

Full Length Research Paper

Textile azo dye degradation by *Candida rugosa* INCQS 71011 isolated from a non-impacted area in Semi-Arid Region of Brazilian Northeast

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The yeast *Candida rugosa*, deposited in the Collection of Reference Microorganisms on Health Surveillance from Oswaldo Cruz Foundation under accession number INCQS 71011, was isolated from a sediment sample from Caldeirão Escuridão, a pristine water reservoir in the surroundings of Serra da Capivara National Park, and was identified based on molecular, physiological and morphological characterization. In addition, it was tested regarding its capacity to degrade three textile azo dyes, namely Reactive Red 198, Reactive Red 141, and Reactive Blue 214 at a concentration of 100 mg l⁻¹ during 7 days of incubation. *C. rugosa* INCQS 71011 was highly efficient towards two azo dyes tested, Reactive Red 198 and Reactive Red 141, demonstrating potential as a biological treatment agent of textile effluent. These results are pioneers for the yeast *C. rugosa*, since its degradation capacity of textile azo dyes has not yet been described. In addition, this study provides important evidence that fungi from non-impacted areas can efficiently degrade azo dyes.

Key words: Fungi, taxonomic characterization, degradation, textile azo dyes.

INTRODUCTION

Wastewaters from textile industries are a complex mixture of several pollutants such as heavy metals, pigments, and dyes (Coulibaly et al., 2003). The potential of pollution from textile dyes has been acknowledged primarily due to possible problems towards human and environmental health (Banat et al., 1996; Meehan et al., 2000; Stolz, 2001). Effluents discharged by textile industries, if not treated properly, can contain large quantities of hazardous chemical compounds (Chen and

Hwang, 1997; Aksu and Dönmez, 2003). There is a necessity to develop better treatment technologies to remove color from industrial effluents that prove technically and economically satisfying as treatment technology. Therefore, several emerging approaches such as advanced oxidation process, membrane filtration, photocatalysis, and sonication, are being proposed and tested at different stages of commercialization (Anjaneyulu et al., 2005).

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Abbreviations: YMA, Yeast extract-malt extract agar; PCR, polymerase chain reaction; RR198, reactive red 198; RR141, reactive red 141; RB214, reactive blue 214; PDA, potato dextrose agar; PDB, potato dextrose broth.

A number of studies have focused on some microorganisms that are able to biodegrade and biosorb dyes in wastewaters. Some bacteria, fungi, and algae are capable of decolorizing a wide range of dyes (Fu and Viraraghavan, 2001). The treatment using fungi is a promising alternative to replace or complement conventional treatment processes due to the efficiency of dye decolorization by fungi, mainly because of the fungal enzymatic apparatus that enables the degradation of these compounds and for not producing material to be discarded. Additionally, fungal treatment has a good public acceptance (Fu and Viraraghavan, 2001). However, more studies on the ability of fungi to decolorize dyes used by the textile industry are needed. Little is known about the degradation of dyes and color removal by yeasts (Singh, 2006); nonetheless, most studies show positive results. Several genera of yeast have been studied such as *Kluyveromyces*, *Schizosaccharomyces*, *Issatchenkia*, *Debaryomyces*, *Pseudozyma*, and *Saccharomyces* (Aksu and Dönmez, 2003; Ramalho et al., 2004; Yang et al., 2003, 2005; Yu and Wen, 2005; Kumari and Abraham, 2007). *Candida* species have been used in some works and good results were presented for removal of reactive dyes in concentrations ranging from 10 - 700 mg l⁻¹, nevertheless, these studies showed adsorption, besides degradation (Gönen and Aksu, 2009; Ertugrul et al., 2009) and absorption (Dönmez, 2002; Aksu and Dönmez, 2003).

Compared with bacteria and filamentous fungi, the yeasts display attractive characteristics. Despite the fact that yeasts do not always grow as fast as bacteria, they grow faster than filamentous fungi and have the ability to resist to unfavorable environments (Pajot et al., 2007). Based on this context, the isolation of fungal species from an environment with potential bioremediation has been spreading (da Silva et al., 2003; Junghanns et al., 2008; Passarini et al., 2011) and different ecosystems have been exploited for this purpose.

Brazil has ecosystems and biomes that are not yet fully known, such as the semi-arid Caatinga, an area of uncertain rainfall situated in the northeastern state of Piauí. This biome represents a valuable biodiversity reservoir, in which there is an important reserve for the preservation of its specific environment, the Serra da Capivara National Park (Pessis, 1998; Gusmão and Maia, 2006).

From the surroundings of Serra da Capivara National Park, sediment samples of pristine water reservoirs where collected and mixed fungal cultures were selected for dye degradation. From the most efficient mixed fungal culture, Caldeirão Escuridão (CE), a yeast strain (CE-9) was isolated (Nascimento et al., 2011). Therefore, the aim of the present work was to identify by phenotypic and molecular methods the yeast CE-9 as well as to test its capacity to degrade three textile azo dyes.

MATERIALS AND METHODS

Yeast isolation and preservation

The yeast strain CE-9 was isolated from a mixed fungal culture

collected from Caldeirão Escuridão (CE), a natural water reservoir situated in the surroundings of the Serra da Capivara National Park, at an altitude of 423 m (xUTM - 769341, yUTM - 9022784 (Nascimento et al., 2011). The yeast is currently deposited as *Candida rugosa* INCQS 71011 in the Collection of Reference Microorganisms on Health Surveillance (Fiocruz-CMRVS) at the National Institute for Quality Control in Health (INCQS) from Oswaldo Cruz Foundation (Fiocruz). The American Type Culture Collection (ATCC) strain of *C. rugosa* ATCC 10571 was used as a reference strain. The strains are maintained lyophilized and cryopreserved at -70 and -150°C. For routine work at laboratory scale, the selected yeasts were maintained on yeast extract-malt extract agar (YMA) slants at 4°C and subcultured at 15 day regular intervals.

Yeast identification

The isolate CE-9 was characterized by standard methods (Yarrow, 1998) and the identification followed the keys of Kurtzman and Fell (1998). Concurrently, the characterization by the Vitek[®] 2 using the Yeast Biochemical Card (YBC) (bioMérieux, Durham, NC) was conducted. Genomic DNA of CE-9 and of the reference strain *C. rugosa* ATCC 10571 was prepared after 2 days of incubation on YMA using the methodology described by de Barros Lopes et al. (1998). The D1/D2 variable domains of the large-subunit rDNA were amplified by polymerase chain reaction (PCR) using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') according to Lachance et al. (1999). Amplification was performed as follows: 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 54°C for 25 s, and 72°C for 20 s, and a final extension at 72°C for 10 min. The amplified DNA was concentrated and cleaned (Kit Wizard Plus SV Minipreps DNA Purification System—Promega, USA). Sequencing was conducted using an ET dynamic terminator kit in a MegaBACE 1000/ automated 96 capillary DNA sequencer (GE Healthcare, Buckinghamshire, UK). The quality of each electropherogram was evaluated using Phred-Phrap software and consensus sequences were obtained by alignment of both sequence strands using CAP3 software available on the Eletropherogram quality analysis web page (<http://asparagin.cenargen.embrapa.br/phph>). The nucleotide sequences and other related sequences were aligned using the CLUSTALW software package (EMBL-EBI) (<http://www.ebi.ac.uk/clustalw/>). Phylogenetic relationships were estimated using the MEGA program Version 4.0 (Tamura et al., 2007). The phylogenetic trees were constructed using the neighbor joining (NJ) algorithm with bootstrap values calculated from 1,000 replicate runs. The Maximum Composite Likelihood model was used to estimate evolutionary distance.

Dyestuff

Three commercial reactive dyes were used: Reactive Red 198 (RR198), Reactive Red 141 (RR141), both from DyStar (Brazil) and Reactive Blue 214 (RB214) from Clariant (Brazil) with maximum wavelength of 520 nm, 548 nm and 608 nm, respectively. All of them are azo dyes and the chemical structures are shown in Figure 1. Stock solutions (5 g l⁻¹ for RR198 and RR141; 4 g l⁻¹ for RB 214) were filter-sterilized (Millipore filter, 0.22 µm, Millipore Corp., Bedford, USA).

Yeast growth

CE-9 was grown on potato dextrose agar (PDA) with 200 mg l⁻¹ of each of the three dyes separately and incubated for 48 h. Aliquots from these cultures were transferred to sterile purified water and the turbidity was adjusted to 2 McFarland standard (6 x 10⁸ CFU ml⁻¹). From this suspension, 2 ml were used to inoculate 100 ml of potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks in duplicate. The Erlenmeyer flasks were incubated under agitation for 2 days in the dark (Heinfling-Weidtmann et al., 2001; Máximo et al., 2003) on a

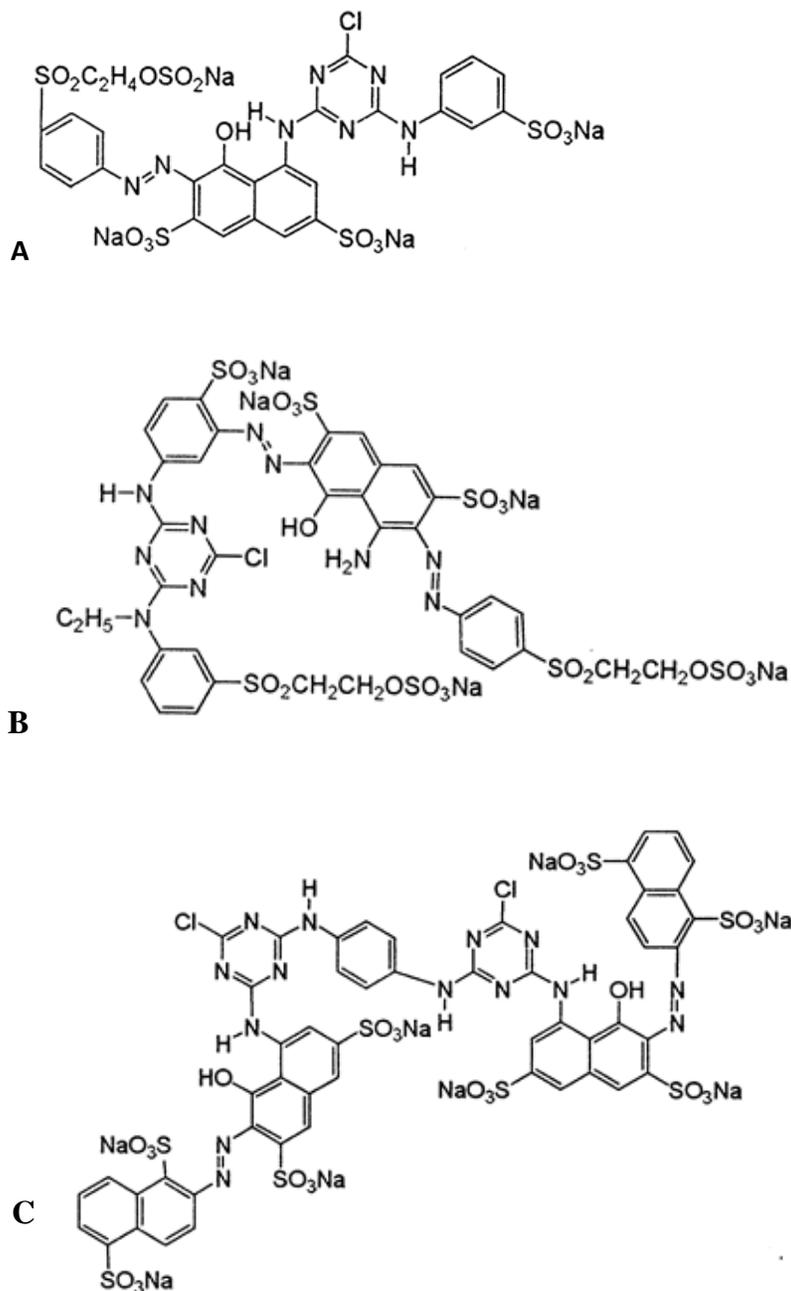


Figure 1. Chemical structures of the dyes tested: A, Reactive Red 198; B, Reactive Blue 214; C, Reactive Red 141.

rotary shaker (New Brunswick, Innova 4335, USA) at 140 rpm. After this incubation period, 100 mg l⁻¹ of the dyes were added separately in all flasks. The cultures were kept incubating under the same conditions during 7 days. Control experiments were conducted by means of incubating PDB medium without yeast and with the same concentrations of the dye tested as well as with PDB without either dye or yeast.

Evaluation of dye decolorization

The evaluation of dye decolorization by CE-9 was conducted during the 7-day incubation at the following times: 24, 48, 72, 96, 120, 144 and 168 h. After centrifugation at 10,000 rpm for 10 min of 2 ml

aliquots of the cultures, 0.5 ml of supernatant was diluted 10 times in purified water in order to be analyzed using spectrophotometer UV/VIS (Shimadzu - UV-1601, Japan). The culture medium with dye and without inoculum was used as a negative control whereas medium without either dye or inoculum was used as blank. The spectra were scanned between 200 to 700 nm of absorbance (Máximo et al., 2003) in order to analyze dye disappearance. Decolorization activity (%) was calculated for the two flasks (duplicate) of each culture according to the formula below, considering the maximum wavelength of 520 nm for RR198, 548 nm for RR141, and 608 nm for RB214.

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

Table 1. Physiological results of isolate CE-9 identified as *C. rugosa*.

Compound	Result
Assimilation of carbon compounds	
Glucose	+
D-galactose	+
L-sorbose	-
D-glucosamine	-
D-ribose	-
D-xylose	+
L-arabinose	-
L-rhamanose	-
Sucrose	-
Maltose	-
Trehalose	-
α -methyl-D- glucoside	-
Cellobiose	-
Salicin	-
Melibiose	-
Lactose	-
Raffinose	-
Melezitose	-
Glycerol	+
Erythritol	-
Ribitol	-
Galactitol	-
D-mannitol	+
D-glucitol	+
Inositol	-
D-gluconate	-
DL-Lactate	+
Succinate	+
Citrate	-
Assimilation of nitrogen compounds	
KNO ³	-
KNO ²	-
Others	
Vitamin-free	-
Cycloheximide 0,1 %	-
Cycloheximide 1 %	-
Urease	-
Amyloid compounds	-
Fermentation	
D-glucose	-
D-galactose	-
Maltose	-
Sucrose	-
α - α -trehalose	-
Lactose	-
Cellobiose	-
Raffinose	-

Where, A is the Initial absorbance and B is the observed absorbance (Özsoy et al., 2005). All assays were conducted in duplicate

and results were expressed as the mean values.

RESULTS

Phenotypic characterization

The macro-morphological observations on YMA medium after 2 days at 25°C of the isolate CE-9 demonstrated a butyrous texture, colored white to cream. From the micro-morphological analysis, cells were elongate, single and in pairs and pseudo-hyphae were produced. These are morphological characteristics typical of *C. rugosa*. In Table 1, the physiological results obtained from CE-9 were present. The yeast was negative for urea hydrolysis and formation of extracellular amyloid compounds. It did not ferment carbohydrates, it did not grow in the absence of vitamins, it was sensitive to cycloheximide, it did not assimilate the NaNO₂ and KNO₃ as nitrogen source, but assimilated the following carbon compounds: glucose, D-galactose, D-xylose, glycerol, D-mannitol, D-glucitol, lactic acid, and succinic acid. The other carbon compounds used in the test were not assimilated by the yeast. Hence, the isolate was identified biochemically and physiologically as *C. rugosa*.

The automated method of identification using Vitek[®]2 was also used for the biochemical characterization whereas the YBC was applied to confirm the conventional method. As the result, the identification was 97% of probability for *C. rugosa*. The D1/D2 sequence of strain *C. rugosa* ATCC 10571 has 527 nucleotides, where 24 and 19 correspond to the nucleotide primers NL1 and NL4, respectively. Thus, the sequence to be obtained from isolate CE-9 should be 484 nucleotides.

Forward and reverse strands of D1/D2 domain of isolate CE-9 were sequenced using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). Consensus sequence had 447 nucleotides and was aligned with the sequences of *C. rugosa* ATCC 10571. Manual editing of the sequence was performed based on the chromatograms obtained and no differences between the sequences were observed. When performing blast, it had 99% of identity with only one Gap of difference. Therefore, these results confirm the phenotypic characterization, leading to the identification of the isolate CE-9 as the species *C. rugosa*.

Decolorization

C. rugosa INCQS 71011 decolorized 78.46% of RR198 at 100 mg l⁻¹ dye concentration after 24 h of incubation and reached 100% decolorization of this same dye after 48 h of incubation (Table 2), respectively. Regarding RR141 dye, decolorization reached 75.60% after 24 h of incubation, and complete decolorization was achieved within 144 h of incubation (Table 2). In both cases, there was no adsorption by the biomass but degradation, as demonstrated by the results of spectrophotometric analyses

Table 2. Rate of decolorization of the dyes RR198, RR141 and RB214 by *C. rugosa* INCQS 71011 during 24 – 168h of incubation.

Sample	Decolorization (%)						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Treatment of RR198	78.46	100	100	100	100	100	100
Treatment of RR141	75.60	86.46	88.86	88.58	89.39	100	100
Treatment of RB214	50.20	59.21	69.01	70.43	73.82	73.91	76.05

that showed the disappearance of the characteristic peak of those two dyes (Figures 2a and 2b). The other dye tested, RB214, was less efficiently decolorized by *C. rugosa* INCQS 71011. In the first 24h incubation, decolorization achieved 50%. From 48 to 144 h, decolorization increased progressively, however slowly; and at the end of the incubation period (168 h), the decolorization rate was 76.05% (Table 2). The adsorption to the biomass just occurred with this dye, which decreased up to the end of the incubation period (data not shown), while the characteristic peak of this dye was transformed into a new peak (Figure 2c).

DISCUSSION

In some investigations, positive results for textile dye decolorization were obtained using fungi isolated from non-impacted areas (Pajot et al., 2011; Martorell et al., 2012), as similarly obtained in the present study. The ascomycetous yeast *C. rugosa* INCQS 71011 showed a very good result mainly in the decolorization of RR198, a monoazo, decolorizing 100% of the dye at a concentration of 100 mg l⁻¹ after 48 h, while within 24 h the decolorization was 75.60%. Regarding RR141, the results were also satisfactory 75.20% was decolorized after 24 h incubation and the complete decolorization was achieved within 144 h incubation. For RB214, however, the decolorization took longer, 76.05% was decolorized at the end of the incubation period (168 h) (Table 2). The decolorization of a number of simple azo dyes derived from 2-naphtholaminoazobenzene and p-N,N-dimethylaminoazobenzene in concentrations of 10-50 mg l⁻¹ were tested in liquid aerated batch cultures using a strain of the yeast *Candida zeylanoides* UM2, by which method a 90% adsorption was obtained from the best result after seven days of incubation (Martins et al., 1999). This same strain was used by Ramalho et al. (2005) which, after changing some parameters, such as medium, for example, *C. zeylanoides* UM2 showed 100% efficiency of decolorization without traces of absorption after 60 h.

Dye degradation occurs when the biomass remains with its original color and the medium becomes decolorized (Chen et al., 2003; Yu and Wen, 2005). It can be monitored during cultivation of the fungus in liquid medium through the ratio between two characteristic

wavelengths of the dye. Adsorption occurs when the characteristic peak of absorption of the dye remains constant during the incubation period, while degradation occurs when this peak disappears completely or a new peak appears (Glenn and Gold, 1983). In the present study, *C. rugosa* 71011 succeeded in promoting the disappearance of the characteristic peaks of absorption of visible light from both dyes, namely RR198 (520 and 370 nm) and RR141 (548 and 522 nm) (Figures 2a and 2b), with no dye adsorption by the biomass, thus typifying this fungal activity as the degradation of these dyes.

Degradation was also determined by Yang et al. (2003) when using *Candida tropicalis* Y2-0814 and *Debaryomyces polymorphus* Y1-0813 to decolorize 100 mg l⁻¹ of seven synthetic dyes. Within 16-48 h of incubation the color disappeared completely without residual color on the biomass in the case of only one of these dyes, the Reactive Black 5. The metabolic profile species-specific, such as enzymatic activity (Ramalho et al., 2005; Martorell et al., 2012), media culture (Ramalho et al., 2002; Kaushik and Malik, 2010) and the structure complexity of the dye (Fu and Viraraghavan, 2001; Bergsten-Torralba et al., 2009) can greatly influence the final response of fungal degradation. In the present work, *C. rugosa* INCQS 71011 was much more efficient degrading RR198, a single azo class dye, than it was towards RR 141, a double azo class dye, which took longer to be degraded and towards RB 241, another double azo class dye, which was not completely degraded under the conditions established in this study.

The present results demonstrated the efficiency of *C. rugosa* INCQS 71011 to degrade textile azo dyes and indicates its potential as a biological treatment agent of textile effluent, despite its origin, a non-impacted area in Semi-Arid Northeastern Brazil. To our knowledge this is the first report of a strain of *C. rugosa* with the ability to degrade textile azo dyes. Further studies on the optimization conditions for this yeast to degrade azo dyes, nonetheless, are needed.

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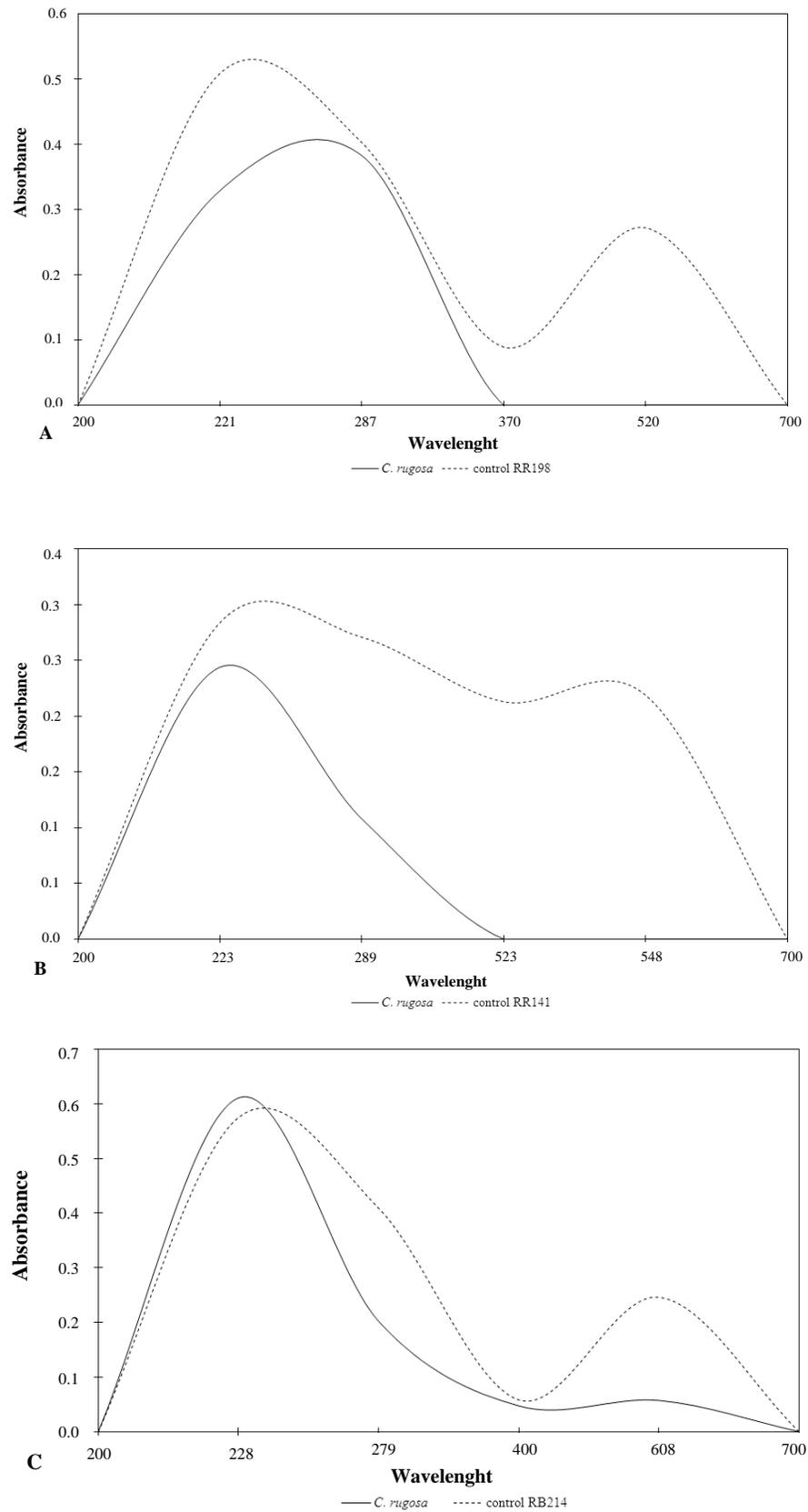


Figure 2. Espectra resulting from the dyes RR 198 (a), RR 141 (b) and RB 214 (c) degradation by the *C. rugosa* INCQS 71011.

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