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Removal of azo dyes from water by sol-gel immobilized *Pseudomonas* sp.

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Abstract

Water pollution control is presently one of the major scientific research areas. Sol-gel immobilized *Pseudomonas* sp. able to enzymatically reduce azo groups was used for the decolourization of water containing azo dyes. It was observed that immobilized bacteria produced more than seven times higher amounts of extracellular enzymes involved in the biodegradation of azo dyes. The reusability of the immobilized bacteria was successfully evaluated with repeated-batch decolourization experiments. Indeed, after four repeated experiments, the decolourization was over 75%, 79% and 83% for remazol black, methyl orange and bencyl orange, respectively. The herein sol-gel immobilized bacteria offer advantages such as high viable cell densities, high stability and extended reaction times. Thus it would be applied as a cost-effective and efficient treatment to remove dyes from effluents.

Keywords: sol-gel, immobilization, azo dyes, bioremediation

1. Introduction

Water pollution control is presently one of the major scientific research areas. Particularly, coloured organic compounds generally represent a minor fraction of the organic components of wastewaters but their colour renders them aesthetically unacceptable. The colour of waste effluents is due to the presence of phenolic compounds such as tannins or lignins (2–3%), organic colorants (3–4%) and especially dyes and dye intermediates [1]. Dyes are difficult to be decolourized due to their complex structure, synthetic origin and recalcitrant nature, which makes it obligatory to remove them from industrial effluents before being disposed into hydrological systems [2]. These dyes include several structural forms such as acidic, reactive, basic, disperse, azo, diazo, anthraquinone based and metal-complex dyes [3]. In this sense, Government legislation imposes strict regulating measures that compel industries to treat their waste effluents to increasingly high quality levels. During the past two decades, several decolourization techniques have been reported, few of which have been accepted by industries. Thus, there is a need to find alternative cost-effective and efficient treatments to remove dyes and colorants from effluents [4]. Among the different methods to treat effluents, the advantage of biological treatments over certain physico chemical treatment methods is that over 70% of the organic material present may be converted to biosolids [5]. In this aspect, numerous bacteria capable of dye decolourization [6-9] have been reported [10-14].

Immobilized microorganisms are being increasingly used for wastewater treatment bioreactors as they offer advantages such as high cell densities, high stability, absence of

47 cell washout, and extended reaction times [15]. Among the different immobilization
48 techniques, sol–gel chemistry is an interesting domain because it allows obtaining
49 materials with desirable new chemical and mechanical properties [16, 17]. Moreover, it
50 was early identified as an eco-friendly process compared to traditional synthesis routes to
51 ceramics and glasses, thus improving sustainability in product developments [18]. During
52 the last 15 years several works reported the encapsulation of living cells in sol-gel silica
53 matrices[19-23]. Indeed, since Carturan *et al*., [24] pioneered the encapsulation of living
54 microorganisms in sol-gel silica matrices several works were reported extending the
55 process to other cell types [25, 26] such us bacteria, yeast, algae and mammalian cells
56 which were successfully immobilized in silica matrices[27-32]. In most cases it was
57 demonstrated that the employment of biocompatible molecules such as glycerol,
58 polyethylene glycol or glycine betaine further improve the biocompatibility of the
59 immobilization process [33-37].
60 Nowadays, this technology is well established for the development of immobilization
61 matrices and its application in different processes is growing fast. Particularly, the
62 immobilization of bacteria in sol-gel matrices for environmental biotechnological
63 processes constitutes an active area of research [38-46]. Especially, because it will allow
64 using them in environments that are normally hostile to biosystems [47, 48]. Herein we
65 report the immobilization of *Pseudomonas* sp. in sol-gel silica matrices and its
66 application for water treatment. Indeed, the immobilized bacteria were successfully
67 applied to decolorize remazol black (RB), methyl orange (MO) and benzyl orange (BO),
68 which are azo dyes commonly used in industrial processes. To the best of our knowledge,

it is the first time that sol-gel immobilized *Pseudomonas* sp. with excellent decolorizing ability against azo dyes has been reported.

2. Experimental methods

2.1. *Bacterial strains, culture conditions, and viability determination*

Pseudomonas sp. was gently provided by the Higiene y Sanidad group from the Microbial Culture Collection of Facultad de Farmacia y Bioquimica (CCM 29), University of Buenos Aires, Argentine. Cells were grown for 24 hours at 35 ºC and maintained in Luria–Bertani (LB) medium (yeast extract, 5 g l^{-1}; NaCl, 10 g l^{-1} and tryptone, 10 g l^{-1}) up to OD (600 nm) 0.800, centrifuged and resuspended in LB medium. The number of colony-forming units (cfu) per milliliter of this suspension was determined by the plate count technique before its utilization in immobilization studies. The bacterium expressed azoreductase activity and was able to decolorize a variety of azo dyes.

2.2. *Cell immobilization methods*

For immobilization experiments, a 25 ml aliquot of the cell culture in stationary phase was centrifuged at 5000 rpm for 10 minutes and the pellet was washed and resuspended in an equal volume of 0.2 M sodium phosphate buffer, pH 7.00. The resulting cell suspension was used for cell immobilization in silica matrices. *Pseudomonas sp*. cells were immobilized into sol–gel silica matrices using citric acid in the sol-gel process [49]. For this purpose, 1 g of sodium silicate in 6 ml water was heated up to 80 °C until sol formation. The pH was then fitted to 6.50 using 0.75 M citric acid and the cell suspension

92 was added. The preparation (0,2 ml) was poured in wells of a 96 well plate and left at
93 room temperature until gelation (2 min). Finally, the gels were stored 24 h at 35 °C for
94 the formation of mechanically resistant pearls. For the determinations of living bacteria,
95 decimal dilutions in physiological saline of the bacteria suspension were plated in
96 duplicate.
97
98 2.3. Silica matrices characterization
99 The specific surface area, total pore volume, and pore size were measured for silica
100 matrices by nitrogen sorption at 77 K using an automatic gas adsorption analyzer (Gemini
101 2360 V2.00). Prior to the measurement, the gels were reduced to powder and degassed
102 for 24 h at 150 ºC. The specific surface area was calculated according to the Brunauer–
103 Emmett–Teller (BET) theory, while the pore size distribution and the total pore volume
104 were calculated by the Barrett–Joyner–Halenda (BJH) method.
105 Silica matrices samples for scanning electron microscopy (SEM) were fixed using 3.63%
106 glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) with 0.3 M saccharose for 1
107 h at 4°C. Following fixation, samples were washed three times in the same buffer and
108 then dehydrated in a graded series of ethanol (70%, 95% and two changes of alcohol
109 100%). Finally, the samples were subjected to supercritical drying and were gold sputter-
110 coated for analysis using a Zeiss Supra 40 microscope for Scanning Electron Microscopy
111 (SEM).
112
113 2.4. Enzyme activity measurements
114 The non immobilized cells were harvested by centrifugation at 5,000 rpm for 5 min and
115 the supernatants were used to assay azo dye reduction by measuring residual absorption

116 at the appropriate wavelength for each azo dye. In the case of immobilized cells the cell
117 free supernatants were obtained after removing the silica pears. The azoreductase activity
118 of the cell free supernatant was determined based upon the procedures described by
119 Zimmermann *et al*., [50]. In general, 1 ml of supernatants (typically at 0,1 g protein ml^{-1})
120 was added into 12 ml of reaction mixture containing 0.1 M phosphate buffer, 24 mM
121 remazol black, and 0.35 mM NADH (Sigma). The residual dye concentration in the
122 reaction mixture was detected as a function of time, and the enzyme activity was
123 determined from initial rate of dye disappearance.
124 Activities of lignin peroxidase, laccase and tyrosinase were assayed
125 spectrophotometrically in cell free supernatants. Lignin peroxidase activity was
126 determined by monitoring the formation of propionaldehyde at 300 nm in a reaction
127 mixture (pH 3.5) containing 2.5 ml 100 mM n-propanol, 250 mM tartaric acid, 10 mM
128 H_2O_2 as described by Shanmugam *et al*. [51] Laccase activity was determined in a
129 reaction mixture of 2 ml containing 10% of 2,2'-azino-bis(3-ethylbenzothiazoline-6-
130 sulphonic acid) in 0.1 M acetate buffer (pH 4.9) and measuring the increase in optical
131 density at 420 nm with the method of Hatvani and Mecs [52]. Tyrosinase activity was
132 determined in a reaction mixture of 2 ml, containing 0.01% catechol in 0.1 M phosphate
133 buffer (pH 7.4) at 495 nm as described by Zhang *et al*. [53]. All enzyme assays were
134 carried out at 25°C where reference blanks contained all components except the cell free
135 supernatants. All enzyme assays were run in triplicate and average rates were calculated.
136 One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg^{-1} of
137 protein. Protein concentrations were estimated by Biuret method.
138

139 2.5. Colour removal by immobilized bacteria

140 The reactive azo dyes used in this study were Remazol Black 5 (RB), Methyl Orange
141 (MO) and Benzyl Orange (BO) which were obtained from Sigma-Aldrich. (St. Louis,
142 USA). The concentration of azo dye in samples was determined by measuring the
143 absorbance of the supernatant at 595 nm (RB), 495 nm (MO) and 435 nm (BO).

144 Immobilized *Pseudomonas sp.* cells (3 pearls) were placed in 10 ml medium containing
145 0.1% glucose, 0.1% $NH_4(SO_4)$, 0.01% $MgSO_4$, 0.25 KH_2PO_4, 0.05% sodium citrate and
146 0.7% K_2HPO_4 and the designated concentrations of RB, MO and BO for the removal of
147 dye colour. The resulting solution was then statically incubated at 35°C for colour
148 removal. After complete colour removal, the immobilized-cell pearls were collected,
149 rinsed twice with sterile 0.9% NaCl solution and transferred into fresh medium for the
150 second bioremediation experiment. The same procedures were repeated four times in four
151 weeks.

152

153 2.6 Statistical analysis

154 All the experiments were performed 3-4 times and the average values were used in
155 calculations. Data are means $\pm$ SD. The differences were analyzed using unpaired t test. p
156 < 0.05 was considered significant.

157

158 **3. Results and discussion**

159

160 3.1. Activity of extracellular enzymes

Microbial extracellular enzymes have a potential to degrade a wide range of complex aromatic dyestuffs. Thus, the analysis of the activity and release of these enzymes from immobilized bacteria is highly important when foreseeing industrial applications. In this sense, the activity of laccase, tyrosinase, azoreductase and lignin peroxidase were measured in supernatants of free and immobilized bacteria cultures in the presence of the dye. It was observed that the activity of the enzymes lignin peroxidase and tyrosinase increased in the presence of remazol black (data not shown). Particularly, the activity of lignin peroxidase, tyrosinase and laccase were significantly higher for immobilized bacteria than for free bacteria, while there were no statistical significant differences in azoreductase activity between free and immobilized bacteria. Another interesting result is that the total amount of proteins in the supernatants of immobilized bacteria is significantly higher than in free bacteria. This result further confirms that the immobilized bacteria were able to produce extracellular proteins, especially the enzymes involved in the degradation of the dye (**Table 1**). Silica materials possess nitrogen adsorption–desorption isotherms similar to the isotherms characteristic of microporous materials. They have low porosity with a total pore volume of 0.033 cm^3 g^{-1} with a specific surface area equal to 72 m^2 g^{-1}. This porous structure allows the diffusion of the azo dyes from the supernatant to the interior of the silica pearl or the release of the extracellular enzymes involved in the biodegradation of the dyes. These results allow us to envisage at least two real advantages of the sol-gel immobilized bacteria. In one hand, the employment of sol-gel immobilized bacteria would facilitate its reutilization and would avoid the dissemination of bacteria in the effluents. On the other hand, the higher

183 levels of proteins and enzymes produced and released would allow performing faster
184 bioremediation processes.
185
186 3.2. Remazol black colour removal with immobilized bacteria
187 Immobilization is one of the great tools for developing economically and ecologically
188 available biocatalysts and can be applied for both enzymes and whole cells [54]. The
189 above results indicate that sol-gel matrices were suitable for maintaining high levels of
190 viable bacteria, allowing protein release from the sol–gel silica matrix. However, the
191 performance of immobilized bacteria for dye colour removal must be addressed. In this
192 sense, it was observed that the immobilized bacteria were able to decolorize remazol
193 black and that this activity was modified by the different concentrations assayed. Indeed,
194 immobilized bacteria were able to decolorize 80% of the dye after 24 h of incubation
195 when 25 or 50 μg ml^{-1} was added to the solution (**figure 1**). The decolourization was
196 almost complete after 48 h for these concentrations. Meanwhile, when 75 or 100 μg ml^{-1}
197 was added to the solution the immobilized bacteria decolorized 75% after 24 h and near
198 90% of the dye after 48 h. These results show that dye concentration has an important
199 effect on the reduction activity of the bacteria which is also illustrated in **figure 2**. Indeed,
200 the amount of dye decolorized per day decreased from 85 μg day^{-1} to 75 μg day^{-1} when
201 dye concentration increased from 25 to 100 μg ml^{-1}. Thus, it is probable that at higher
202 RB concentrations, the dye exerts an inhibition effect over bacteria. It is worth
203 mentioning that bacteria appear randomly dispersed within the gel and the bacteria reach
204 a mean value of around 10^7 cfu per gel in all conditions (**figure 3**). Moreover, it indicates
205 that the dye did produce a deleterious effect over immobilized bacteria. This bacteria

density is in agreement with previous reported works that suggest that gels might have a maximum capacity to host bacteria [55]. In this sense, it was suggested that the production of quorum sensing molecules involved in intercellular communication are responsible of this bacteria stationary state [56].

3.3. Reutilization of immobilized bacteria

Immobilized cells exposed to various remazol black concentrations were incubated for 24 h and then silicate pearls were washed and reused for three more cycles of biodegradation. It was observed that the immobilized bacteria possessed almost the same initial efficiency after the reutilization cycles. Even in the presence of the higher dye concentration the reutilization could be performed without activity loss. One of the main advantages of bacteria immobilization for the treatment of polluted aqueous solutions compared to the utilization of free cells is the real possibility of recycling silicate pearls with immobilized bacteria for consecutive cycles of dye biodegradation without significant efficiency loss, as shown in **figure 4**.

3.4. Effect over other industrial dyes

The silica pearls with the immobilized bacteria were successfully employed to decolorize other dyes. The decolourization activity of the immobilized bacteria in the presence of various concentrations of methyl orange and benzyl orange is shown in **figure 5**. Similarly to the results obtained with remazol black, the activity of the immobilized bacteria was influenced by the concentration of bencyl orange and methyl orange. After 24 h with low dye concentration, the biodegradation was higher for remazol black (85 %)

229 than for methyl orange (77%) and benzyl orange (66%). While with a concentration of
230 100 μg ml^{-1}, the biodegradation was higher for benzyl orange (83%) followed by methyl
231 orange (79%) and remazol black (75%). These results further confirm that dye
232 concentration influenced the biodegradation process (**Table 2**). As it can be seen in table
233 2, the maximum degradation of remazol black was obtained with the lowest
234 concentration, while for methyl orange and benzyl orange the highest biodegradation was
235 obtained at 50 μg ml^{-1}.
236 When comparing the biodegradation of the three dyes under study, it was observed that
237 during the first 6 hours of incubation the biodegradation of methyl orange and benzyl
238 orange dyes was higher than the decolourization of remazol black in the same period.
239 These results could be related to the structure of each dye and its diffusion through the
240 sol-gel matrix. Indeed, methyl orange is the smallest molecule and it is supposed to have
241 the lowest diffusion limitation and thus the higher biodegradation. Moreover, the
242 biodegradation in the short term (i. e.: 6 h) perfectly correlates with the size of the dyes.
243 Another thing to take into account is the charge of the dyes at the pH used for this work,
244 as it can be seen in **figure 6**. While MO and BO possess only one negative charge
245 provided by the SO_3^- group, RB has 4 negative charges, which could imply a higher
246 electrostatic repulsion given by the pearl's silica shell. After 48 h, diffusion limitation is
247 not very significant and the decolourization of 100 μg ml^{-1} of the three dyes was higher
248 than 90% in all cases (**figure 7**). In addition, the immobilized bacteria were successfully
249 employed in colour removal of the three dyes without a significant diminution of their
250 activities. In fact, near 80% of decolourization of each dye was observed even after 4
251 cycles of reutilization in the presence of 100 μg ml^{-1} of the three dyes assayed (**figure 8**).

4. Conclusions

Herein, bacteria have been immobilized in silicate pearls without losing their viability or ability to decolorize the three dyes assayed. In comparison with other cells, soil bacteria survive best sol-gel process of encapsulation into silica gels [57, 58]. Moreover, immobilized bacteria have gained certain advantages. One important advantage of the herein presented biodegradation system is the production of higher levels of extracellular enzymes involved in the biodegradation of the dyes. Indeed, three azo dyes were successfully biotransformed by sol-gel immobilized *Pseudomonas sp*. Moreover, colour removal is not related to the adsorption of the dyes to the silica matrix. The advantages of bacteria encapsulation include protection of the bacteria from the external aggressive media, higher protein production per bacteria and the possibility of reutilization of the pearls. Our results indicate that silica- encapsulated *Pseudomonas* sp. could be used as an effective system for environmentally safe biotransformation of the various textile dyes assayed in this work.

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447 Table 1: Total amount of proteins and activity of extracellular enzymes in the

448 supernatants

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	Immobilized bacteria	Non immobilized bacteria
Proteins (mg/ml)	130,0 ± 0,2 *	50,3 ± 0,9
Azoreductase activity (µmol/min)	7,91 ± 0.9	6,79 ± 1,0
Laccase activity (µmol/min)	0,20 ± 0,01 *	0,036 ± 0,01
Lignin peroxidase activity (mmol/min)	6,9 ± 0,4 *	0,9 ± 0,1
Tyrosinase activity (µmol/min)	29,6 ± 1,9 *	2,8 ± 1,0

451

452 * Statistical significant different from non immobilized bacteria, $p < 0.001$

453

454 Table 2: Percentage of dye biodegradation after 24 h.

Dye concentration ($\mu g\ ml^{-1}$)	Biodegradation %		
	Remazol black	Benzyl orange	Methyl orange
25	85± 2	66± 5	77± 4
50	81± 2	86± 3	86± 3
75	77± 4	72± 4	84± 2
100	75± 4	83± 3	79± 1

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Legends to figures

Figure 1: Remazol black colour removal with immobilized bacteria. The initial Remazol black concentration was: ● 25 μg ml^{-1}, ■ 50 μg ml^{-1}, ▲ 75 μg ml^{-1} and ▼100 μg ml^{-1}.

Figure 2: Remazol black colour removal. A: 25 μg ml^{-1} and B: 100 μg ml^{-1}. Tubes 1: silica matrices without bacteria, tubes 2 silica matrices with immobilized bacteria.

Figure 3: SEM image of silica matrices with immobilized bacteria.

Figure 4: Remazol black colour removal by immobilized bacteria in aqueous media after four cycles of reutilization when exposed to various RB concentrations.

Figure 5: Benzyl orange and Methyl orange colour removal with immobilized bacteria. The initial dye concentration was: ● 25 μg ml^{-1}, ▲50 μg ml^{-1}, ▼75 μg ml^{-1} and ♦100 μg ml^{-1}.

Figure 6: Chemical structure of Remazol black, Benzyl orange and Methyl orange.

Figure 7: Decolourization of 100 μg ml^{-1} of ▲Remazol black, ■ Benzyl orange and ● Methyl orange.

479 Figure 8: Colour removal by immobilized bacteria in aqueous media after four cycles of
480 reutilization when exposed to of 100 μg ml^{-1} of Remazol black, Benzyl orange and
481 Methyl orange.
482

Figure 1
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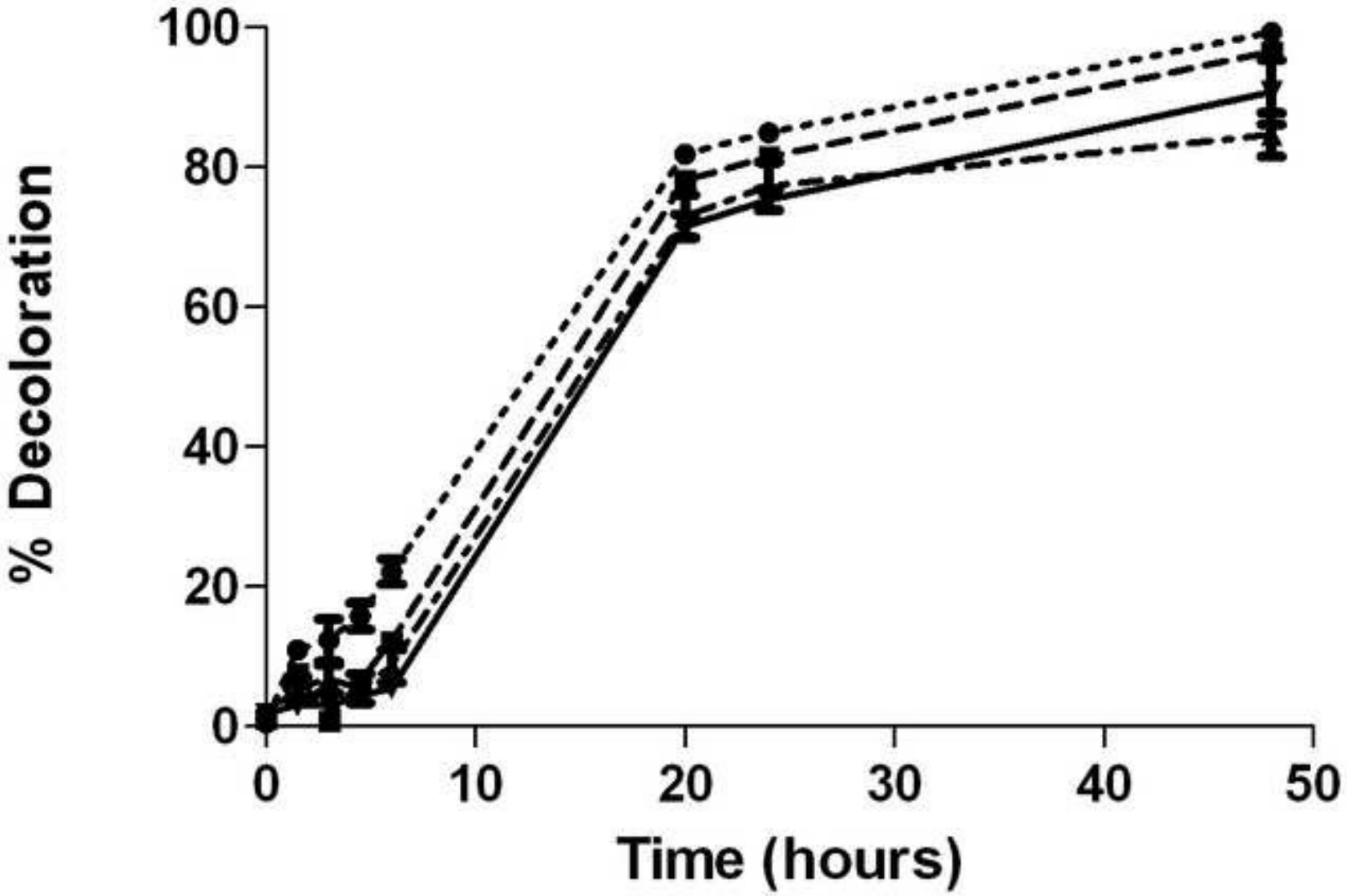

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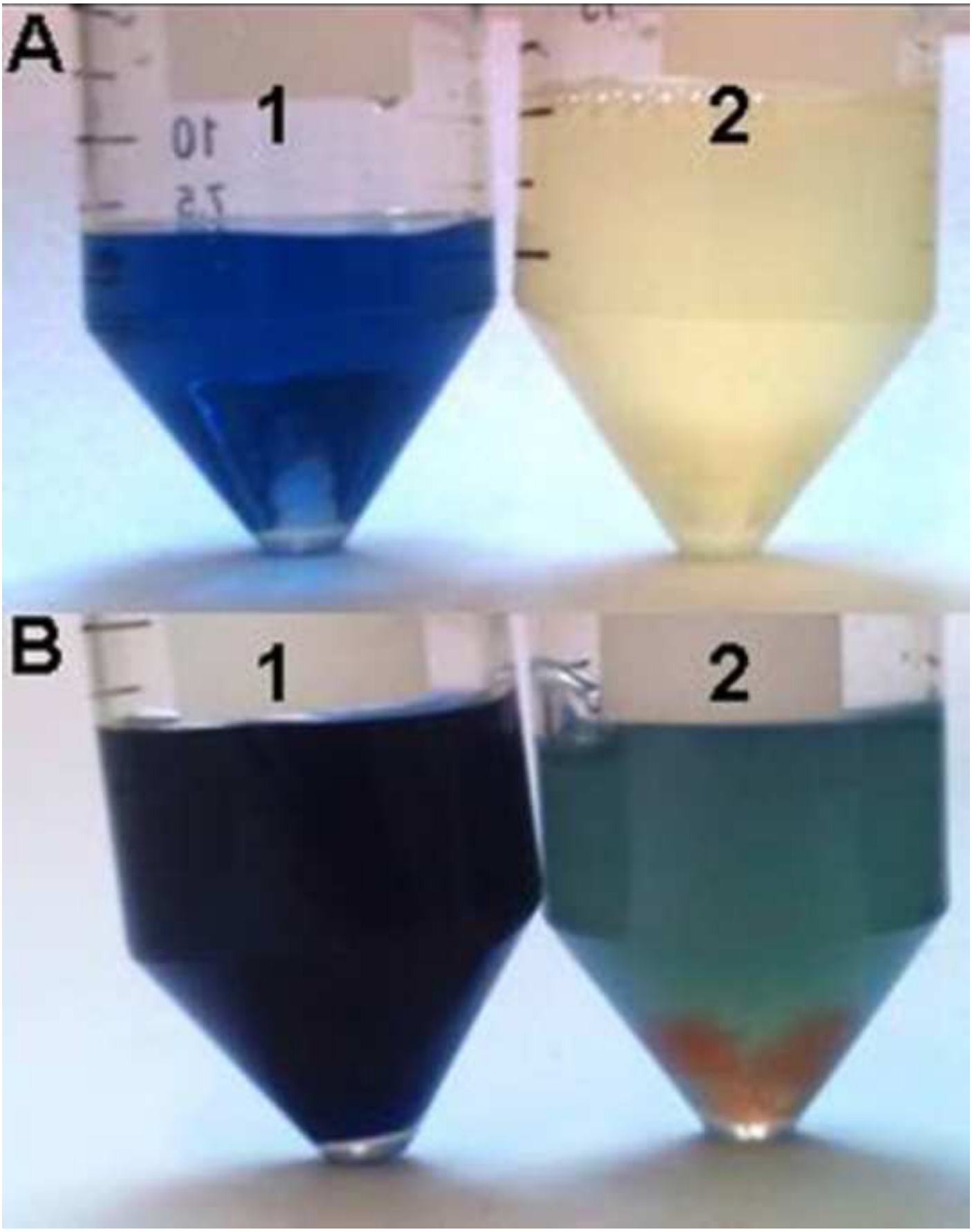

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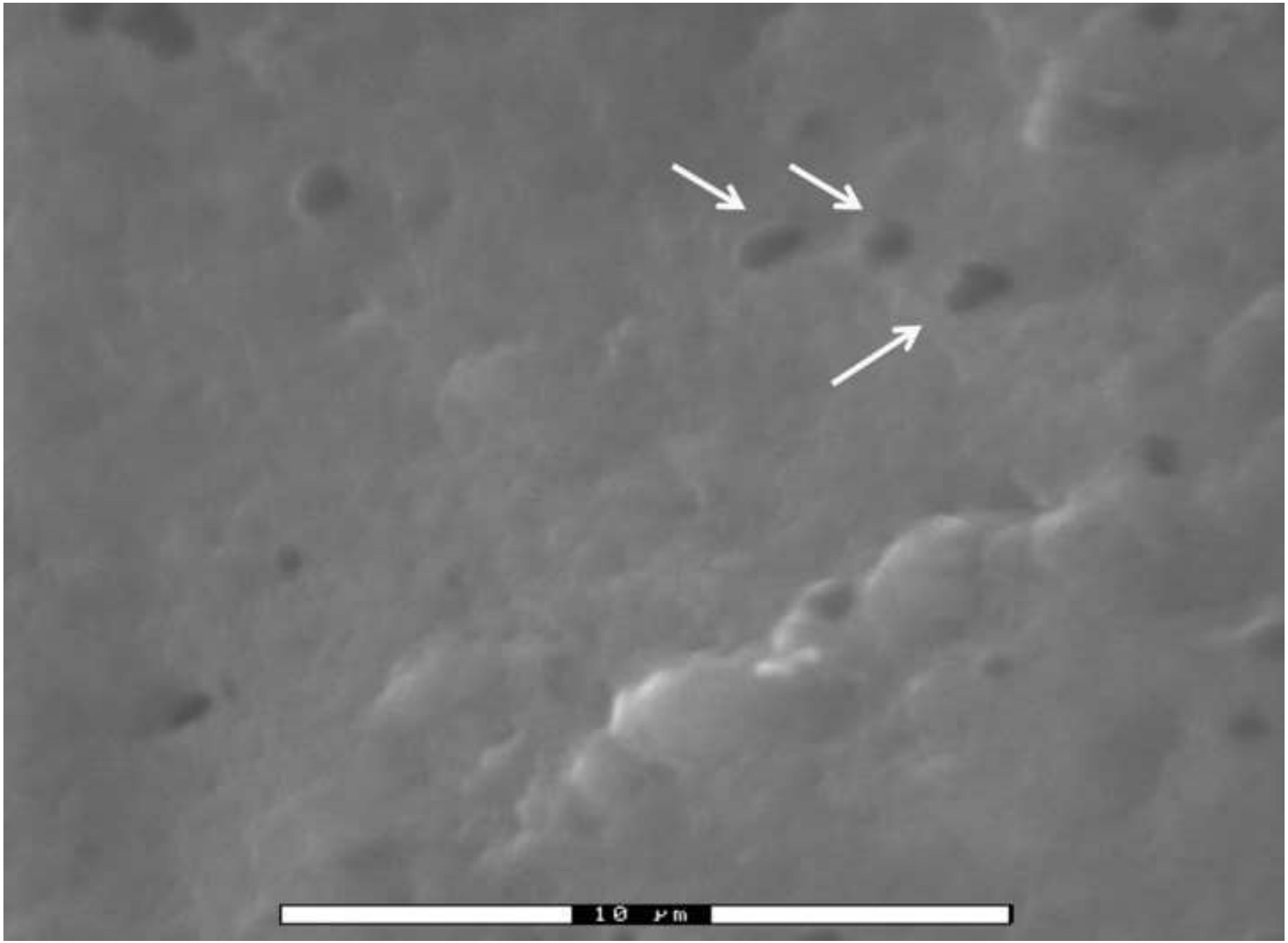

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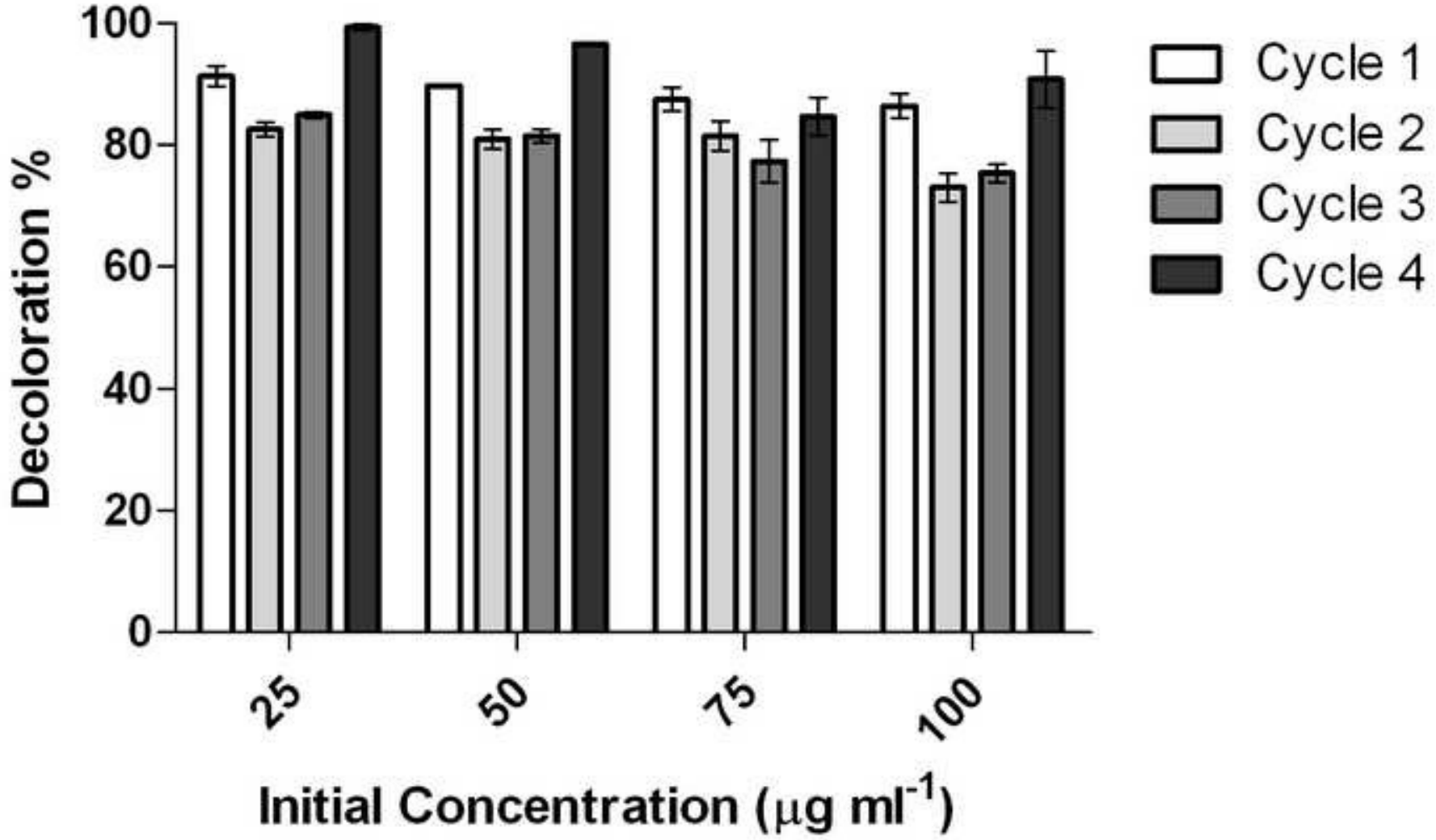

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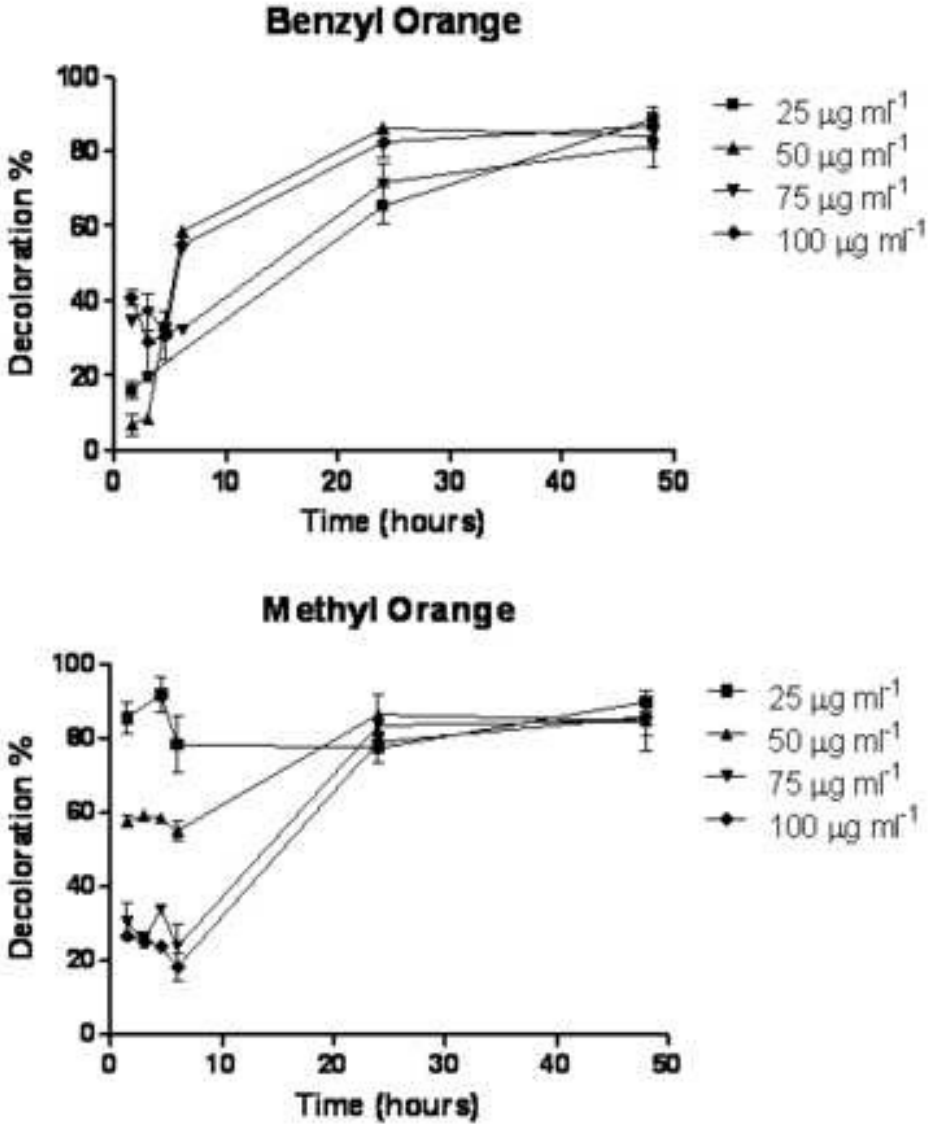

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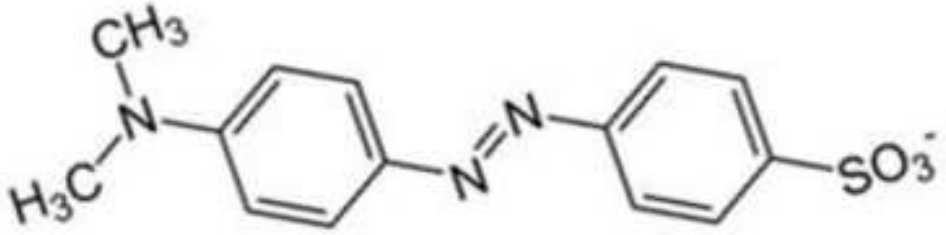

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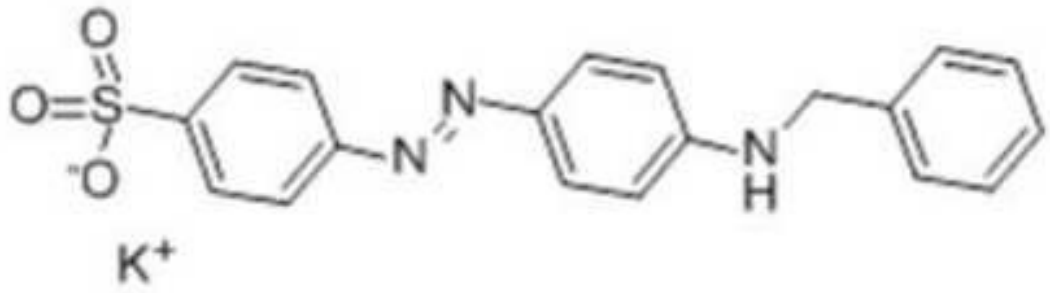

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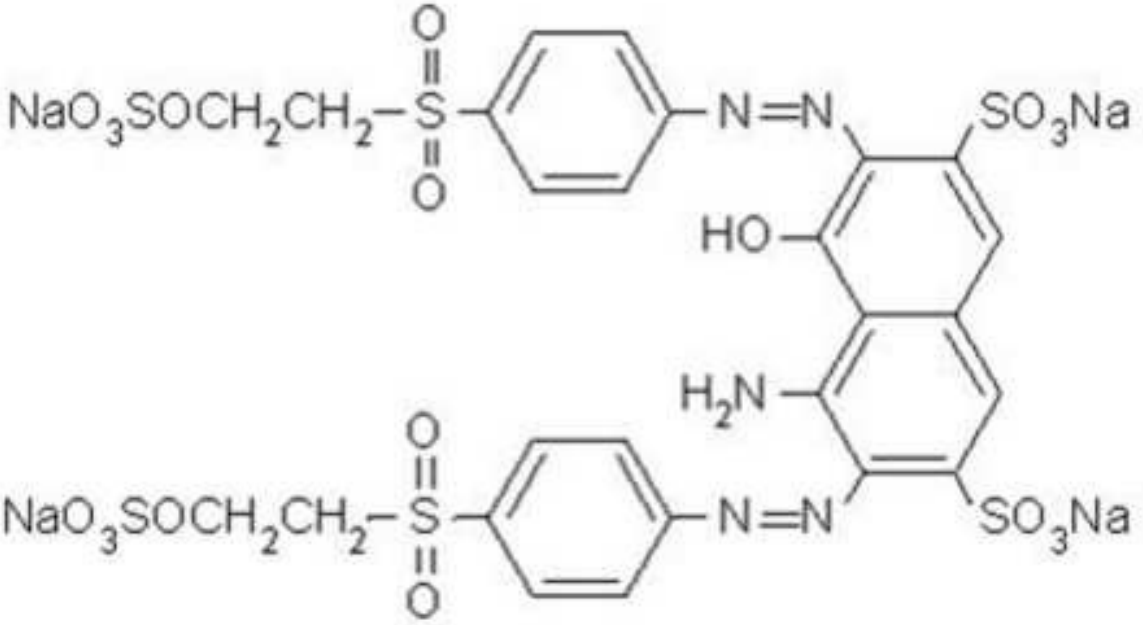

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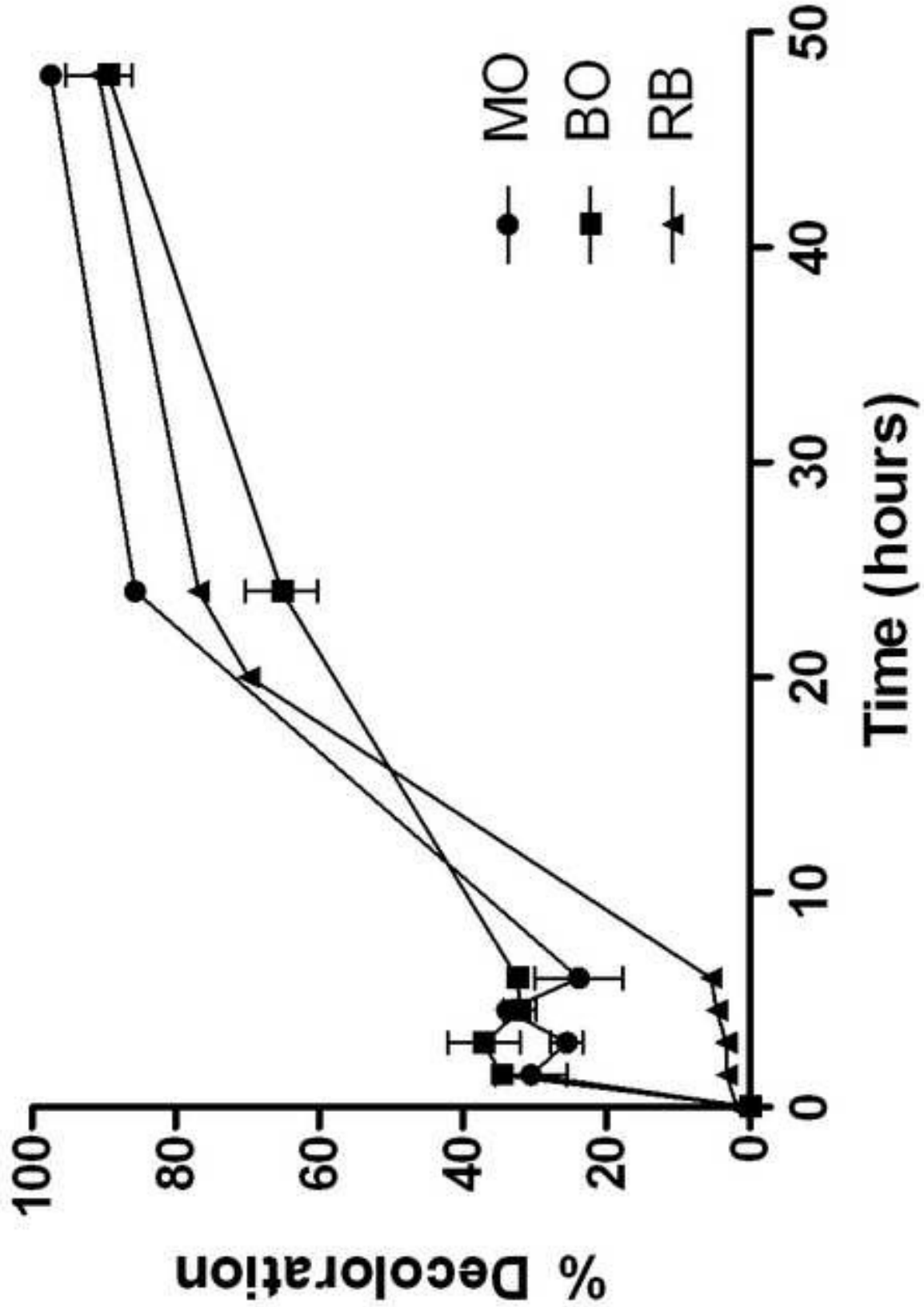

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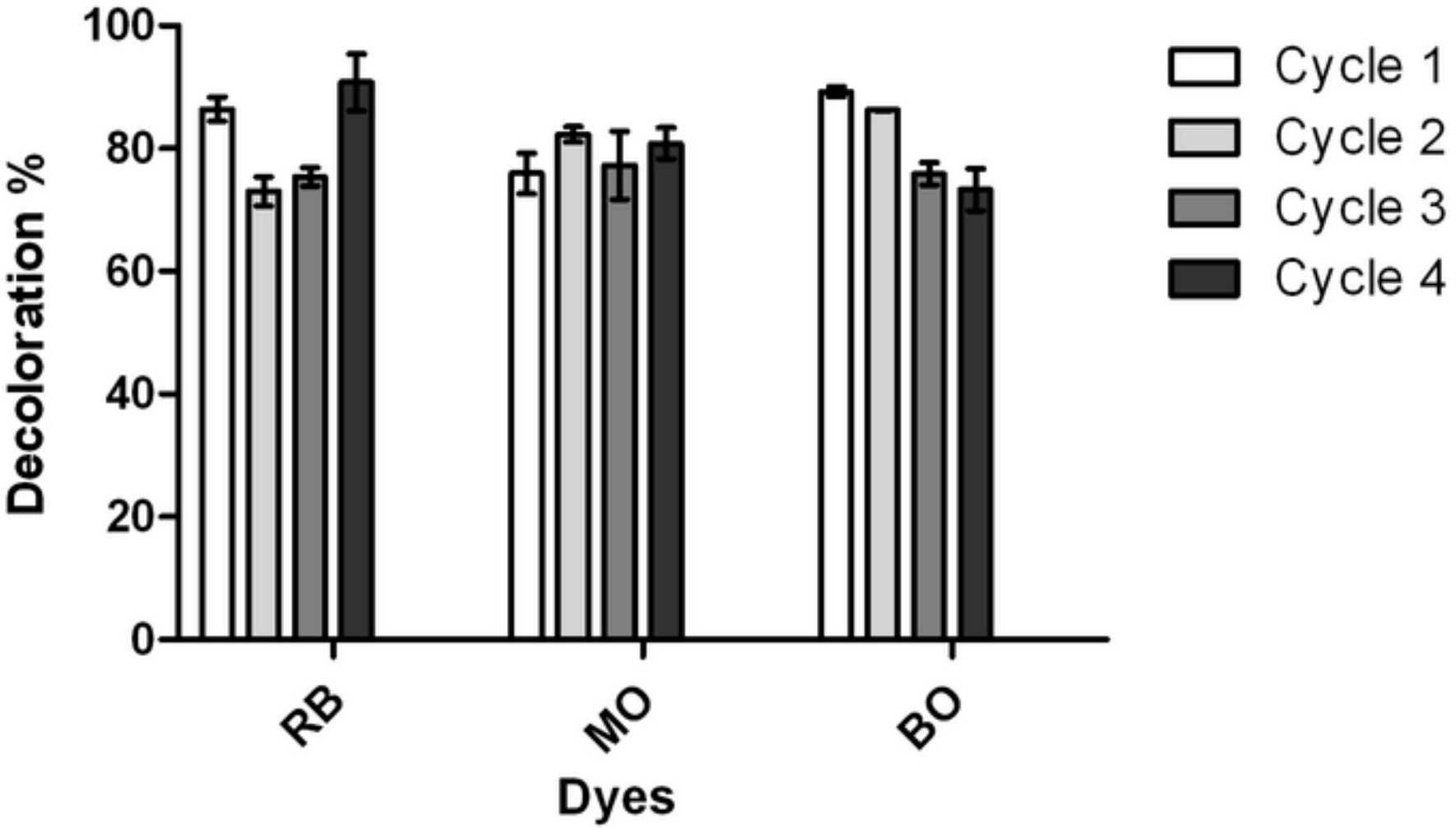

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