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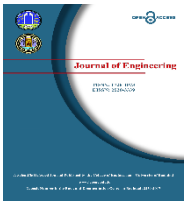

Chemical, Petroleum and Environmental Engineering

Decolorization, Biodegradation and Detoxification of Reactive Blue Azo Dye Using Immobilized Mixed Cells

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ABSTRACT

Drastic threat to the natural system is caused by the uncontrolled release of synthetic pollutants, including azo dyes. This study centered on the decolorization and biodegradation of water soluble azo dye reactive blue (RB) in a batch mode sequential anaerobic-aerobic processes. A local sewage treatment plant was the source where activated sludge was collected to be used as non-adapted mixed culture with both free and the alginate immobilized cells for RB biodegradation. Under anaerobic conditions, the free and immobilized mixed cells were proved to completely decolorize 10 mg/ L of RB within 20 and 30 h, respectively. Alginate-immobilized mixed cells, resulted in 88%, 87%, and 87% maximum COD removals with samples containing RB at initial concentration of 10, 20, and 40 mg/L, respectively. UV-vis spectra showed the biological cleavage of the azo bond in the anaerobic phase. Estimation of the phytotoxicity of the degraded metabolites suggested that the non-adapted immobilized mixed bacterial cells successfully detoxified RB azo dye.

Keywords: reactive dye, immobilized cells, biodegradation, Decolorization

ازالة اللون، التحلل العضوي و أزالة سمية صبغة الازو الزرقاء الفعالة باستخدام مزيج للخلايا الحية المقيدة

الخلاصة

أدى الطرح العشوائي للملوثات الصناعية ومنها صبغات الازو الى تهديد عالي لمنظومة البيئة الطبيعية. تتركز هذه الدراسة حول ازالة اللون والتحلل العضوي لصبغة الازو الزرقاء الفعالة من خلال النظام المتعاقب اللاهوائي– الهوائي بواسطة تجارب الدفعات. تم اعتماد مزيج الاحياء المجهرية المستخرج من حوض التهوية لمحطة معالجة محلية لمياه الصرف الصحي – للخلايا الحرة والمقيدة على حد سواء– بشكل مباشر من دون المرور بمرحلة التأقلم للملوث المستهدف (الصبغة الزرقاء الفعالة). أدى استخدام كل من الخلايا الحرة والمقيدة على حد سواء تحت ظروف لاهوائية الى الازالة الكاملة للصبغة الزرقاء بتركيز 10 مغ/ لتر خلال 20 و 30 ساعة على التعاقب. مزيج الخلايا المقيد للصبغة الزرقاء الفعالة ادى الى انقاص نسبة الحاجة الكيميائية للاوكسجين بنسبة 87، 88، 87% للتراكيز 40,20,10 ملغم/لتر على التعاقب. اظهر تحليل الطيف الخاص بالصبغة بعد المعالجة اللاهوائية للصبغة الزرقاء زوال القمة المرتبطة بأصرة الازو المسؤولة عن ظهور اللون. من

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ناحية أخرى، أظهر فحص السمية لمزيج الاحياء المجهرية المقيد وغير المؤقلم أن فعالية الخلايا البكتيرية قد ادت الى أزالة سمية الصبغة الازو الزرقاء الفعالة.

الكلمات الرئيسية: الصبغة الفعالة، الخلايا المقيدة، التحلل البايولوجي، أزالة اللون

1. INTRODUCTION

The profuse and abundant use of synthetic dyes resulted in exaggerating the problem of wastewater discharge, leading to a challenging and urgent issue in many parts of the world. Despite the hardship associated with dye biodegradation, dye-containing wastewater decolorization has been treated as a top priority goal in a response to the continuous public demand, **Hao, et al., 2000**. The carcinogenicity of the parent dye and their degradation products enhances the decline and worsening of the well-being of aquatic life, not to mention the impact of colored wastewater to the aesthetic aspect, even with presumably very low concentrations, **Nigam,** and **Marchant, 1995; Weisburger, 2002**. During the dying process, a high portion of reactive dyes hydrolyze and wash off with discarded wastewater effluent, **Fitzgerald,** and **Bishop, 1995.** The frequent application of physical and chemical treatment methods was disrupted by the many discrepancies between their efficiencies and the challenges linked with their use, the reason why a great deal of attention was directed towards the biological treatment, a method that offers an encouraging and auspicious aspect for organic pollutants, **Pandey, et al., 2007**; **Kalyani, et al., 2016**; **Yagub, et al., 2014**; **Mahmoodi, et al., 2011**. Immobilization assisted and elevated the endurance of microorganisms against the most recalcitrant pollutants. It has also enhanced the overall biotreatment throughout facilitating the separation and reuse of the applied biocatalysts, **Das,** and **Chandran, 2011**; **Ismail,** and **Khudhair, 2015**. Several studies have included the employment of free bacterial cells for the biotreatment of different dye types. On the other hand, limited researches reported using immobilized bacterial cells targeting the decolorization and biodegradation of dyes. **Saratale, et al., 2011** detected the decolorization and degradation of C.I Reactive Blue 172 employing the ability of *Proteus vulgaris* at initial concentration of 50 mg/L. In 8 h, about 80 % COD reduction was achieved. **Patil, 2016** studies the decolorization of the textile dye Navy blue HE2R using the consortium PMB11. The results showed that the decolorization process for the 50 mg/L in the broth medium within 6 h reached 91% decolorization. The immobilized consortium of PMB11 accomplished complete decolorization of dye solution in the flask within 15h. **Akpor, 2018** investigated the decolorization of crystal violet and bromothymol blue under batch process figuring out the relationship between the decolorization of the dyes and glucose concentration. The used cultures were *Bacillus subtills* and *Pseudomonas aeruginosa* in both free and immobilized forms. The optimum concentrations of glucose were 5 and 10 g/L for free *Bacillus subtills* cells. On the other hand, the immobilized cells needed 10 and 5-10 g/L for bromothymol blue and crystal violet, respectively.

2. MATERIALS AND METHODS

2.1 Dye and Microbiological Media

Azo dye reactive blue (RB) is widely used in textile industries. RB is polyaromatic di-sulphonated dichloro- triazene high molecular weight dye as shown in **Table 1**. The other chemicals and reagents employed in the study were of analytical grade. Mineral salt medium (MSM) along with different concentrations ranging from 0.1 to 2 g/L of yeast extract supplied with predefined RB concentration ranged from 10 to 40 mg/L was used for the development of mixed cultures. MSM was prepared according to the procedure outlined in, **Ghangrekar, et al.,**

2005 by dissolving in grams: 0.2 $MgSO_4.7H_2O$, 0.015 $CaCl_2$, 0.001 $FeCl_3.6H_2O$, 0.02 $MnSO_4.H_2O$, 1.825 NaH_2PO_4, 0.35 KH_2PO_4, and 0.42 $NaHCO_3$ in liter distilled water. Glucose was used as a co-metabolite for azo dye decolorization.

2.2 Microorganisms

Activated sludge was freshly granted from a local wastewater treatment plant in Baghdad. It was employed, without any prior adaptation to the selected reactive blue (RB), as the source of free and immobilized mixed bacterial cells throughout the sequential anaerobic-aerobic treatment

2.3 Bio-carrier Materials

In the current study, sodium alginate was used as an available and friendly bio-carrier. It is known for having little water stability, but this problem can be controlled by its cross-linking with a synthetic polymer, especially polyvinyl alcohol (PVA), a widely used polymer

2.4 Immobilization Protocol

Polyvinyl alcohol- sodium alginate (PVA-SA) matrix

PVA is used for the entrapment of living cultures by the classical freezing-thawing method primarily as a result to its high efficiency and nontoxic nature towards living cells. The preparation technique included embedding the PVA matrix with the cultures, **Bai, et al., 2010**. This technique is advantageous in producing elastic, rubber like hydrogel with an expected limitation of bead agglomertion. The latter problem can be overpowered by mixing PVA with 2% sodium alginate and solidifying the mixture with a solution of mixed boric acid (6%) and calcium chloride (4%), **Wu,** and **Wisecarver, 1992**. 5 ml of biomass inoculum was inserted to the PVA-SA mixture, and shaken without the formation of any bubbles. Then the mixture was poured into sterile micro-plates and placed in the freezer. The formed beads were washed 3 to 4 times with distilled water, **Kumar, et al., 2014**.

2.5 Analytical Methods

The UV–vis spectrum of RB starting from 200 to 1200 nm wavelength using a UV–vis spectrophotometer (ADVANCED MICROPROCESSOR UV-VIS SPECTROPHOTOMETER SINGLE BEAM LI-295) equipped with a quartz cell of 1.0 cm path length. The concentrations of the RB samples were measured as the absorbance values at λ_{max}= 580 nm along with the standard curve. Chemical oxygen demand (COD) was detected by COD analyzer (Model: Lovibond, RD 125). Volatile suspended solids (VSS) measurements were measured according to the, **Standard Methods APHA, 2005**. All measurements were performed in duplicate.

2.6 Experimental Procedure

Reactive blue decolorization and biodegradation was accomplished by applying a sequential anaerobic-aerobic biotreatment. The inoculation of 50 mL liquid volume of RB-loaded solution with immobilized mixed cells in 100 mL flasks was accomplished for the decolorization process. At room temperature (27-33°C), the anaerobic conditions were achieved by nitrogen flushing followed by sealing the flasks. At specific time intervals, 2 mL samples volumes of the supernatant were taken from each Erlenmeyer flask, centrifuged at 6000 rpm for 20 min, filtered through 0.4 micron filters and quantify the maximum absorption wavelength (λ_{max} = 580 nm) for the remained RB concentration. The percentage decolorization was measured according to the following formula:

$$\% \text{ Decolorization} = \frac{A_i - A_f}{A_i} x\ 100 \quad (1)$$

Where: A_i is the absorbance of the initial concentration of RB; A_f is the absorbance of the final RB concentration at time t.

The influence of multiple parameters on the decolorization was detected. The effect of yeast extract dose on decolorization process was investigated by inoculating the MSM solution with predetermined concentration of RB, with different concentrations of yeast extract: 0.1, 0.3, 0.5, 0.7, 1, 1.5, and 2 g/l. The effect of RB initial concentration (10, 20, 40 mg/l) on decolorization was assessed. In addition, the capacity for RB decolorization was executed with different free bacterial cells percentages, which were 3%, 5%, 7%, 10% at constant initial dye concentration and yeast extract dose. Control experiments were done in triplicate with autoclaved biomass for possible dye removal due to adsorption.

2.7 Reuse of Beads for Excessive Treatment Cycles

Bead recycling, an advantageous step resulted from using immobilized cells, offers superior features over using free cells. Immobilized mixed cells were applied for repetitive decolorization and aerobic biodegradation. Immobilized cells were collected and washed with deionized water after their use and applied for a second cycle of RB decolorization and biodegradation. The same procedure was performed during the aerobic phase for the used immobilized cells for subsequent cycles.

2.8 Phytotoxicity Test

Phytotoxicity tests were carried out to evaluate the influence of the treated supernatant on the plant life when it is discarded to the ecosystem as well as to find out the likelihood of the treated solution reuse in irrigation domains. Phytotoxicity of the loaded dye solution, and treated samples after each of the sequential treatment phases were estimated. Many assays are associated with phytotoxicity involving *Triticum aestivum* are performed as described by **Gomare‹** and **Govindwar, 2009** and, **Zhao**, **et al., 2014**. Crop seeds sterilization was accomplished with 3% hydrogen peroxide solution followed by washing them with distilled water. Four Petri-plates were assembled; each plate has 10 healthy seeds of *Triticum aestivum*, and was daily watered with 5-ml of solution as follows: plate (a) with tap water (as a control); plate (b) with 10 mg/L RB-laden solution; plate (c) with anaerobically treated solution; and plate (d) with aerobically treated solution. Percentage germination, plumule and radicle length were recorded and compared. Each experiment was carried out in duplicate.

3. RESULTS AND DISCUSION

3.1 Decolorization and Biodegradation Analysis

At different intervals, the reduction in dye concentration can be clearly observed by the UV–vis scan (200-1100 nm) of RB-loaded supernatants using mixed free and immobilized cells. The peak at 580 nm observed with the untreated RB-loaded solution was decreased after the anaerobic biotreatement without shift in λ_{max} up to complete decolorization using free and immobilized mixed cells **Fig. 1 A&B**. The chromophore group of RB azo dye was evidenced by the detected peak in the visible range at 580 nm. Another wide-range peak at the UV range was also detected between 285 and 300, which was mentioned by **Franciscon, et al., 2012** to be consistent with substituted benzene compounds. As given by the UV scan in **Fig.1**, a shift of the wide band to a sharp peak at 280 nm with a total disappearance of the peak was noticed in the

visible region referring to the azo bond cleavage. The absorbance reduction of the band in the range of 285 -300 nm and the formation of a new peak at 280 nm reflected transformations to the aromatic structure, in particular the substituents on the aromatic groups, consistent with azo bond cleavage. This result confirmed that complete decolorization. However, the amines resulted from the biodegradation of RB were not degraded during the anaerobic phase, and thus a secondary aerobic treatment step was required to accomplish complete biodegradation. Similar observations were reported by **Jonstrup, et al., 2011**.

Adding to the detection of the main and prominent mechanisms, the involvement of biosorption process along with biodegradation of RB, was also investigated. An experiment was conducted using autoclaved mixed cells. The UV-Vis spectrum analysis reflected that the original peaks at 580 and the wide band between 285 and 300 nm appeared with the RB-loaded sample have been reduced without the formation of any new peaks **Fig.1C** This result proves that biosorption was initially responsible for the slight RB reduction that supports the biodegradation process. Similar observation was reported by **Cheng, et al., 2012** about the immobilization of *Burkholderia vietnamiensis* C09V strain in PVA–alginate–kaolin gel beads to enhance crystal violet degradation.

3.2 Effect of Initial Dye Concentration on Decolorization

The decolorization effect that comes from changing the initial RB concentration was investigated and resulted in a reduction to the decolorization rate in response to increasing the initial concentration of RB. Using free mixed cells, the increase in RB initial concentration from 10 to 20, and then up to 40 mg/L, extended the duration for complete decolorization from 20 to 30, and 40 h, respectively, as shown in **Fig. 2**. Increasing RB initial concentration resulted in reducing the decolorization rate, but keeping the overall removal efficiency the same. This may be interpreted in terms of a decrease in the growth of cultures in response to an increase in initial dye concentration. However, a relatively longer duration for RB decolorization occurred as a consequence to the application of immobilized mixed cells up to 30, 50, and 60 h at RB initial concentrations of 10, 20, and 40 mg/L, respectively. Increasing initial RB concentration up to 100 mg/L inhibited the free mixed cells' activity, dissimilar to the immobilized cells, which maintained their degrading capacity towards RB at high concentration (data not shown). Complete decolorization indicated that RB did not possess and consequently pose any inhibition influence over immobilized cells and the immobilization gels have a potential capacity to host the bacterial cells. **Fig. 3** shows the profiles of RB and COD removals over the sequential anaerobic and aerobic phases. During the anaerobic phase, it can be clearly observed that RB was totally decolorized and biodegraded nevertheless the change in initial concentration value. These results are in disagreement with, **Vaigan, et al., 2009** when increasung the initial concentration of Brill Blue KN-R from 20 to 40 mg/L reduced the removal efficiencies of from 57 to 31%. The anaerobic phase was distinguished in reducing COD values leading up to 42 %, 52 %, and 76% using PVA-SA as the bio-carrier for mixed cells at initial concentrations of 10, 20, and 40 mg/L, in 30h, 50h, and 60h, respectively **Fig. 3**. The lower COD removal in the aerobic phase, in comparison to the anaerobic phase, could be explained in terms of the insufficient presence of substrates that are readily available for biodegradation which resulted from the anaerobic stage.

3.3 Effect of Yeast Extract Dose on Decolorization Process

Yeast extract (YE) has a remarkable capability of enhancing enzyme activity in comparison to other commonly used sources **Imran, et al., 2016**. It has been mentioned in many researches that YE is the most efficient growth supplement for bacteria, especially the ones responsible for azo

dye degradation **Khalid، et al., 2008**. It has been reported that YE is the best organic nitrogen source for the growth of bacterial cell with many growth stimulants and amino acids leading to considering it an effective mixture of amino acids, with superior advantages than individual amino acid. The result variating YE concentrations on decolorization of RB at 10 mg/l initial concentration by immobilized mixed bacteria are revealed in **Fig. 4**. A notable increase in decolorization efficiency occurred upon increasing YE concentration from 0.1 to 0.7 g/L and then to 1g/L within 35 h. Further increase in YE concentration assisted a decline in RB decolorization efficiency, which might be interpreted in terms of enzyme saturation upon 1 g/L of YE. Similar results have been reported by **Imran, et al., 2016** who declared that increasing YE above 1 g/L slowed the enzyme response which shows a trend of enzyme saturation near 1g/L. This might also be reclined to the fact that bacterial cultures have an inclination to use yeast extract as a readily available nitrogen source for their growth as a replacement to encountering the complexity of biodegrading the used azo dye

3.4 Effect of Inoculum Size

The influence of changing inoculum sizes on RB decolorization was accomplished by inoculating the MSM with 3%, 5%, 7%, and 10% (v/v) mixed cells. As given in **Fig. 5**, it can be noticed that using inoculum sizes of 7% and 10 % resulted in of 94 % and 100 % decolorization percentages, while both using 3 % and 5 % reached 83 % and 82 % decolorization, after 25 h, indicating insufficient culture concentration that ultimately necessitated increasing the used sludge concentration.

Sani, and **Banerjee, 1999** have observed similar behavior of increasing decolorization with increasing the inoculum size. However, beyond 10% (v/v) inoculum size, the change in decolorization rate was not mentionable which shows no proportionate increase between decolorization and *Kurthia* sp. inoculum size of

3.5 Recycling of Immobilized Mixed Cells

Bead Recycling is considered as an encouraging step for immobilized cultures' applications. As given in **Fig. 6**, the time duration needed for a complete decolorization throughout the anaerobic treatment of RB was about 30 h with approximately similar COD profiles during the second cycle. The second cycle, during the aerobic phase, showed a higher COD removal efficiency that consequently resulted in complete mineralization, and a reduction in the needed time course. The highest COD removal percentage for PVA-SA was 86% after 67 h, whereas the second cycle resulted in complete COD removal within only 47 h. The interpretation of this behavior could be the induced ability of the used immobilized cultures for aerobic enzymes ejection (mainly responsible for the cleavage of aromatic ring). **Yaşar, et al., 2012** have noted that upon increasing the exposure period of the immobilized cultures to the aerobic phase, an increase of the ejection level of the aerobic enzyme, catechol 2, 3-dioxygenase (C_{23}DO), which is used as aromatic amines aerobic biodegradation.

3.6 Phytotoxicity Test

Table 2 shows the results of toxicity test. The germination of *triticum aestivum* was 20% with RB-loaded solution as an irrigation source. It is very low compared to 100% resulted from tap water as the control solution. The results indicated that by the end of the anaerobic treatment stage and the use of its solution for plant watering, the germination rate increased to 30 %. An outstanding results were observed by the implication of aerobically-treated solution with a germination rate up to 100%. The lengths of plumule and ridicule were 0.4 cm and 0.26 cm with the RB-loaded solution, where the anaerobically treated solution showed better results with

0.68 cm and 0.5 cm, respectively. The aerobically treated solution stood out over tap water, showing superior results with 1.06 cm and 0.51 cm, compared to 0.9 cm and 0.63 cm in terms of plumule and ridicle lengths, respectively.

4. CONCLUSIONS

The biodegradation of azo dye reactive blue (RB) was accomplished by the use of mixed bacterial cultures cultivated from activated sludge resulted from a local wastewater treatment plant. The decolorization potential for the RB, under anaerobic conditions, was found to be 100% using free and immobilized mixed cells during 20 and 30 h, respectively. The results of using non-adapted immobilized mixed cells showed that 88, 87, and 87% maximum COD removals at RB initial concentration of 10, 20, and 40 mg/L, respectively. In the current study, immobilized mixed cells have not lost their ability to decolorize RB even after the second treatment recycle.

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Table 1. Chemical structure of RB.

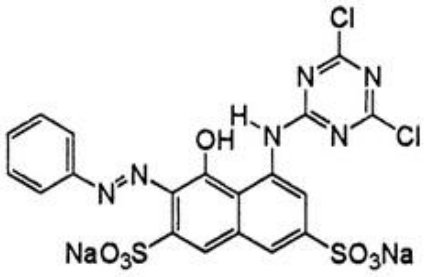
Dye	Structure	Chemical class	λ_{max}
Reactive blue (RB)		Azo	580 nm

Table 2. Phytotoxicity test results for watering *Triticum aestivum* with tab water, RB-loaded solution, anaerobically-treated solutions, and aerobically-treated solutions.

Parameters	**Tap water**	**RB-loaded solution**	**Anaerobically-treated solution**	**Aerobically- treated solution**
Germination (%)	100	20	30	100
Plumule (cm)	0.9	0.4	0.68	1.06
Radicle (cm)	0.63	0.26	0.5	0.51

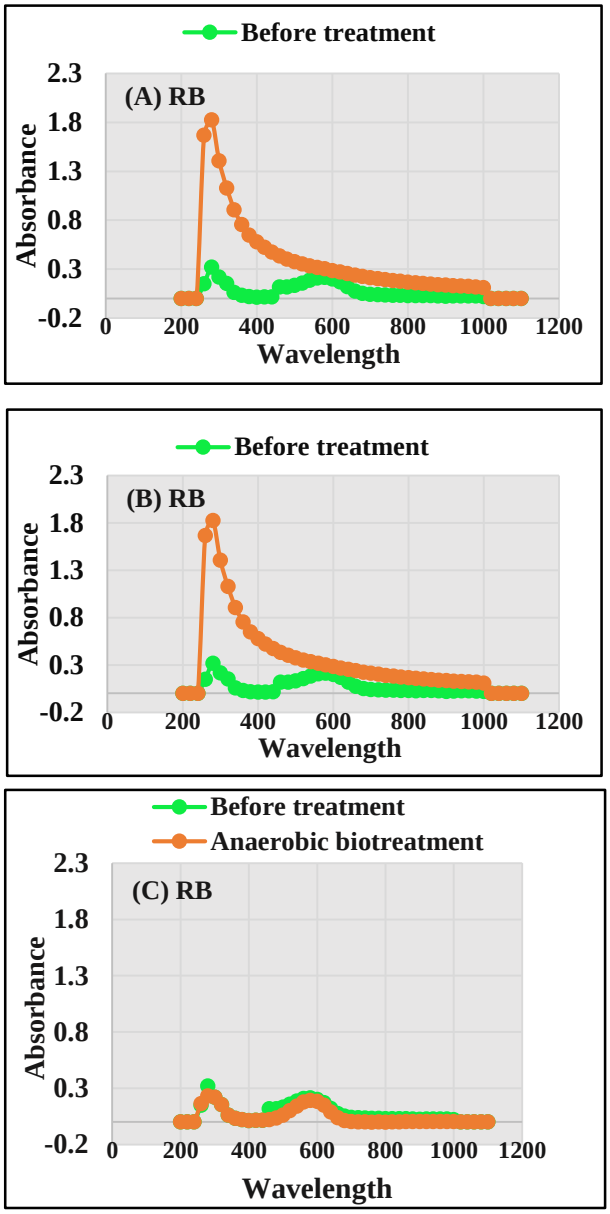


Figure 1. UV-vis spectra; (A) Free mixed cells, (B) immobilized mixed cells, (C) blank beads (free of cells).

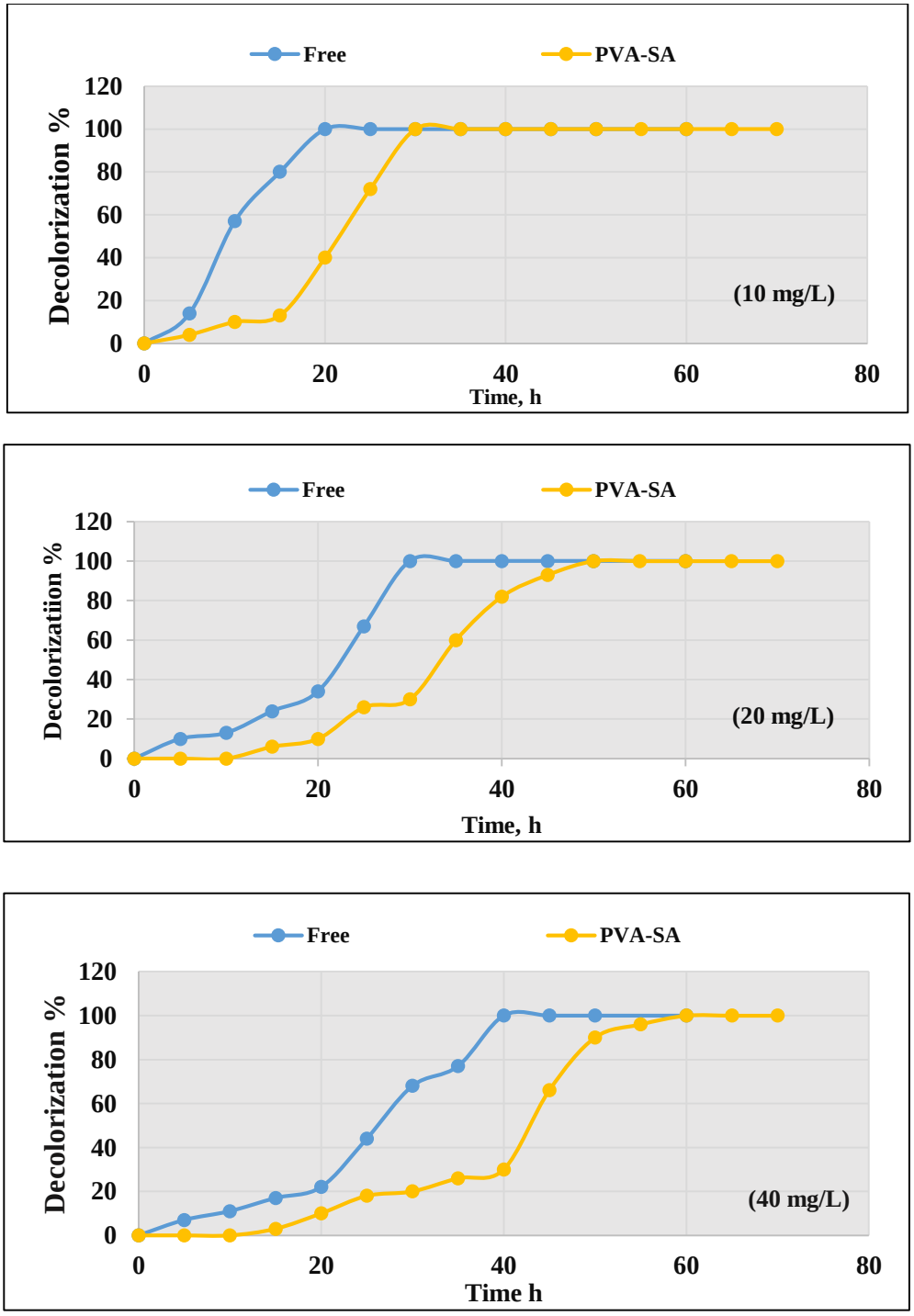


Figure 2. Effect of different initial concentrations on the decolorization of RB using free mixed cells

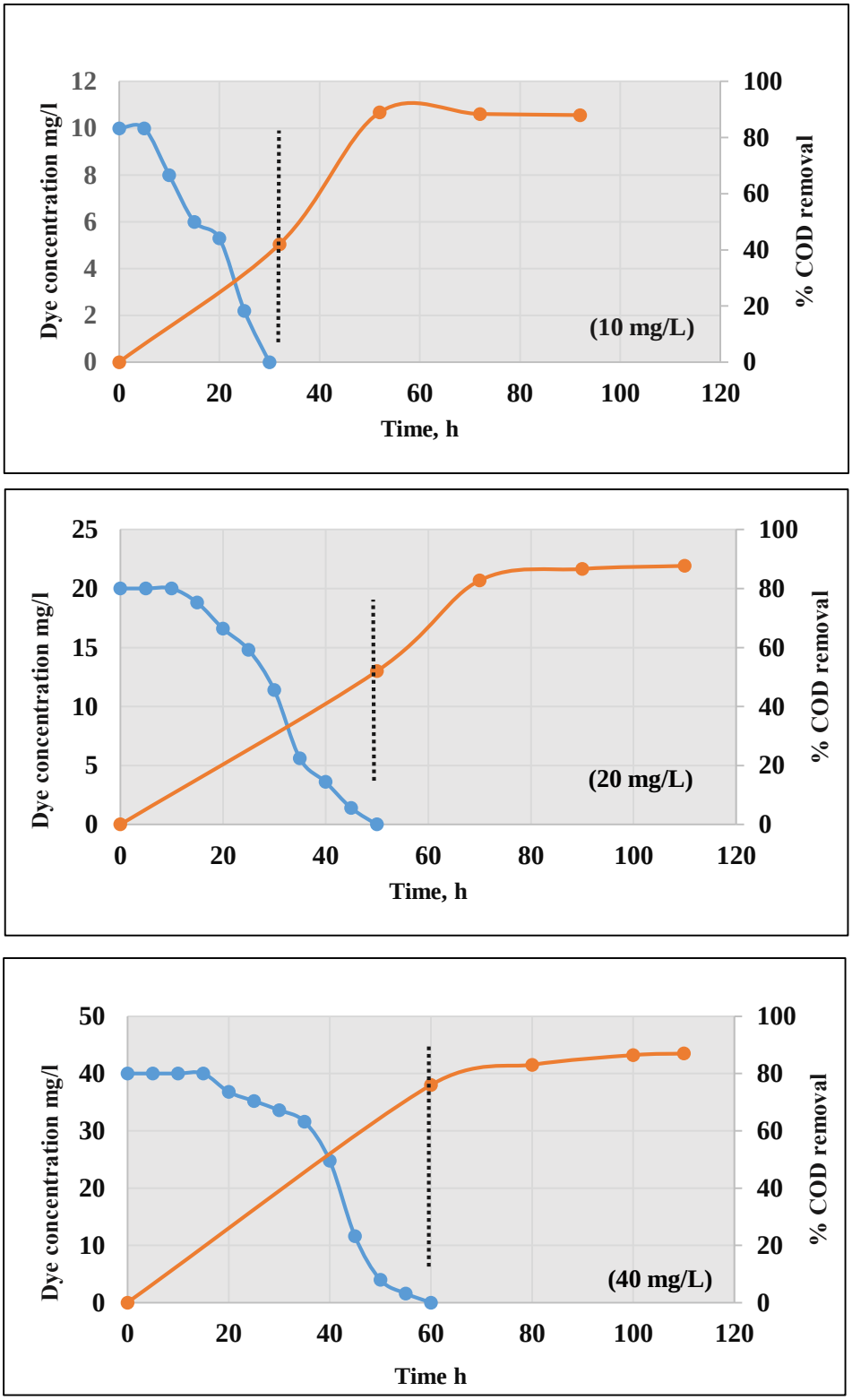


Figure 3. Profiles of dye and COD removals of RB using PVA-SA.

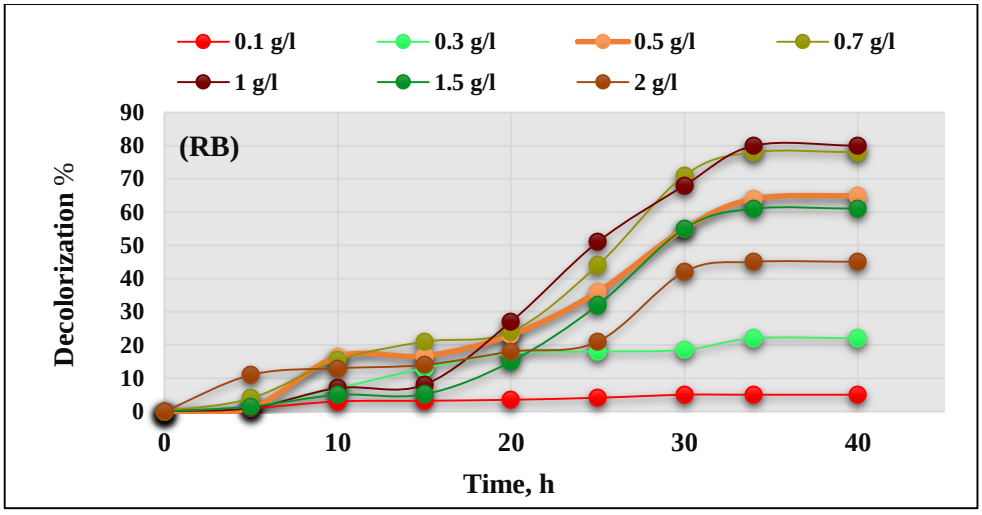


Figure 4. Effect of YE on the decolorization of RB.

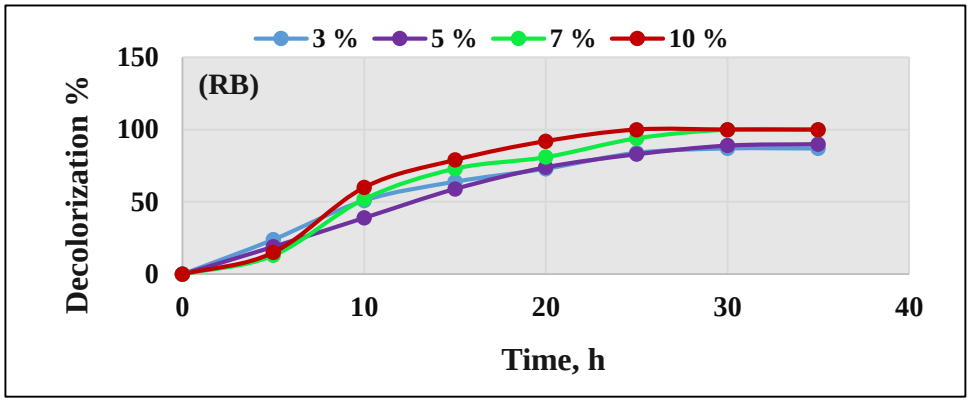


Figure 5. Effect of inoculum size on the decolorization of RB

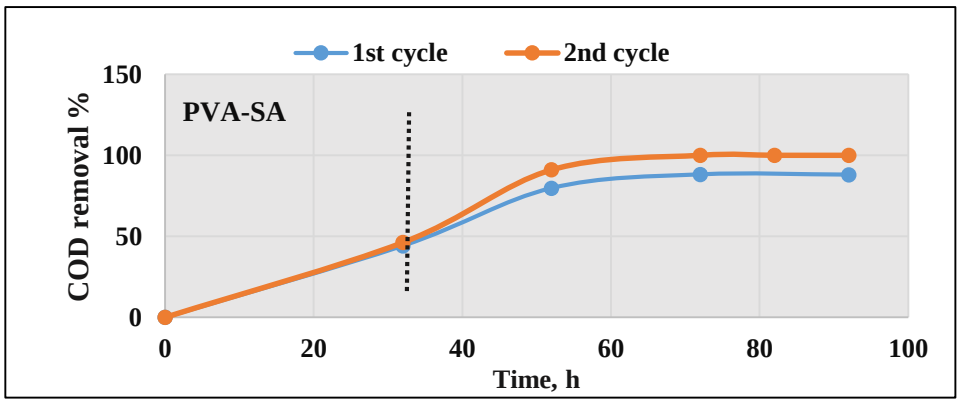


Figure 6. RB profiles of COD removal using recycled immobilized cells.

NOMENCLATURE

MSM: mineral Salt Medium
PVA: polyvinyl alcohol
SA: sodium Alginate
VSS: volatile Suspended Solids
RB: reactive Blue

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