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Discoloration of Reactive black 5 by individual and consortium of *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* and *Escherichia coli* free and immobilized

Decoloración del Negro reactivo 5 por un consorcio, y los microorganismos que lo constituyen: *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* y *Escherichia coli* libres e inmovilizados

M. A. Flórez-Restrepo[1], M. García-Jiménez[2], D. F. López-Lugo[3], L. M. Múnera-Porras[4], N. J. Pino-Rodríguez[5], and G. A. Peñuela-Mesa[6]

ABSTRACT

The textile industry is a generator of high volumes of waste water with a high content of pollutants such as azo dyes, which are recalcitrant and persistent in the environment, these ones have been of interest in the last decades for the entities in charge of the care of the environment. This study evaluated the ability to discolor of reactive black 5 (NR5) by a consortium and the microorganisms that constitute *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* and *Escherichia coli* free and immobilized in calcium alginate, coffee husks and bagasse of sugar cane. The results show discoloration was evidenced, where the highest percentage corresponds to **G.** *pseudocandidum* (90,05%) and the lowest to *R. mucilaginosa* (79,31%). When comparing the percentages of discoloration between the free microorganisms and the immobilization matrices, it is observed that the former exhibit the highest percentages of discoloration. In addition, there are no significant differences between using cane bagasse or coffee husks as immobilization matrix.

Keywords: Calcium alginate beads, bagasse, coffe hucks, immobilized cells, percentage of discoloration.

RESUMEN

La industria textil es un generador de altos volúmenes de aguas residuales con un alto contenido de contaminantes como los colorantes azoicos, que son recalcitrantes y persistentes en el medio ambiente. Estos han sido de interés en las últimas décadas para las entidades a cargo del cuidado del medio ambiente Este estudio evaluó la capacidad de decoloración del negro reactivo 5 (NR5) por un consorcio y los microorganismos que constituyen *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* y *Escherichia col*i libres e inmovilizados en alginato de calcio, cascarilla de café y el bagazo de la caña de azúcar. Los resultados mostraron una decoloración, donde el porcentaje más alto corresponde a *G. pseudocandidum* (90,05%) y el más bajo a *R. mucilaginosa* (79,31%). Cuando se comparan los porcentajes de decoloración entre los microorganismos libres y las matrices de inmovilización, se observa que los primeros exhiben los porcentajes más altos de decoloración. Además, no hay diferencias significativas entre el uso de bagazo de caña o cascarilla de café como matriz de inmovilización.

Palabras clave: Alginato de calcio, bagazo de caña, cascarilla de café, células inmovilizadas, porcentaje de decoloración.

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Introduction

The textile industry is characterized by the use of large amounts of water and chemical compounds during the processes; especially, during the dyeing of fabric, in which a high volume of wastewater is generated with a significant number of pollutants with acids, bases, dissolved solids, toxic compounds and residues of dyes that do not bind to the fibers (Ghaly *et al.*, 2013). Effluents produced are

[1] Industrial and environmental microbiologist, School of Microbiology, University of Antioquia, Colombia. Grupo de Control y Diagnóstico de la Contaminación (GDCON). Faculty of Engineering. E-mail: mariaa.florez@udea.edu.co.

[2] Industrial and environmental microbiologist, School of Microbiology, University of Antioquia, Colombia. Grupo de Control y Diagnóstico de la Contami- nación (GDCON). Faculty of Engineering. E-mail: madeleiner.garcia@udea.edu.co.

[3] Industrial and environmental microbiologist, School of Microbiology, University of Antioquia, Colombia. Grupo de Control y Diagnóstico de la Contaminación (GDCON). Faculty of Engineering. E-mail: diego.lopezl@udea.edu.co.

[4] Teacher,School of Microbiology, University of Antioquia, Colombia. Grupo de Control y Diagnóstico de la Contaminación (GDCON), Faculty of Engineering. A. A. 1226. Medellín (Antioquia), Colombia. E-mail: luisam.munera@udea.edu.co.

[5] Teacher, School of Microbiology, University of Antioquia, Colombia. Grupo de Control y Diagnóstico de la Contaminación (GDCON), Faculty of Engineering. A. A. 1226. Medellín (Antioquia), Colombia. E-mail: nancy.pino@udea.edu.co.

[6] Teacher, Faculty of Engineering, University of Antioquia, Colombia. Grupo de Control y Diagnóstico de la Contaminación (GDCON), Faculty of Engineering. A. A. 1226. Medellín (Antioquia), Colombia. E-mail: gustavo.penuela@udea.edu.co.

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an environmental problem due to the contamination that generates, especially by the dyes discharged (Morales & nvironmental microbiologist, School of Microbiology, University of Antioquia, Colombia. Grupo de Control y water collection, recreation) and reduces the penetration of sunlight, affecting photosynthesis processes and generating a loss of biological diversity (Khan *et al.*, 2012, Santos & Boaventura, 2015). An example of an azo dye is the reactive black 5 which is one of the most commonly used azo dyes in the textile industry (Pérez, 2009).

Several methods have been evaluated to eliminate dyes from wastewater, which include physical processes such as membrane filtration, adsorption, coagulation and chemical processes such as oxidation and ozonation. However, most of these techniques present drawbacks, such as high operating cost and an intensive labor use (Ferreira *et al.*, 2014). Because of this, biological methods such as the use of microorganisms for the removal of azo dyes have proved to be an attractive alternative for the treatment of waste water from the textile industry. A wide variety of species have been reported with the ability to remove dyes, all of which were evaluated under controlled conditions (Solís *et al.*, 2012). However, the use of microbiological methods in the field is limited mainly by disadvantages such as the uncontrolled growth of microorganisms, the almost total loss of biomass, and the susceptibility of microorganisms to environmental factors such as temperature, pH, among others (Garzón & Barragán, 2008).

Immobilization consists of the physical retention of catalytically active cells in a specific space, can occur naturally or has to be induced, it has been proposed to overcome the limitations of biological methods in the field. The techniques used to immobilize cells include adsorption, aggregation, confinement and entrapment (Karel *et al.,* 1985). Multiple synthetic supports such as calcium alginate, glass beads, polyacrylamide gel have been used to immobilize cells and enzymes by entrapment, while natural supports that include some agroindustrial residues, such as the husks generated in the coffee and bagasse industries were used for the passive immobilization of cells by aggregation (Garzón, 2009, Orzua *et al.*, 2009; Man *et al.*, 2015).

Alzate *et al*. (2016) isolated from a biofilm formed in 3 subsurface flow wetlands of a textile industry in the municipality of Rionegro (Antioquia), the microorganisms *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* and *Escherichia coli*, in which the fading capacity was demonstrated within a consortium. However, it has not been done using the microorganisms individually or using matrices to immobilize them. Therefore, the objective of the present investigation was to determine the ability of discoloration of reactive black 5 by a consortium and the microorganisms that constitute *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* and *Escherichia coli* free and immobilized in calcium alginate, coffee husks and bagasse of sugar cane.

Materials and methods

Microorganisms and culture conditions

The microorganisms which formed the consortium *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* and *Escherichia coli* were previously isolated and identified from a biofilm formed in the inlet and outlet gravel of three subsurface flow wetlands lo cated in a textile company in the municipality of Rionegro, Antioquia (Colombia) (Alzate *et al.*, 2016). In order to determine the percentage of discoloration by each of the microorganisms and the free consortium, axenic cultures were taken to obtain biomass in the culture medium Agar Tripticasa Soya (TSA) (Oxoid, UK). Two colonies were inoculated into 100 mL of Tryptic Soy Broth (TSB) broth (Oxoid, UK), were incubated at 30 °C, 100 rpm for 48h. Subsequently, the cultures were centrifuged at 5 000 rpm for 15 min, the supernatant discarded, two washes were performed with a 0,9% sterile saline solution for the total removal of the TSB medium, and the biomass was resuspended in the culture medium for the discoloration tests containing: 1 g/L K_2HPO_4 (Merck, Germany); 0,5 g/L $MgSO_4.7H_2O$ (Honeywell, Germany); 1 g/L $(NH_4)_2SO_4$ (Mallinckrodt, Mexico) and 4 g/L anhydrous glucose (Panreac, Spain) and commercial NR5 at 100 mg/L. Subsequently, the optical density of the culture was adjusted to one (optical density (OD) =1) at a wavelength of 600 nm by spectrophotometry (Thermo UV Vis 300).

Free state Discoloration tests

Suspensions were adjusted to OD=1 of the different microorganisms individually and in consortium were exposed to 100 mg/L of the dye in 60 mL of culture medium, for discoloration tests, and incubated at 30 °C, 100 rpm under dark conditions to evaluate their discoloration ability (El Bouraie & El Din, 2016).

Samples of 1 mL were taken daily for six days, which were centrifuged at 5 000 rpm for 10 min and analyzed by spectrophotometry at 592 nm. The percent discoloration was determined by the formula described by Chen et al. (2015). Finally, the residual concentration was found in a calibration curve of the dye at serial dilutions of 100 to 1 mg / L.

All treatments were performed by triplicate.

Evaluation of the immobilization of microorganisms in the three matrices

Three immobilization matrices: calcium alginate, cane bagasse and coffee husk were selected for cellular immobilization. The agroindustrial raw materials were subjected to a conditioning process using the protocol described by Basak *et al.*, 2014. The average sizes of the pieces were on average 0,6x0,4 cm for coffee husks and on average 0,9x0,2 cm for cane bagasse. The microorganisms

separately and the consortium, corresponded to the treatments for each agroindustrial matrix along with their respective controls.

Growth kinetics were performed in TSB at 30 °C, 100 rpm for 48 h. Where the time was set to reach the exponential phase of each microorganism in order to determine the amount of biomass to be immobilized in the calcium alginate. For the consortium began the growth of yeasts and missing 6 h to reach the exponential phase found for these promoted the growth of *E. coli*.

Immobilization was performed on calcium alginate following the protocol recommended by Dong *et al*. (2014). The immobilized biomass in 6 g of calcium alginate was evaluated by the counting of colony forming units in TSA medium.

The biomass immobilized in the agroindustrial residues coffee husk and bagasse cane was measured by the dry weight method, using 6 g of coffee husks in 60 mL of TSB and 3 g of cane bagasse in 50 mL of TSB. After the immobilization process, a protocol variant was used to measure the amount of biomass immobilized by dry weight described by Garzón (2009), using distilled water instead of phosphate buffered saline. All treatments were performed in triplicate.

Discoloration tests with immobilized microorganisms

The microorganisms were inoculated in TSB medium and incubated at 30°C and 100 rpm until the exponential phase. After this time the medium was washed and 60 ml of culture medium were added to the bleaching tests plus 6% of coffee husk and to 100 ml of culture medium 6% of cane bagasse. The media were incubated for three days, time necessary for the colonization of the substrate as described by Garzón (2009).

Immobilization with the calcium alginate was performed as is described above.

The microorganisms immobilized individually and in consortium, were inoculated in the medium described for the discoloration tests at the same concentration of dye, retaining the volumetric quantities of the medium and mass of the matrices. Discoloration tests were incubated at 30 °C, 100 rpm for six days under dark conditions. Negative controls were made, that consisted in the matrices in the culture medium without the microorganims at the same conditions of incubation to evaluate the effect of the matrices on decolorization. The percentage of discoloration was evaluated as in the test with the microorganisms in the free state.

All treatments were performed in triplicate.

Statistical analysis

The immobilization matrices and the discoloration capacity of the individual and consortium microorganisms were compared by ANOVA using the statistical program STATGRAPHICS® Centurion XVI with a confidence level of 95% ($P < 0,05$). In addition, multiple range tests were performed to observe the significance between the matrices. Finally, all treatments were performed in triplicate to obtain statistical validity of the data obtained experimentally.

Results and discussion

Ability to decolorize by the microorganisms in free state

The microorganisms that interact constantly with dyes in a habitat can present a natural adaptation to these compounds, allowing degradation processes (Khehra et al., 2005; Guerrero et al., 2014). The environmental conditions of the environment in which both the yeasts and the bacteria evaluated were isolated, favor this adaptation due to the varied concentrations and the different types of dyes present in the wetlands. This is evidenced by the kinetics of discoloration (figure 1), which shows the percentage of discoloration that the evaluated microorganisms have the ability to discolor, where the highest percentage corresponds to *G. pseudocandidum* (90,05%) and the lowest to *R. mucilaginosa* (79,31%) (Figure 2a). The variations between the microorganisms can be presented by the different microbial mechanisms used for the discoloration in each of the microorganisms; these mechanisms can be: enzymatic, physical as biosorption and a combination of these (Solís et al., 2012). Enzymes such as laccase, azoreductase, riboflavin reductase and tyrosinase have been reported in species of the genus *Galactomyces*, which may be involved in the discoloration process (Solís *et al.*, 2012).

With respect to *R. mucilaginosa*, Yang et al (2013) report species of *Rhodotorula* producing extracellular enzymes as LiP, MnP, laccase, or oxygenases that are responsible for dye decolorization and degradation of aromatic substances (Yang *et al.*, 2013).

In addition, Charumathi & Das (2012) suggest that this process could carried out biosorption and attributes this process to heteropolysaccharide and cell wall lipid components, which contain different functional groups, including amino, carboxy, hydroxy, phosphate and other charged groups, causing strong attraction forces between the azo dye and the cell wall.

The percentage of dye removal by *E. coli* was 89,02% (Figure 1 and 2a). The decolorization value of the microbial consortium was lower (87,87%) than that presented by *G. pseudocandidum* and *E. coli* (Figure 2a). These data are in contrast with previous studies that report higher percentages of discoloration in consortia than in individual microorganisms because the process can be

made by combining different metabolic processes of each microorganism, which allows an increase in speed and degree of discoloration (Ayed *et al.*, 2010).

Another valuable point to consider is that the use of a consortium of microorganisms isolated from environments that contains contaminants from the textile industry ensures a high tolerance to dye concentrations and resistance to environmental variations (De Almeida et al. 2017). However, the operation of a native consortium changes when it is brought to in vitro conditions, because in situ phenomena such as environmental variation occur which can exert a significant influence on metabolic exchange, energy flow and cycle of nutrients (Ding et al., 2016).

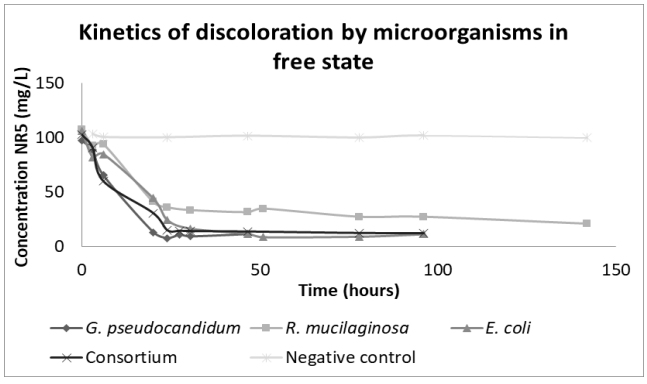

Figure 1. Discoloration kinetics of *R. mucilaginosa*, *G. pseudocandidum*, *E. coli* and the consortium in free state in culture medium for discoloration tests with initial concentration of 100 mg/L of NR5.
Source: Authors

Evaluation of immobilization of microorganisms in matrices

Once the discoloration capacity of the microorganisms has been determined under the conditions described above, the amount of biomass retained by the calcium alginate and the colonization of the agroindustrial matrices by the species yet studied is then evaluated. The results for the microorganisms immobilized in calcium alginate are obtained by sowing in solid medium, evidencing growth in all the sowing performed.

When comparing the percentages of retention of the microorganisms in the coffee husk, it was obtained that *E. coli* was immobilized 97,3%, 88,9% and 11% more than *G. pseudocandidum*, *R. mucilaginosa* and the consortium, respectively. This variability can be attributed to the differences in the time of duplication of the evaluated microorganisms, since the yeasts have had longer time to the one of the bacteria, and as it has been demonstrated in other studies, the colonization of the matrices depends on the rate of growth of the microorganisms (Orzua et al., 2009). With reference to the immobilization in the cane bagasse, *G. pseudocandidum* and *R. mucilaginosa* present negative values of dry weight (Table 1), which can be attributed to the degradation of the matrix by the yeasts that use it as a source of carbon (Garzón, 2009). On the other hand, is important to consider the loss of biomass due to the agitation process necessary to avoid sedimentation of the microorganisms and favor their contact with the matrix, since the passive immobilization process does not ensure a complete fixation of the cells (Garzón, 2009).

Table 1. Dry weight of the biomass immobilized in cane bagasse and coffee husks after three days of colonization

microorganisms	husk coffee	Bagasse of sugar cane
	Weight of dry biomass (g)	
R. mucilaginosa	0,01455	-0,26285
G. pseudocandidum	0,00355	-0,18275
E. coli	0,13075	0,40935
Consortium	0,1177	0,12925

Source: Authors

Decolorizing capacity by immobilized microorganisms

When comparing the percentages of discoloration between the free microorganisms and the immobilization matrices, it is observed that the former exhibit the highest percentages of discoloration (Figure 2) and the time required for the process is lower (Figure 1). This is attributed to the fact that the immobilization matrices have mass transfer limitations, so the required time for discoloration in them increases, since the immobilized cells need more time to contact the culture medium to discolor (Rodriguez & Toca, 2006; Basak *et al.*, 2014). However, there are no statistically significant differences (P = 0,1319) between treatments with immobilized microorganisms or in the free state, so immobilization is presented as a viable alternative, since it allows the stability of microorganisms against adverse conditions, in addition to the reuse of biomass (Quek *et al.*, 2006).

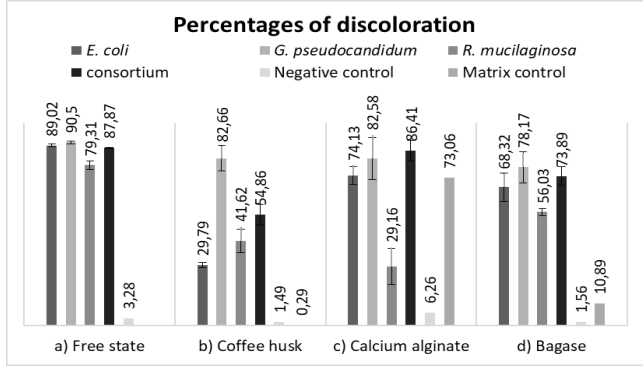

Figure 2. Percentages of discoloration of the microorganisms of *R. mucilaginosa*, *G. pseudocandidum*, *E. coli* and the consortium in a) free state. b) immobilized in coffee husks. c) immobilized in calcium alginate d) Immobilized in cane bagasse. This procedure was performed in culture medium for bleaching tests with initial concentration of 100 mg / L of NR5.
Source: Authors

Calcium alginate was selected because it is one of the most used matrices to perform cell trapping, since it allows a

high immobilized biomass load (Chen & Lin, 2007) and allowed a comparison with non-conventional matrices, like cane bagasse and coffee husks. Also, agroindustrial matrices are a viable alternative for the immobilization of microorganisms, because they are low cost, friendly to the environment and have a high porosity, which facilitates the transfer of mass, greater colonization, protection and source of nutrients (Dos Santos *et al*, 2000; Orzua *et al.*, 2009).

In this study, there were no statistically significant differences between immobilization matrices and percentages of discoloration (P = 0,6211). However, when analyzing each matrix, statistical differences were observed in the percentages of discoloration of the microorganisms evaluated in calcium alginate (P = 0,0001), coffee husk (P = 0,0) and cane bagasse (P = 0 , 0035); these differences are observed in table 2, where *G. pseudocandidum* presents the greatest differences with respect to the other microorganisms on the percentage of discoloration of NR5. This agrees with the previously mentioned theory about the enzymatic machinery of the genus *Galactomyces*. Finally, although the calcium alginate matrix and agroindustrial matrices use a different immobilization method, both are presented as a viable option in the NR5 bleaching process.

Table 2. Test of multiple ranges for percentage of discoloration by immobilized microorganisms

Testing of Multiple Ranges for percentages of discoloration by microorganisms						
Contrast	Calcium Algnate		Husk coffee		Cane Bagasse	
	Sig	*Difference*	*Sig*	*Difference*	*Sig*	*Difference*
G. pseudocandidum - *R. mucilaginosa*	*	53,4167	*	41,03	*	25,14
G. pseudocandidum - *E. coli*		8,44	*	52,8667		9,85
G. pseudocandidum - consortium		-3,83333	*	27,7967		4,27667
R. mucilaginosa - *E. coli*	*	-44,9767	*	11,8367	*	-15,29
R. mucilaginosa - consortium	*	-57,25	*	-13,2333	*	-20,8633
E. coli - consortium		-12,2733	*	-25,07		-5,57333

*Indicates a significant difference.
Source: Authors

Regarding to the consortium, the discoloration profiles can be determined under controlled conditions for each species; however, when these are subjected together, it would be expected that the discoloration of the consortium would be enhanced or additive. However, it is found that the consortium has discoloration profiles similar to those of the individual microorganisms or even smaller; suggesting that there are ecological interactions that could affect the microorganisms constituting the consortium, including competition, amensalism and antagonism (Ding *et al.*, 2016).

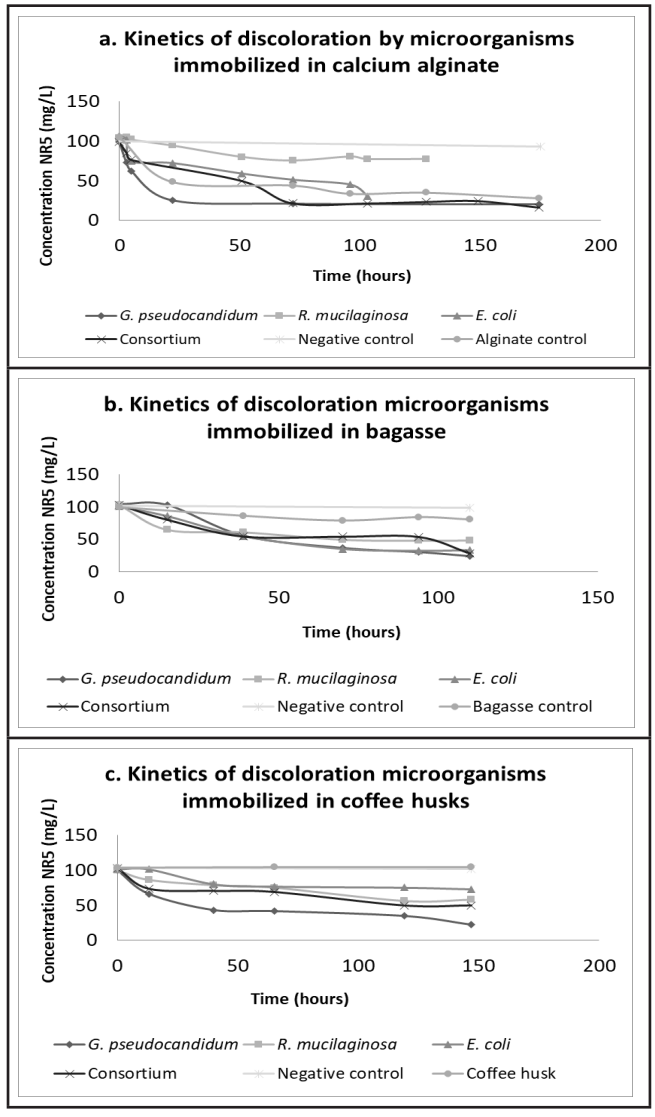

Figure 3. Kinetics of discoloration of the microorganisms *R. mucilaginosa*, *G. pseudocandidum*, *E. coli* and the consortium immobilized in a) alginate, b) cane bagasse and c) in coffee husks. In culture medium for discoloration tests with initial concentration of 100 mg / L of NR5.
Source: Authors

Analyzing the controls of the different immobilization matrices without microorganisms are found to have an effect on discoloration (Figure 3). Each matrix has characteristics that allow it to perform sorption phenomena (Quek *et al.*, 2006). In the case of calcium alginate its ability to adsorb dyes has been studied, where it is suggested that it absorbs and immobilizes these contaminants (Jeon, Lei & Kim, 2008). For agroindustrial matrices it was found that they have the ability to discolor, possibly as calcium alginate has the ability to adsorb the dye (Oliveira & Franca, 2015; Tahir *et al.*, 2016). However, these sorption phenomena do not ensure the breakdown of the chemical bonds of the dyes and this is why biological systems are an attractive alternative for the treatment of wastewater with different contaminants such as dyes (Bai *et al*., 2010).

Conclusions

The present study showed that the consortium and the constituent microorganisms *Rhodotorula mucilaginosa*, *Galactomyces pseudocandidum* and *Escherichia coli* in free culture have the capacity to decolorize the NR5, therefore, they are presented as potential for its possible application. In addition, it was demonstrated that the immobilized microorganisms in the different matrices did not lose their viability and their fading ability; however, in comparison with the free microorganisms, a lower percentage of discoloration of NR5 was obtained, both individually and in a consortium. However, the immobilization is presented as a viable alternative because of the many of its advantages. The present investigation allowed to determine that the use of agroindustrial residues as immobilization matrices have proven to be viable for use in dye treatment processes using microorganisms, leaving the door open for future studies of application of these matrices in the field.

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