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# Sequestration of Reactive Blue 4 by free and immobilized *Bacillus subtilis* cells and its extracellular polysaccharides

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ABSTRACT

*Bacillus subtilis* a gram positive bacteria and its extracellular polysaccharide were used in free form as well as immobilized form as biosorbent for sequestration of an anionic dye, Reactive Blue 4 (RB) in aqueous phase. The dye uptake enhanced with decrease in pH. Extracellular polymeric substances (EPS) and free cells were found to be better adsorbents when compared to alginate immobilized cells (IC) and EPS (IEPS). The presence of functional groups in free cells and EPS was confirmed by FT-IR analysis. Immobilization resulted in poor adsorption performance due to increase in mass transfer resistance by the polymeric matrix. High $Q_{max}$ and $b$ values were noted in the case of free cells and free EPS in contrast to IC and IEPS. From the kinetic experiments, the adsorption system was found to be a pseudo-first-order reaction at low dye concentration. Desorption of RB was found to be 100% in 1N NaOH. However, the alginate beads were found to be unstable under high alkaline conditions of NaOH.



## 1. Introduction

Synthetic dyes are extensively used in textile dyeing, paper, printing, color photography, pharmaceutics, cosmetics and other industries. Among these, azo dyes represent the largest and most versatile class of synthetic dyes [1]. Approximately 10–15% of the dyes are released into the environment during manufacturing and usage [2]. Since some of the dyes are harmful, dye-containing wastes pose an important environmental problem [3]. These dyes are poorly biodegradable because of their chemical structures. Treatment of wastewater containing dyes usually involves physical and/or chemical methods [4] such as adsorption, coagulation–flocculation, oxidation, filtration and electrochemical methods [5,6]. However, these methods are expensive and have operational problems. Treatment costs are very high for most textile industries, explaining the growing need to develop more efficient and economic methods, which consume less chemicals and energy.

The use of microorganisms as biosorbents is an attractive alternative to the existing methods for toxicity reduction and removal of dyes from industrial effluents because of their good performance and low cost. Dye adsorption studies using microorganisms has been carried out by many researchers [7]. Some microorganisms that have been used as dye adsorbents are *Trametes versicolor* [8,9], *Rhizopus arrhizus* [10], *Aspergillus fumigatus* [11], and bacterial consortium [12]. On review of literature, a stark revelation of lack of research in bacterial adsorbents compared to other microorganisms is seen [13]. Unfortunately, the use of native biomass (such as bacteria, yeast, fungi and algae) in freely suspended state is limited owing to their inherent disadvantages such as small particle size, possible clogging and low mechanical strength of the biomass. In addition, a density similar to that of the suspending medium may complicate biomass/effluent separation. Immobilized biomass overcomes some of these problems and offers greater potential applications. Benefits being better control of particle size, easy separation of biomass and effluent, capability of regeneration, high biomass loadings and minimal clogging under continuous flow [14]. Furthermore, such systems may be better defined mathematically and could be effectively used in a manner similar to conventional technology involving ion-exchange resins or activated carbon [15]. Keeping the above reasons in mind, microbes have been immobilized using calcium alginate [16,17], inactive carboxylmethylcellulose (CMC) [11], polyurethane foam, nylon sponge and stainless steel sponge [18] and polysulphone [19]. Apart from free and immobilized cells, bacterial extracellular polymeric materials (EPS) secreted by bacterial strains may also serve as excellent adsorbents. EPS are heterogenous mixtures composed mainly

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of polysaccharides and proteins, with nucleic acids and lipids as minor constituents [20]. Although there are few reports of metal biosorption by EPS [21–23] research in this area is lacking in the field of dye removal.

The present study involves the exploitation of *Bacillus subtilis*, a ubiquitous soil bacteria for the removal of RB from aqueous solutions. In our study we used four forms of adsorbents from this bacterial species, namely, free cells (FC), calcium alginate immobilized cells (IC), extracellular polymeric substances (EPS) and calcium alginate immobilized EPS (IEPS). The model dye used as adsorbate was Reactive Blue 4 (RB), which is an anthraquinone-based chlorotriazine dye very important in dyeing of cellulosic fabrics. Dye adsorption studies were carried out in batch mode.

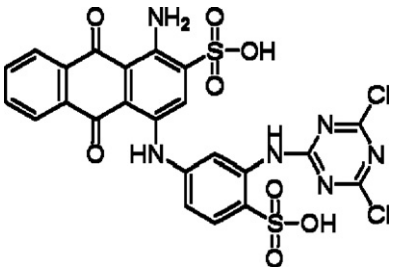

**Fig. 1.** Structure of Reactive Blue 4 (RB).

## 2. Materials and methods

### 2.1. Adsorbent preparation

*B. subtilis* (KCCM 11316) was incubated in 1000 mL nutrient broth on a rotary shaker at 100 rpm at 30 °C for 24 h. At the exponential phase it was autoclaved at 121 °C for 15 min. The cells were harvested by centrifugation at 10,000 × *g* for 20 min and freeze-dried. The freeze-dried autoclaved cells were used as biosorbent. To immobilize the freeze-dried cells, they were resuspended in 2% sodium alginate. The sodium alginate—*B. subtilis* slurry was added dropwise in to 5% $CaCl_2$ solution with a syringe under magnetic stirring at 4–7 °C and pH 7. The alginate beads were harvested after 2 h, washed thrice with distilled water and used for biosorption experiments. Alginate beads devoid of *B. subtilis* served as control.

### 2.2. EPS extraction using soya bean as media

500 g of soyabean was washed and soaked overnight at 15 °C. It was then autoclaved at 121 °C for 30 min. After cooling to 30 °C the steamed soyabean was inoculated with *B. subtilis* and allowed to ferment for 48 h at 30 °C. In a large vessel, 300 g of fermented soyabean was homogenized with 2.4 L of distilled water for 10 min. It was then centrifuged at 10,000 × *g* for 10 min. The supernatant was collected. Centrifugation was continued with addition of sterile distilled water thrice and the supernatant collected. Once the supernatant was collected 1 part was added to 3 parts of ethanol, shaken well and kept at 4 °C overnight. The precipitated extra cellular polymeric material (EPS) was freeze-dried and used for biosorption experiments. The percent amount of ash, crude protein and crude fat were analysed. To incorporate EPS in ca-alginate, 1.5% aqueous solution of sodium alginate was mixed with 0.5% EPS with continuous magnetic stirring. Beads were formed by adding the alginate-EPS slurry dropwise in to 5% $CaCl_2$ solution with a syringe at 4–7 °C and pH 7. The beads were harvested after 2 h by filteration, washed thrice with distilled water and used as adsorbent.

### 2.3. Adsorbate

Reactive Blue 4 (RB) was procured from Sigma–Aldrich, South Korea. The dye structure is given in Fig. 1. Molecular formula: $C_{23}H_{14}Cl_2N_6O_8S_2$, molecular weight: 637.43, CAS number: 13324-20-4; $\lambda_{max}$ 595 nm. Standard solutions containing 10–80 mg/L of the dye were prepared by diluting a stock solution of 1000 mg/L of RB.

### 2.4. Estimation of ash, crude protein and crude fat in EPS

Total ash content, percentage of crude protein and crude fat in EPS were measured according to AOAC (1995) standards. To determine the ash content of EPS, 3 g of EPS was taken in a crucible and placed in a furnace at high temperature (550–600 °C) for 12 h, such that all the water, volatile compounds, and organic matter either evaporate or burn. After that, the remaining material was weighed. Crude protein and crude fat were measured by micro-Kjeldahl and Soxhlet method, respectively.

### 2.5. SEM analysis

Scanning electron microscopy (SEM) of autoclaved bacteria as free cells and alginate immobilized bacteria prepared as mentioned above were carried out using JEOL, JSM-5200 (Japan) scanning electron microscope. The free cells and bacteria laden alginate beads were dried overnight at 60 °C. The dried adsorbents were then glued separately on a brass stub using 'spot-o-gold' labels and coated with gold–palladium using a JEOL, JFC-1200 (Japan) fine coater. The images were then captured at various magnifications (500×, 1000× and 3500×) using an electron beam high voltage of 15 kV at a 45° tilt on left side.

### 2.6. FT-IR spectra

The IR spectra of free cells of *B. subtilis* and the extracellular polysaccharide (EPS) produced by the bacteria were obtained by IR spectroscopy (Shimadzu 8201PC, Japan). The autoclaved free cells and EPS were dried overnight at 60 °C. Approximately 0.01 g of the dried biomass/EPS was mixed with 0.1 g of KBr and pressed into a tablet form by pressing the ground mixed material with the aid of a bench press. The resulting pellet was transparent and was used to test the surface functional groups by IR spectroscopy where it was scanned between 4000 and 400 $cm^{-1}$ at a resolution of 4 $cm^{-1}$.

### 2.7. Biosorption studies

Dye uptake studies were conducted at room temperature and involved contacting 50 mL of dye solution with 1 g biomass/L for 5 h on an orbital shaker at 150 rpm. All studies were carried out in duplicate and biomass-free controls were run concurrently. The flasks were withdrawn at predetermined time intervals. The adsorbent was removed by centrifugation at 7500 rpm for 10 min and the dye concentration of the supernatant analysed. The amount of dye adsorbed by the adsorbent ($q$, mg/g) was calculated. Dye concentrations were determined by monitoring the absorbance at 595 nm using a UV–vis spectrophotometer (Shimadzu, UV-1601). The optimal pH for dye removal by the biosorbent was determined by measuring uptake from 50 mL of 50 mg/L dye solutions over a range of pH values from 2 to 10. The solution pH was adjusted using 1 M HCl and 1 M NaOH. Adsorption isotherm experiments were carried out with a series of solutions of initial dye concentration ranging from 10 to 80 mg/L at the optimal pH value determined as described above.