHEIRO et al., 2004; BAFANA et al., 2011; SOLÍS et al., 2012). The presence of 4-aminoacetanilide and 5-acetamido-2-amino-1-hydroxy-3,6-naphthalene disulfonic acid was reported to be responsible for the increased toxicity in rats after Violet dye degradation by the bacterium *Pseudomonas putida* (BEN MANSOUR et al., 2010).

According to the literature, the Orange G dye is not easily degraded by microorganisms. Fungi such as *Thelephora* sp. and *Pleurotus sajor-caju*, which present a recognized potential for the decolorization of different azo dyes, removed only 30% and 50% of the color, respectively (CHAGAS; DURRANT, 2001; SELVAN et al., 2003).

The Orange G decolorization rate by *Bacillus megaterium* was above 94.5% (TRIPATHI; SRIVASTAVA, 2014). However, the metabolites produced were identified as naphthalene, aldehyde derivative compounds, and phenylhydrazine, which are known for their toxicity by oral, dermal, and

inhalation routes, in addition to causing skin and eye irritations and being directly involved in hemolytic anemia in vertebrates (SHUKLA et al., 2012).

Longer-time fermentation could contribute to increased dye mineralization and reduce or eliminate the metabolite toxicity. In addition, the use of microbial consortia is highly recommended to obtain complete dye degradation.

CONCLUSIONS

The yeast *C. cylindracea* SLJ6 was efficient in Orange G dye decolorization, showing good tolerance of high dye concentrations. However, the results suggested incomplete biodegradation, and the metabolites produced after decolorization were more toxic than the original dye molecule, underscoring the need for additional studies to identify the metabolites produced. In addition, new approaches are necessary to reduce the production of potentially toxic metabolites, such as the use of microbial consortia.

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RESUMO: A biodescoloração do corante Alaranjado G foi investigada utilizando um novo isolado de *Candida cylindracea* SJL6, isolado de amostras de água do Rio Subaé, Bahia, Brasil. A linhagem SJL6 foi identificada como *Candida cylindracea* com base na região 26S do rDNA. Os parâmetros estudados na descoloração do corante e

crescimento celular foram: concentração do Alaranjado G (100 a 500 ppm), temperatura (20 a 40 °C), concentração de glicose (0 a 5%) e pH inicial (3 a 8). Os testes de biotoxicidade foram realizados utilizando o microcrustáceo *Artemia salina* para determinar a concentração letal (L₅₀) e bulbos de cebola (*Allium cepa*) para determinar os efeitos citotóxicos e genotóxicos tanto do corante alaranjado G quanto dos metabólitos produzidos após a descoloração. Uma taxa de descoloração acima de 90% foi atingida a 500 ppm por *C. cylindracea* SJL6 a 30 °C, pH 3 e 1% de glicose. Entretanto, os testes de biotoxicidade mostraram que ocorreu um aumento da toxicidade após a descoloração, o que sugere uma degradação parcial da molécula do corante Alaranjado G e produção de metabólitos tóxicos.

PALAVRAS-CHAVE: Biodegradação. Fungos. Corantes

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