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Removal of Reactive Orange 16 Dye using *Casuarina equisetifolia* seeds as Packing Media for Microbial Biofilm Formation

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Abstract. Dyes effluent mostly is toxic and mutagenic to living organism. *Casuarina equisetifolia* seeds are usually found in coastal and have potential as a packing media for microbial biofilm formation. The present study was designed to evaluate the performance of three laboratory scale reactors namely, sequencing batch biofilm reactor (CES-SBBR), a combination of adsorption and biological process, sequencing batch biofilm reactor (FC-SBR), and packed bed reactor (CES-PBR) on the removal of RO16 dye. The CES-SBBR and CES-PBR were packed with *Casuarina equisetifolia* seeds. The microorganism was collected from textile industry and undergo the preadaptation period with the 2 mg/L of RO16 solutions in CES-SBBR and FC-SBR. The biomass concentration initially reduced from 0.12 to 0.03 mg/L. Upon the addition of nutrients, the biomass concentration was increased to 0.22 mg/L. The performance study of three reactors was performed with the initial concentration of RO16 solution and hydraulic retention time (HRT) of 2 mg/L and 24 hr, respectively. The RO16 dye removal efficiencies were in range of 80.00-82.26%. The removal efficiencies were reduced to 70.00-71.28% when the initial concentration of RO16 dye increased to 4 mg/L. The removal efficiencies were continued to reduce with only 48.06-50.00% as the initial concentration of RO16 dye increased to 6 mg/L. The removal efficiencies were improved when the HRT was extended to 48 hr. The biomass concentration of CES-SBBR and FC-SBR increased up to 0.50 and 0.33 mg/L, respectively. The RO16 removal percentage for FC-SBR and CES-PBR were found to be lower compared to as for CES-SBBR. This study shows that the combination of adsorption and biological process enhanced the removal efficiency of RO16 dye.

1. Introduction

Dyes have extensive application in textile, paper, equipment, cosmetic, pharmaceutical, aquaculture, food and beverage, as well as plastic industries [1]. The invention of synthetic dyes solved the natural dyes problems which normally fade when exposed to sunlight and washing, however new issues arise from improper treatment or untreated dyes by dye-utilizing industries [2]. More than 280,000 tons of untreated synthetic dyes are expected to have been discharged into water bodies globally [3].

Synthetic dyes are made from complex organic substances to strengthen their capability to withstand degradation when contact with water and detergents even when under extreme heat [2]. Synthetic dye



molecules are complex which contain auxochromes and chromophores which is water soluble bonding compound and colour giving compound respectively allowing them to have stable structure.

Casuarina equisetifolia also known as Rhu or Beach She-Oak and are commonly found in Southeast Asia which grows at coastal habitat such as sandy coasts and coastal forests. *Casuarina equisetifolia* is a plant which is cultivated for applications in paper manufacturing, firewood, furniture and building timber since its woods are extremely hard and dense [4]. However, *Casuarina equisetifolia* seeds are considered as biowaste and have potential as a packing media for bioreactor. It served as a packing media for the formation of microbes and helped to adsorb the pollutant on its surface. Benefits of using *Casuarina equisetifolia* seeds includes the easily accessible, provide a high surface area and most crucially its suitability for attachment and growth of microorganisms.

Biological dye removal methods were an appealing approach compared to conventional methods. Generally, removal of dyes by bacteria strains are more effective compared to fungi and algae [5]. Besides, biofilm process for dye removal is proved it's among the most effective methods. The steps in biofilm development start with the initial attachment of the bacteria to the surface, establishment to the biotic and abiotic surfaces, biofilm maturation and lastly detachment of cells from surface. Extracellular polymeric substances (EPS) help the biofilm community to adapt towards the changes of pH, concentration of toxic substances and temperature thus play important roles in biofilm growth and function as a part of protection mechanisms for the biofilm community. The benefits of packing media with biofilm formation are to increase the chance of extended usage of the absorbent media because microbial degradation helps extend the saturation point. Microorganisms that usually found in batik wastewater are *Bacillus* sp., *Brevibacillus parabrevis* and *Alcaligenes faecalis* [6][7]. Biofilm reactor configurations applied in dye wastewater removal are tricking filters, high-rate plastic media filters and membrane immobilized cell reactors.

Thus, the aim of this study is to monitor the performance of three laboratory scale reactors namely, sequencing batch biofilm reactor (CES-SBBR), a combination of adsorption and biological process, sequencing batch biofilm reactor (FC-SBR) and packed bed reactor (CES-PBR) on the RO16 dye removal. This study also conducted to determine the effect of two operational parameters which are initial concentration of dye and hydraulic retention time (HRT) on RO16 dye removal.

2. Materials and Methods

2.1. Chemicals

Reactive Orange 16 dye and glucose were purchased from Sigma-Aldrich Chemicals. Ammonium nitrate and potassium dihydrogen phosphate were purchased from Merck Chemicals. Hydrochloric acid, sodium hydroxide, magnesium chloride (Hexahydrate), and calcium chloride (Dihydrate) were purchased from R&M Chemicals. Iron (III) chloride-6-hydrate, manganese (II) chloride-4-hydrate, and sodium bicarbonate were purchased from HmbG Chemicals.

2.2. Microorganisms

The source of microorganisms in this study was collected from a textile industry located in Pantai Cahaya Bulan, Kelantan, Malaysia. The sample was culture in a reactor. Growth of microorganisms was monitored using mixed liquor suspended solids (MLSS). The MLSS were measured by filtering 10 mL of samples through the 0.45 μm Whatman® cellulose nitrate membrane filter. The samples were heated in oven at 105 °C for 1 hr and the weight of sample before and after the heating process was determined [8].

2.3. Preparation of Packing Media

Casuarina equisetifolia seeds were collected from Pantai Batu Burok, Kuala Terengganu, Terengganu, Malaysia. *Casuarina equisetifolia* seeds were washed with distilled water and soaked for 24 hr. Next, the seeds were dried in oven at 70 °C for 5 hr. The specific surface area and pore size distribution were determined by Brunauer, Emmett and Teller (BET).

2.4. Design of Laboratory Scale Reactors

Three laboratory scale reactors namely, CES-SBBR, FC-SBR and CES-PBR, with the working volume of 1.0 L were operated in this study (figure 1). The reactors were maintained at the condition as in table 1. The CES-SBBR, FC-SBR and CES-PBR were operated in aerobic condition in which oxygen was pumped into the reactor by a peristaltic pump. The CES-SBBR and CES-PBR were packed with the 80 g of *Casuarina equisetifolia* seeds as the packing media for microbial biofilm formation (figure 1). The CES-SBBR and FC-SBR were supplied with the nutrients for the microbial growth. The composition for the nutrient supplied were 300 mg/L glucose as carbon sources, ammonium nitrate as nitrogen sources and potassium dihydrogen phosphate as phosphorus sources with the ratio C:N:P of 100:5:1. During performance studies, the reactors were fed with the concentration of RO16 dye solutions ranged from 2 to 6 mg/L. In order to determine the effect of the initial concentration of RO16 dye solution, the HRT was maintained at 24 hr. In order to determine the effect of HRT on the RO16 removal the HRT was varied from 24 to 48 hr while the initial concentration of RO16 dye solutions was maintained at 6.0 mg/L.

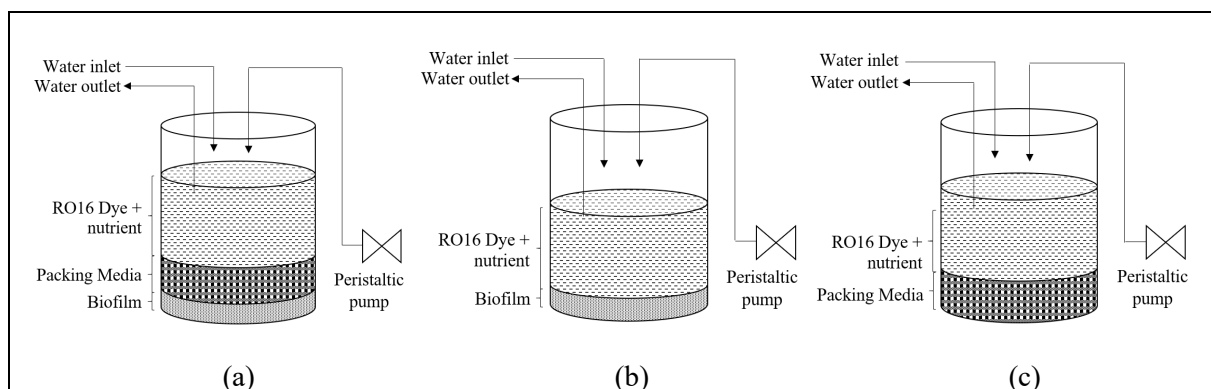


Figure 1. Schematic diagram for the laboratory scale reactor. (a) CES-SBBR; (b) FC-SBR and; (c) CES-PBR.

Table 1. Operation conditions of the reactors.

Parameter	Value
Working volume (L)	1.0
Temperature (°C)	25.0±1.0
pH	7.0±0.7
HRT (hr)	24-48

2.5. Determination of RO16 dye removal

The spectrophotometer (Thermo Scientific™ GENESYS 20) was used to analyse the UV-Visible spectra and was set to the wavelength at maximum absorbance (λ_{max}) of RO16 dye [9]. The samples were filtered using a syringe filter with a pore size of 0.45µm. The absorbance reading of before and after the removal of RO16 dye was determined using spectrophotometer at wavelength of 493 nm.

3. Results and Discussion

3.1. Characterization of CES Media

Through BET result the surface area of CES Media is 0.731 m²/g (table 2).

Table 2. Specification of CES Media using BET.

BET	Value
Surface area	0.731 m ² /g
Average pore radius	4.78545e ⁺⁰¹ Å
Total pore volume	1.749e ⁻⁰³ cc/g for pores smaller than 1285.1 Å (radius) at P/Po = 0.99249

3.2. Preadaptation of Microorganisms

The preadaptation period is completed within 19 day. The purpose of the preadaptation period is to allow attachment of microbial biofilm formation on packing media. 2 mg/L of RO16 dye was added into the CES-SBBR and FC-SBR reactors to retain the microbe be able to withstand RO16 dye solutions. Initially, the MLSS value for CES-SBBR was 0.12 mg/L, before reduced to 0.05 mg/L on day 4 and 0.03 mg/L on day 7 (figure 2). The reduction in biomass concentration is due to insufficient nutrients in both reactors. The source of energy for the synthesis of new cellular material is carbon and inorganic nutrient for example nitrogen, phosphorus, sulphur, potassium, calcium, iron, sodium, magnesium, and chloride are required for microbial growth and reproduce [10]. On day 7, the nutrients were added to both reactors with C:N:P ratio of 100:5:1. On day 10, the MLSS reading slightly increased to 0.06 mg/L. This increment in MLSS reading could be explained that nutrients are required for the microbial growth. The biomass concentration in CES-SBBR and FC-SBR were continued to increase up to 0.22 and 0.18 mg/L, respectively on day 19. From the results obtained, the microbe grew faster in CES-SBBR compared to FC-SBR (figure 2 and figure 3). Both reactors were then supplied with higher concentration of RO16 dye in order to study their performance in the removing of RO16 dye.

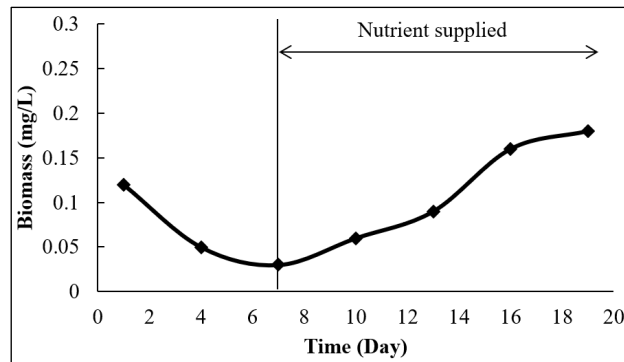


Figure 2. Biomass growth in CES-SBBR.

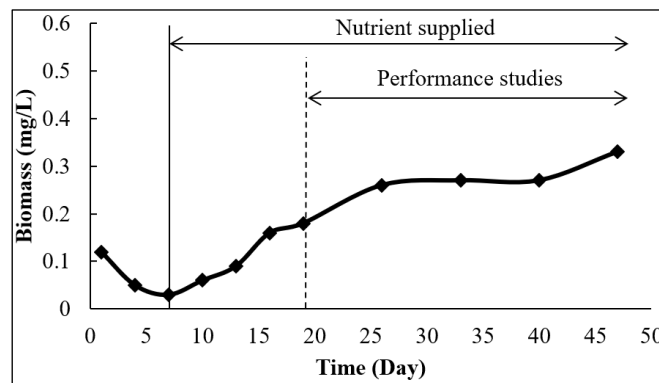


Figure 3. Biomass growth in FC-SBR.

3.3. Performance studies of Reactive Orange 16 dye removal process

During performance study, the effect of initial concentration of dye and HRT to the RO16 removal have been monitored. The performance of reactors was analyzed on the basis of the removal efficiency. The study was carried out for 32 days. The nutrients were supplied into the CES-SBBR and FC-SBR reactors. The concentration of biomass (mg/L) was also been monitored throughout the study.

3.3.1. CES-SBBR. Initially, the CES-SBBR reactor was operated at HRT of 24 hr and supplied with the initial concentration of RO16 dye of 2 mg/L. At this stage, the removal efficiency was in the range of 80.0-82.3% at HRT 24 hr. Next, the reactor was supplied with the 4 mg/L of RO16 dye, and the removal efficiency was in the range of 69.1-71.3%. The initial dye concentration was further increased to 6 mg/L and the removal percentage was reduced to the range of 48.1-50.0%. As shown in figure 4, the removal efficiencies of the CES-SBBR reactor were decreased when the initial dye concentration was increased. However, the removal efficiency was improved when the HRT was extended to 48 hr (figure 4). The removal efficiency was in range of 74.6-76.6%. Similar results were found in the previous study conducted by [11]. It is noted the dye removal efficiency was increased with HRT. For the microbial growth, the concentration of biomass was 0.29 mg/L at the first week of the study prior been increase to 0.36 mg/L at the second week of the study. The concentration of biomass was gradually increased to 0.48 mg/L at week 4 of the study. An even thickness of the biofilm effects the removal efficiency and simultaneously shorten the HRT, the CES Media is an ideal carrier since it has porosity and rough surface were improving attachment and growth of biofilm [12]. According to previous studies, the dye removal percentage increased with HRT which recorded $52.2 \pm 3.1\%$ RO16 removal at HRT of 24 hr and increased to $60.6 \pm 3.9\%$ at HRT of 48 hr and further improved to $68.1 \pm 3.4\%$ at HRT of 72 hr [11].

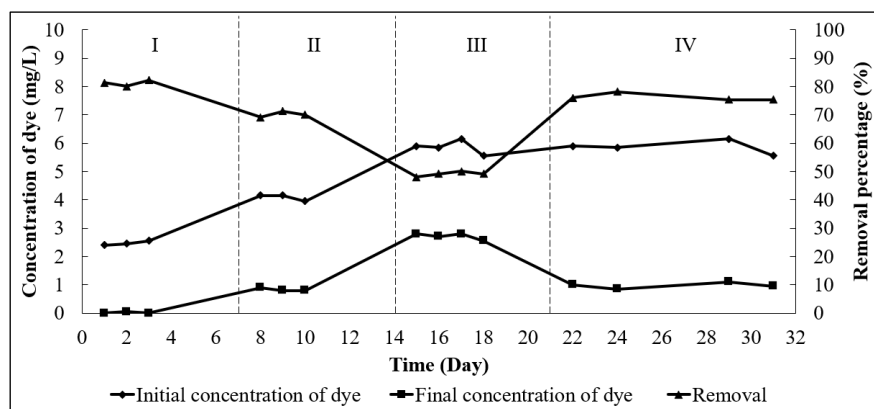


Figure 4. Performance study monitoring for CES-SBBR. Initial concentration (◆); Final concentration (■); and Removal Efficiency (▲).

3.3.2. FC-SBR. Initially, the FC-SBR reactor was operated at HRT of 24 hr and supplied with the initial concentration of RO16 dye of 2 mg/L. At this stage, the removal efficiency was in the range of 60.0-62.9% at HRT 24 hr. Next, the reactor was supplied with the 4 mg/L of RO16 dye, and the removal efficiency was in the range of 39.4-40.4%. The initial dye concentration was further increased to 6 mg/L and the removal percentage was reduced to the range of 34.4-42.6% at HRT of 24 hr and showed improvement during HRT of 48 hr with 52.5-55.9% dye was removed. As shown in figure 5, the removal efficiencies of the CES-SBBR reactor were decreased when the initial dye concentration was increased. However, the removal efficiency was improved when the HRT was extended to 48 hr (figure 5). The removal of the azo dye is most likely to happen under microaerophilic condition which means under low oxygen concentrations condition since aerobic respiration dominates the use of NADH, thus preventing electron transfer from NADH to the azo bond [12]. Isolated microbe *Bacillus* sp able to degrade 100 mg/L of RO16 at HRT of 72 hr with 90% removal

under microaerophilic condition [6]. Isolated microbe *Proteus sp.* under microaerophilic condition able to remove 150 mg/L of RO16 dye at HRT of 72 hr with $68.1 \pm 3.4\%$ removal [11].

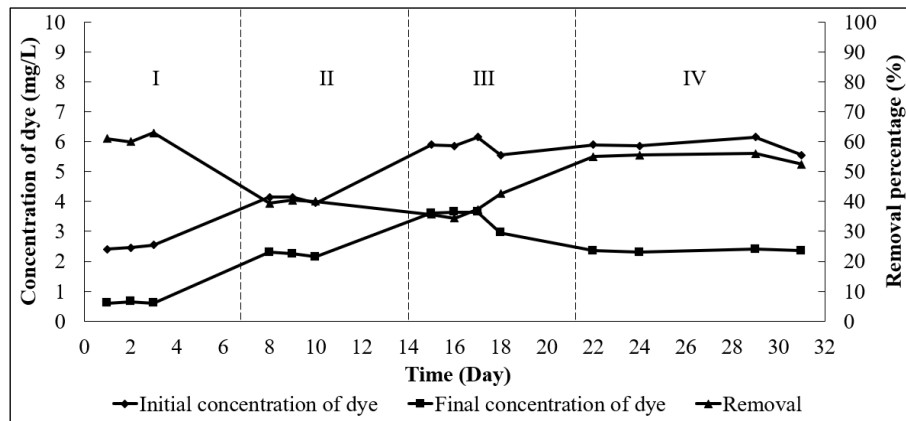


Figure 5. Performance study monitoring for FC-SBR. Initial concentration (◆); Final concentration (■); and Removal Efficiency (▲).

3.3.3. *CES-PBR*. Initially, the CES-PBR reactor was supplied with the initial concentration of RO16 dye of 2 mg/L and operated at HRT of 24 hr. At this stage, the removal percentage was in the range of 3.2-5.0% at HRT of 24 hr. When the reactor was supplied with 4 mg/L of RO16 dye with HRT of 24 hr, the removal percentage of dye was only in the range of 1.1-4.4%. When influent dye solution was increased to 6 mg/L, the removal percentage of dye recorded decreased to range from 0.0-2.2%. Although the HRT was increased to 48 hr, the removal percentage was only 0.78-2.99%. This result indicated that CES Media alone is inefficient in removing RO16 dye. Figure 6 shows overall performance of CES-PBR in removal of RO16 dye. At lower concentration of dye, the ratio of surface-active sites to the total dye molecules in the solution is high, therefore all the dye molecules can interact with the absorbent and be removed from the solution [13].

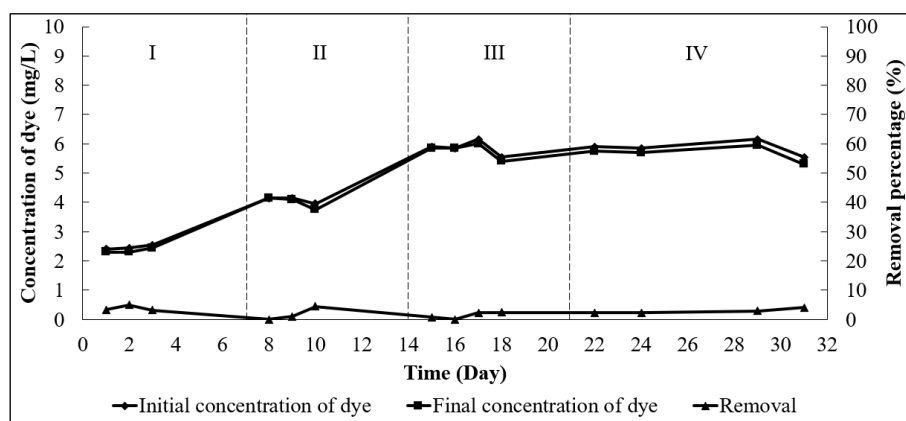


Figure 6. Performance study monitoring for CES-PBR. Initial concentration (◆); Final concentration (■); and Removal Efficiency (▲).

3.4. Effect of Initial Concentration of Dye

Based on the findings, CES-SBBR is better in terms of effectiveness for RO16 removal compared to both FC-SBR and CES-PBR (figure 7). The result indicates that the combination of microbial biofilm and CES Media in CES-SBBR improved the efficiency of RO16 removal which the maximum RO16 removal for HRT of 24 hr had reached 82.3%. While, CES-PBR is ineffective in removing RO16 dye

which maximum RO16 removal for HRT of 24 hr was in range of 3.2-5.0%, whereas in FC-SBR maximum RO16 dye removal for HRT of 24 hr was in range of 60.0-62.9%.

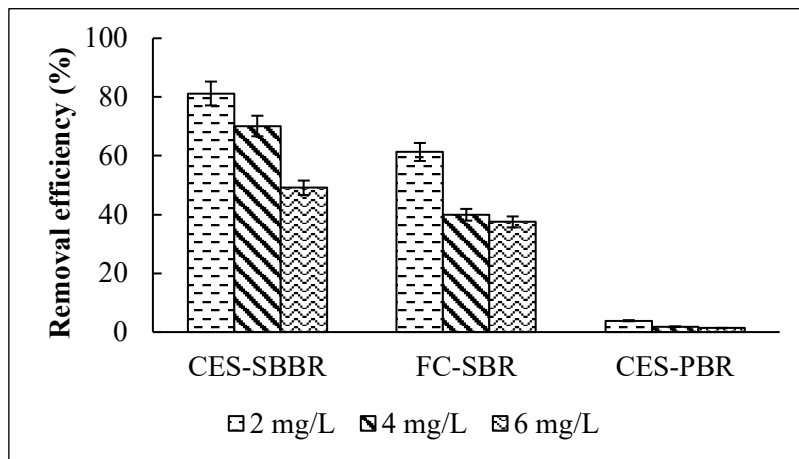


Figure 7. Effect of initial concentration of RO16 dye on removal efficiency of RO16 in CES-SBBR, FC-SBR and CES-PBR.

3.5. Effect of HRT

Removal percentage increased at HRT of 48 hr compared to HRT of 24 hr (figure 8). Removal percentage in CES-SBBR reached 78.13% at HRT of 48 hr but maximum removal in CES-SBBR reached only 50.00% at HRT of 24 hr. Same goes to FC-SBR, removal percentage reached 55.97% at HRT of 48 hr while highest removal only 42.62% at HRT of 24 hr. Also, for CES-PBR, the maximum removal percentage is 2.94% at HRT of 48hr compared to 1.37% at HRT 24 of hr. This indicate that a combined adsorption and biological process is better in terms of efficiency of RO16 dye removal compared to single biological process and single adsorption process.

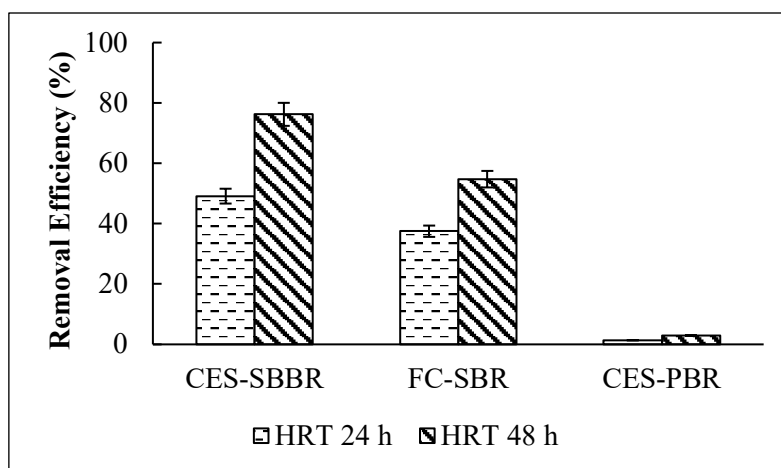


Figure 8. Effect of HRT on removal efficiency of RO16 in CES-SBBR, FC-SBR and CES-PBR.

3.6. Comparison with the Previous Studies

In this study, the performance study of RO16 dye removal using Casuarina equisetifolia seeds as packing media for microbial biofilm formation showed that the maximum removal efficiency was 81.13%. Table 3 shows the comparison on present study with previous studies. The continuous bioreactor packed with Casuarina seeds immobilized with mixed bacteria cultures reached 69.27% removal efficiency for 500 mg/L of RO16 dye [15]. The fungal culture (Pleurotus ostreatus and yeast Candida zeylanoides)

immobilized on polyamide mesh carrier revealed that fungus culture could be applied on removal of RO16 dye [16]. Apart from microbial biofilm from mixed culture, there was a study that using isolated culture of *Halomonas* sp. immobilized in volcanic rocks in a continuous packed bed reactor which was able to remove 95% of 50 mg/L RO16 dye at HRT of 24 hr [12].

Table 3. Comparison with previous studies for RO16 removal.

Microbe	Experimental Condition	Initial concentration (mg/L)	HRT	Removal (%)	Citation
Mixed culture	Biofilm attached on CES media Aerobic, pH 7.3±0.4, 25±1°C	2	24 hr	81.13±1.13	Present study
		4	24 hr	70.22±1.07	
		6	24 hr	49.03±0.97	
		6	48 hr	76.75±1.38	
<i>Halomonas</i> sp.	<i>Halomonas</i> sp. Immobilized in volcanic rocks in continuous packed bed reactor, pH 10, 38°C	50	24 hr	95	[12]
Mixed culture	Cells immobilised on <i>Casuarina</i> seeds Aerobic, pH 7±0.3, 27±5°C	500	10 d	69.27±5	[15]
Mixed culture (<i>Pleurotus ostreatus</i> and yeast <i>Candida zeylanoides</i>)	Fungal culture immobilized on polyamide mesh carrier. Aerobic, pH 6.5, 28°C	150	11 d	87.5	[16]

4. Conclusion

From the findings of the performance study, it revealed that the combination of biological and adsorption processes in CES-SBBR is more efficient in removing dye compared to the single biological process (FC-SBR) and single adsorption process (CES-PBR). It is also noted that the removal efficiencies decreased as the initial concentration of dye increased. When 2 mg/L of RO16 was fed into the reactors, the maximum removal efficiency reached 82.26%. The maximum removal efficiency then reduced to 71.28% when initial concentration increased to 4 mg/L and experienced further reduction to 50.00% as initial concentration of dye increased to 6 mg/L. On the other hand, the removal efficiencies increased as the HRT extended from 24 to 48 hr. With initial concentration of 6 mg/L, the maximum removal efficiency reached up to 78.12% at HRT of 48 hr while only 50.00% at HRT of 24 hr. In conclusion, study on CES-SBBR showed that the combination of biological (biofilm) and adsorption (CES media) able to remove RO16 dye and CES Media was suitable for microbial biofilm formation. Further research on combination of biological and adsorption processes could be the future solution in waste water treatment plants especially for textile industries.

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