Contributions of functional groups and extracellular polymeric substances on the biosorption of dyes by aerobic granules

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The contributions of loosely bound extracellular polymeric substances (LB–EPS), tightly bound EPS (TB–EPS), residual sludge (the sludge left after EPS extraction) and functional groups such as amine, carboxyl, phosphate and lipid on aerobic granules on biosorption of four different dyes ( Reactive Brilliant Blue KN–R (KN–R), Congo Red (CR), Reactive Brilliant Red K–2G (RRB) and Malachite Green (MG) ) were investigated. EPS may be responsible for biosorption of cationic dyes. However, residual sludge always made greater contribution than that of EPS. The biosorption mechanisms were dependent on the functional groups on aerobic granules and dyes’ chemical structures. The lipid and phosphate groups might be the main binding sites for KN–R biosorption. Amine, carboxyl, phosphate and lipid were all responsible for the binding of CR. The lipid fractions played an important role for RBB biosorption. For MG, the phosphate groups gave the largest contribution.

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1. Introduction

Synthetic dyes are widely used in textile, drug, cosmetic, paper and pulp, plastic and printing industries. As a result, about 10–20% of the dyes are lost during the manufacturing and dyeing process, producing large amounts of dye-containing wastewater (Vijayaraghavan and Yun, 2008). Based on the class of chromophore, the three classes of dyes used mostly are azo, anthraquinone and triphenylmethane dyes. These dyes may pose aesthetic problem even in the generation of colorless dead-end aromatic amines, which are generally more toxic than the parent compounds ( Couto, 2009 ). In the past years, biosorption, an ideal candidate for replacing the conventional adsorption by activated carbon, has been investigated as one of the most promising methods for dye removal. Various biological materials have been used as biosorbents such as bacteria ( Uzel and Ozdemir, 2009 ), fungi ( Akar and Divriklioglu, 2010 ), agricultural waste ( Arami et al., 2006 ) and industrial waste or byproducts ( Kılıç et al., 2008 ). However, most biosorbents are in the form of dispersed microorganisms and their characteristics such as small particle size, low density, poor mechanical strength and little rigidity would cause practical difficulties in solid–liquid separation and biomass regeneration, and limit their applications under real conditions ( Xu and Liu, 2008 ). The immobilized types of biosorbents can overcome these drawbacks, but the employment of immobilization procedures is expensive and complex ( Xu and Liu, 2008 ). Aerobic granules have the advantages of excellent settle ability, dense and strong microbial structure, high biomass concentration, and limit their applications under real conditions ( Xi and Liu, 2008 ). Aerobic granules technique has been extensively studied worldwide in the past 10 years ( Adav et al., 2008 ; Liu et al., 2009 ). Aerobic granules contain mainly bacteria, protozoa and extracellular polymeric substances (EPS). EPS in sludge flocs can be stratified into loosely bound EPS (LB–EPS), tightly bound EPS (TB–EPS) and residual sludge (the sludge left after EPS extraction). EPS is a rich matrix of polymeric substances (EPS). EPS in sludge flocs can be stratified into loosely bound EPS (LB–EPS), tightly bound EPS (TB–EPS) and residual sludge (the sludge left after EPS extraction). EPS is a rich matrix of polymeric substances (EPS). EPS may be responsible for biosorption of cationic dyes. However, residual sludge always made greater contribution than that of EPS. The biosorption mechanisms were dependent on the functional groups on aerobic granules and dyes’ chemical structures. The lipid and phosphate groups might be the main binding sites for KN–R biosorption. Amine, carboxyl, phosphate and lipid were all responsible for the binding of CR. The lipid fractions played an important role for RBB biosorption. For MG, the phosphate groups gave the largest contribution.

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effective biosorbent to remove Basic Blue 54 from aqueous solution (Zhang et al., 2009). The adsorption properties of Toluidine Blue on EPS extracted from aerobic sludge and anaerobic sludge have been characterized (Sheng et al., 2008). However, little information can be obtained concerning the biosorption characteristics of dyes onto EPS from aerobic granules.

The functional groups on aerobic granules such as amine, carboxyl, lipid and phosphate may provide binding sites for dye biosorption (Gao et al., 2010a). The contributions of various functional groups on activated sludge in the biosorption of heavy metals have been examined (Kılıç et al., 2008). Several studies have investigated the roles of these functional groups in biosorption of dyes. Through chemical groups modification studies, the carboxyl and amino groups have been demonstrated as the main binding sites for Rhodamine B biosorbed onto Rhizopus oryzae biomass (Das et al., 2008). The different functional groups in the fungal biomass of Aspergillus niger played different roles in biosorption of different dyes (Basic Blue 9, Acid Blue 29, Congo Red and Disperse Red 1) (Fu and Viraraghavan, 2002). To the best of our knowledge, the roles played by these functional groups in biosorption of dyes by aerobic granules have not been studied.

Of the days, aerobic granules have been used as biosorbents for the removal of heavy metals (Xu and Liu, 2008) and Malachite Green (Sun et al., 2008). In our previous works, inactive aerobic granules have been used as biosorbent for the removal of Acid Yellow 17 in single solution (Gao et al., 2010a) and the competitive biosorption of Yellow 2G and Reactive Brilliant Red K–2G in binary system (Gao et al., 2010b). However, the contributions of various functional groups in biosorption of heavy metals have not been examined. A thorough understanding of the complex interactions between EPS, residual sludge and various functional groups on aerobic granules is the step toward comprehensive modeling for the removal and recovery of dyes from contaminated effluents by aerobic granules.

Here aerobic granules were stratified into three parts: LB–EPS, TB–EPS and residual sludge. And the functional groups on residual sludge such as carboxyl, amine, phosphate groups and lipid fractions were chemically modified. The object of this study was to quantify the contributions made by LB–EPS, TB–EPS, residual sludge and various functional groups on the residual sludge in biosorption of four different dyes, Reactive Brilliant Blue KN–R (anthraquinone dye, abbreviated as KN–R), Congo Red (disazo dye, abbreviated as CR), Reactive Brilliant Red K–2G (single–azo dye, abbreviated as RBR) and Malachite Green (triphenylmethane dye, abbreviated as MG). It is expected that the possible mechanisms involved in dyes biosorption by aerobic granules could be disclosed, and novel information could be provided for the application of aerobic granulation technology in wastewater treatment.

2. Methods

2.1. Dyes and analysis

Four types of dye were used in this study, containing KN–R (C.I. Reactive Blue 19, molecular formula = C_{22}H_{16}N_{2}O_{11}S_{2}, \lambda_{\text{max}} = 593 nm), CR (C.I. 22,120, molecular formula = C_{25}H_{22}N_{7}O_{11}S_{2}, \lambda_{\text{max}} = 500 nm), RBR (C.I. Reactive Red 15, molecular formula = C_{25}H_{14}ClN_{7}Na_{4}O_{13}S_{4}, \lambda_{\text{max}} = 512 nm) and MG (C.I. 42,000, molecular formula = C_{23}H_{25}ClN_{2}O_{13}S_{4}, \lambda_{\text{max}} = 617 nm). Their molecular structures are shown in Fig. S1 (see Supplementary material). They were purchased from Tianjin Shengda Chemical Factory (China). The test solutions were prepared by diluting stock solutions to the desired concentrations. The required pH value of the solutions was adjusted with 0.1 mol l⁻¹ HCl or NaOH solutions with a pH–meter (WTW 340i, Germany) for the measurements.

The dye concentration was determined by measuring the absorbance at 593, 500, 512 and 617 nm for KN–R, CR, RBR and MG, respectively, by using a UV–vis spectrophotometer (UV-2802PC, Unico, Shanghai, China).

2.2. Cultivation of aerobic granules

Aerobic granules used for the biosorption tests were collected from a lab-scale sequencing batch reactor (SBR) fed with glucose as the sole carbon source, which is a typical utilizable carbon and energy source for heterotrophic organisms. The setup and operational conditions of the reactor were described elsewhere (Gao et al., 2010a). The mean diameter of the aerobic granules was around 2.0 mm, and the mean settling velocity was 33 m h⁻¹. Prior to use, the aerobic granules were first washed with distilled water three times to remove the surface soluble ions. Generally, aerobic granules suffer intense hydraulic or mechanical shear force in the bioreactors.

2.3. EPS extraction and chemical analysis

As shown in Fig. 1, the aerobic granules were divided into the LB–EPS, TB–EPS and residual sludge, and then the residual sludge was modified by chemical methods for different purpose. The LB–EPS, TB–EPS, residual sludge and the modified residual sludge were all used to adsorb the dyes.

The aerobic granules were taken out the reactor at the end of aerobic phase. First, 15 mL of aerobic granules were dewatered by centrifugation at 3000 rpm for 15 min. The granules in the tube were then re-suspended into 15 mL using 0.05% NaCl solution and centrifuged at 7400 rpm for 15 min, and the supernatants were filtered through 0.20 μm cellulose acetate membranes. The organic substance in the supernatants was LB–EPS. Then TB–EPS

![Fig. 1. Schematic of the EPS extraction from aerobic granules and chemical treatments performed on the residual sludge.](image-url)

of aerobic granules in the sediments of the first step were extracted using cation exchange resin (CER, Dowex Marathon, Sigma 91973) technique (Freund et al., 1996). The sludge left in the tube was smashed, re-suspended into 15 mL with 0.05% NaCl solution and the solution was transferred to an extraction beaker, followed by the CER addition at a dosage of 70 g g⁻¹ SS. The suspension was stirred for 1 h at 600 rpm and 4 °C thereafter. Afterwards, the CER/slugde suspension was settled for 5 min to remove CER, and then TB–EPS was harvested by centrifugation at 12000 rpm at 4 °C for 30 min in order to remove the remaining floc components. The supernatant was filtered through 0.20 μm cellulose acetate membranes and used as the TB–EPS of aerobic granules.

The total EPS content was determined as the sum of carbohydrates, humic substances and proteins. The measurement methods of the contents of carbohydrates, proteins and humic substances could be found elsewhere (Freund et al., 1996). The total organic carbon (TOC) concentration of the EPS solution was measured to characterize EPS using a TOC analyzer (Analytik Jena TOC-Analyzer Multi N/C 3000).

2.4. Chemical treatment of the residual sludge

As shown in Fig. 1, after extraction of EPS from aerobic granules, the residual sludge was treated as following for modification of different functional groups:

- **Type 0**: Residual sludge without chemical treatment (T0).
- **Type 1**: 1 g (dry weight) residual sludge with 20 mL formaldehyde (HCHO) and 40 mL formic acid (HCOOH) was agitated in a water bath shaker with a shaking rate of 125 rpm at room temperature for 6 h (T1). This treatment causes esterification of carboxylic acids present on the cell wall of biosorbent (Kapoor and Viraraghavan, 1997). The reaction occurs as follow:

\[ \text{RCOOH} + \text{CH}_2\text{NH}_2 \rightarrow \text{RCOCH}_2\text{NH}_2 + \text{CO}_2 + \text{H}_2\text{O} \]  

(1)

- **Type 2**: 2 g (dry weight) residual sludge with 130 mL of anhydrous methanol (CH₃OH) and 1.2 mL of concentrated hydrochloric acid (HCl) was agitated in a water bath shaker with a shaking rate of 125 rpm at room temperature for 6 h (T2). This treatment causes esterification of carboxylic acids present on the cell wall of biosorbent (Drake et al., 1996). The reaction occurs as follow:

\[ \text{RCOOH} + \text{CH}_3\text{OH} \rightarrow \text{RCOOHCH}_3 + \text{H}_2\text{O} \]  

(2)

- **Type 3**: 1 g (dry weight) residual sludge with 40 mL of triethyl phosphate (C₆H₁₅PO₃) and 30 mL of nitromethane (CH₃NO₂) was heated under reflux for 6 h (T3). This treatment was reported to result in esterification of phosphate groups present on the cell wall (Fu and Viraraghavan, 2002).

The following two operations were also carried out in order to extract the lipid fractions of the residual sludge.

- **Type 4**: 1 g (dry weight) residual sludge with 75 mL benzene was heated under reflux for 6 h (T4) (Kapoor and Viraraghavan, 1997).

- **Type 5**: 1 g (dry weight) residual sludge with 75 mL acetone was heated under reflux for 6 h (T5) (Fu and Viraraghavan, 2002).

After the chemical treatment, the sludge was washed 10 times using distilled deionized water to remove the excess of chemical reagents, and freeze-dried by using Labconco FreeZone 1L (Labconco, America) to yield biosorbents.

Chemical reagents used to modify different functional groups on aerobic granules including formaldehyde, formic acid, anhydrous methanol, hydrochloric acid, triethyl phosphate, nitromethane, benzene and acetone, were all purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. All the reagents were analytical grade and used without further purification.

2.5. Biosorption experiments

In order to avoid the variability originated from the aerobic granules cultivation and reduce errors, enough aerobic granules were taken out from the reactor at a time, the aerobic granules were then equally divided for EPS extraction. After EPS extraction, the EPS solutions were homogeneously mixed to reduce errors produced during the extraction process. After chemical modification of the residual sludge, the residual sludge modified with the same method was well mixed to reduce errors produced during the chemical modification. Then, the EPS solutions and the modified residual sludge were equally divided for biosorption tests. Biosorption experiments were conducted in 50 mL glass stopper Erlenmeyer flasks which were agitated in a water bath shaker with a shaking rate of 150 rpm at a constant temperature of 20 ± 1 °C until reaching equilibrium. Each test lasted for 24 h. All the biosorption tests were performed twice, and the average value was used. The standard deviation for duplicate samples was 0.20–3.74%.

First, the effect of pH on the biosorption capacity was investigated, initial dye concentration was fixed at 60 ± 5 mg l⁻¹, 0.1 g (dried weight, the same below) aerobic granules was dosed in 50 mL dye solution (aerobic granules dosage was 2.0 g l⁻¹) and pH ranged from 2 to 12. The optimal pH was used for the subsequent biosorption tests.

To compare the contributions of aerobic granules (without EPS extraction), LB–EPS, TB–EPS and residual sludge on the biosorption of four different dyes, in 50 mL dye solution, initial dye concentration was fixed at 100 ± 5 mg l⁻¹, 0.1 g aerobic granules or biosorbent derived from 0.1 g aerobic granules were used. For comparison, the results obtained from dyes biosorption on aerobic granules were used as the control.

To investigate the roles of functional groups on the residual sludge biosorption of dyes, initial dye concentration was fixed at 100 ± 5 mg l⁻¹, biosorbents derived from 0.1 g aerobic granules were used. For the chemical modifications were carried out on the residual sludge, the comparisons were done between residual sludge and different chemical modifications.

Before and after chemical treatment, the residual sludge was characterized by FTIR using a Bruker Vertex 70 FTIR spectrometer. The biosorbent was first freeze-dried by using Labconco FreezeZone 1L, then they were mixed with KBr in the ratio of 1:100 and compacted to pellet form under high pressure. The pellet was immediately analyzed in the range of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹. FTIR spectra of LB–EPS and TB–EPS were also recorded.

After biosorption had reached equilibrium, the samples were centrifuged at 3200 rpm for 2 min, and then the supernatant was analyzed for the residual dye concentration by using a UV–vis spectrophotometer. The amount of dye adsorbed at equilibrium, \( q_e \) (mg g⁻¹), was calculated as:

\[ q_e = \frac{V(C_0 - C_e)}{M} \]  

(3)

where \( C_0 \) is the initial dye concentration in the solution (mg l⁻¹); \( C_e \) is the liquid phase dye concentration at equilibrium (mg l⁻¹); \( V \) is the volume of solution (l); and \( M \) is the weight of biosorbent used (g).

3. Results and discussion

3.1. Effect of pH

The effects of pH on the biosorption of dyes onto aerobic granules were shown in Fig. 2. As seen from the Fig. 2a and c, the optimal pH value of KN–R and RBR biosorption was 2.0, and the maximum \( q_e \) was 23.85 and 32.52 mg g⁻¹, respectively. For the
process of CR biosorption (Fig. 2b), as the pH increased from 2.0 to 6.2, the biosorption capacity obviously increased and reached the maximum value of 24.43 mg g⁻¹ at pH 6.2 (natural, without adjusted); as pH increased from 6.2 to 10.0, the \( q_e \) decreased slowly, and then the biosorption was restricted with a \( q_e \) of 2.10 mg g⁻¹ at pH 12.0. As given in Fig. 2d, at pH 2.0 the lowest \( q_e \) of MG biosorption was obtained and the value was only 7.79 mg g⁻¹. At pH 3.8 (natural, without adjusted), the highest \( q_e \) was achieved as 36.32 mg g⁻¹; as pH increased from 6.0 to 10.0, the biosorption capacity slowly decreased. Therefore, the optimal pH value of CR and MG biosorption on aerobic granules were both natural. All the optimal pH values were used for the subsequent experiment.

It is now recognized that the pH of dye solution plays an important role in the whole biosorption process, influencing not only the solution dye chemistry, but also the surface charge of the biosorbent and the dissociation of functional groups on the active sites of the biosorbent (Crini and Badot, 2008). From the dyes chemical structure (Fig. S1a–c), CR, KN–R and RBR will all produce anionic ion (SO₃⁻/CO₃⁻) in aqueous solutions. MG is a cationic dye, which could produce \( \text{Dye}^+ \) and Cl⁻ in solution (Fig. S1d). The solution pH affects the ionization state of the functional groups like carboxyl, phosphate and amino groups of the bacterial cell wall and EPS. The functional groups are mostly protonated at acidic pH, but become progressively negative-charged with increasing pH due to proton dissociation of carboxyl [pH 2.0–6.0], phospholipids [pH 2.4–7.2], phosphodiester [pH 3.2–3.5], hydroxyl [pH 9.0–10.0] and amino [pH 9.0–11.0] groups (Martinez et al., 2002). The zero charge point of aerobic granules was at a pH of 2.0 (Gao et al., 2010b), the aerobic granules carried positive charges or negative charges when the solution pH is below or above pH 2.0, respectively. The electrostatic attraction allowed greater adsorption of anionic dyes (RBR and KN–R) at lower pH (2.0) and greater adsorption of cationic dyes (MG) at higher pH (3.8). But the optimal pH for CR biosorption was 6.2, suggesting that other mechanism may be involved.

For KN–R, different from RBR, the biosorption capacity increased with pH value increasing from 8.0 to 10.0. The binding mechanism that occurred at acidic pH (pH ≤ 7) may be due to the electrostatic attraction between the positively charged amine groups on aerobic granules and negatively charged sulfonate groups of KN–R molecule. However, under strongly basic pH conditions, the amine groups on aerobic granules did not participate in the biosorption of KN–R because they existed in the –NH₂ form. KN–R has one sulfatoethylsulfone group and one sulfonate group. Weber and Stickney found that the sulfatoethylsulfone group of Reactive Blue 19 (KN–R is another name for Reactive Blue 19) was eliminated on treatment with alkali at room temperature to quantitative form as vinyl sulfone (Weber and Stickney, 1993), which meant that the reactive sulfatoethylsulfone group of KN–R could be converted into the activated vinyl sulfone group, or to the completely hydrolyzed form under alkaline conditions. Moreover, it was generally recognized that reactive dyes of the remazol type, such as Reactive Orange 16, Reactive Blue 19 and Reactive Red 23, reacted with nucleophiles by a nucleophilic addition mechanism (Won et al., 2009). Masri and Friedman explained that protein functional groups could react with alkyl vinyl sulfone via a Michael-type nucleophilic addition reaction (Masri and Friedman, 1988). In the same manner, the KN–R biosorption onto the aerobic granules observed under strongly alkaline conditions (pH = 10.0), as shown in Fig. 2a, was due to the hydroxyl group of aerobic granules reacting with the vinyl sulfone group of KN–R via the nucleophilic addition reaction. Similar results could be found in the biosorption of Reactive Orange 16 under pH 11 by the protonated waste biomass of Corynebacterium glutamicum (Won et al., 2009).

In conclusion, for different dyes the different functional groups played different roles. So the chemical modifications of these functional groups were made to investigate the possible mechanism of biosorption of different dyes on aerobic granules.

Fig. 2. Effects of initial pH value on the biosorption of dyes. (a) Reactive Brilliant Blue KN–R (KN–R); (b) Congo Red (CR); (c) Reactive Brilliant Red K–2G (RBR); (d) Malachite Green (MG).
Fig. 3. FTIR spectra before and after chemical treatments of the residual sludge (a) methylation of amines by formic acid and formaldehyde (T1); (b) esterification of carboxylic acids by methanol and hydrochloric acid (T2); (c) esterification of phosphate groups by triethyl phosphate and nitromethane (T3); (d) lipid extraction by benzene (T4); (e) lipid extraction by acetone (T5). The profile (i) in all the figures was FTIR spectrum of the residual sludge without chemical treatment (T0); profile (ii) in all the figures denoted FTIR spectra of the residual sludge after different chemical treatments.
3.2. FTIR analysis

The FTIR spectra of residual sludge before and after chemical treatment in the range of 4000–400 cm\(^{-1}\) are sketched out in Fig. 3. The compounds deciphered could be identified based on the reports in previous study (Gao et al., 2010a; Gao et al., 2010b). The profile (i) in Fig. 3 portrays the FTIR spectrum of the residual sludge. The strong band at 3437.43 cm\(^{-1}\) reflected N–H and O–H stretching vibrations of hydroxyl and amine groups on the surface of the aerobic granules, while the band at 2924.61 cm\(^{-1}\) and the band at 2852.18 cm\(^{-1}\) represented an asymmetric vibration of CH\(_{2}\). The band at 1394.19 cm\(^{-1}\) could be assigned to the symmetrical stretching vibration of C=O of carboxylate and deformation vibration of O–H of alcohols. A distinct band at 1639.26 cm\(^{-1}\) could be due to a combination of the stretching vibration of C=O and C–N (Amide I) peptidic bond of protein. While a 1549.28 cm\(^{-1}\) band could be due to a combination of the stretching vibration of C–N and deformation vibration of N–H peptic bond of protein (Amide II). The band at 1231.05 cm\(^{-1}\) could be attributed to the C–N stretching of Amide III. Band at 1088.40 cm\(^{-1}\) could be attributed to the stretching vibration of OH of polysaccharides. The finger region demonstrated the existence of sulfur or phosphate groups.

Treatment of biomass with formaldehyde and formic acid changes the methylation of amine group (T1) as shown in Fig. 3a(ii). As seen from Fig. 3a(ii), the band at 3437.43 and the 1549.28 cm\(^{-1}\) band got wider and the apex was lower after this chemical treatment, demonstrating that N–H and Amide II group was reduced. Also, the band at 1639.26 cm\(^{-1}\) was clearly decreased and the 1231.05 cm\(^{-1}\) band disappeared after modification, concluding that Amide I and Amide III groups had been concealed. Fig. 3b(ii) portrays the methanol and hydrochloric acid–treated (T2) FTIR spectrum of the residual sludge.Observed bands at 1394.19 and 1639.26 cm\(^{-1}\) were shifted to 1375.17 and 1637.80 cm\(^{-1}\), respectively, and the two bands became wider and lower which may be attributed to the esterification of carboxyl groups in the biomass. The disappearance of the band at 727.01 cm\(^{-1}\) in Fig. 3c(ii) could be attributed to the esterification of phosphoryl groups as a result of nitromethane and triethyl phosphate treatment (T3). The spectra of residual sludge with acetone and benzene treatment (T4 and T5) were shown in Fig. 3d(ii) and Fig. 3e(ii), respectively. The bands at 2852.18 and 2924.61 cm\(^{-1}\) shifted slightly. The changes could be attributed to the partial elimination of CH\(_{2}\) groups which suggests that lipids were extracted from the biomass.

The FTIR spectra of residual sludge after treatment were shown in Fig. 3b(ii), c(ii), d(ii) and e(ii), respectively. Since the bands at 1639.26 and 1231.05 cm\(^{-1}\) in Fig. S2(i) and Fig. S2(ii) were the result of the stretching vibration of C=O and C–N (Amide I) peptidic bond of protein, while the bands around 2117.71 cm\(^{-1}\) would show the stretching vibrations of C=C. Some bands whose wave number was lower than 1000 cm\(^{-1}\) in Fig. S2 could be attributed to the phosphate and sulfur groups. The FTIR spectra of LB–EPS and TB–EPS had the similar characteristic; however, the TB–EPS had one more band at 1369.18 cm\(^{-1}\) which could be assigned to bending vibration of –CH\(_{2}\).

3.3. Biosorption tests on aerobic granules, EPS and residual sludge

The main contents of EPS are listed in Table 1. The comparisons of dyes biosorption on aerobic granules (without EPS extraction, used as control), LB–EPS, TB–EPS and residual sludge are shown in Fig. 4. As seen from Fig. 4, compared with aerobic granules, the EPS’s contribution to the biosorption of KN–R, CR and RBR could be negligible, while they could adsorb MG very well; different from EPS, the biosorption capacity given by residual sludge for KN–R, CR, RBR and MG was 99.42, 91.11, 90.18 and 89.54% to that of aerobic granules, respectively, indicating that residual sludge played the main role. Fig. 4 portrayed that LB–EPS had no effects in biosorption of CR and RBR, and the values of \(q_e\) were all 0 mg g\(^{-1}\), while the relative higher \(q_e\) values were obtained from biosorption on TB–EPS (2.04 and 14.75 mg g\(^{-1}\), respectively), indicating that TB–EPS gave a higher contribution to CR and RBR biosorption. For KN–R, compared with the biosorption capacity of aerobic granules, the capacity was reduced 93.62% and 95.17% for LB–EPS and TB–EPS, respectively. On the contrary, EPS and residual sludge were all involved in the biosorption of MG and the biosorption capacity was almost similar, but the \(q_e\) value on residual sludge was slightly higher.

According to these results, LB–EPS and TB–EPS had different effects on all the four types of dyes in this investigation and TB–EPS produced a relative higher contribution to biosorption. The residual sludge always played an important role than that of EPS. Aerobic granules are mainly composed of microorganisms, EPS and inorganic component. The EPS which contains polysaccharide, protein, lipids, and nucleic acids provides a three-dimensional, gel-like, highly hydrated and often negative charged polymeric matrix.
for enmeshment of the microbes and cations. EPS was thought to be of considerable importance in bioflocculation, adhesion and sorption of both organic and inorganic substances. Dyes removal by EPS may be explained on the electrostatic interaction between the functional groups and dyes. As seen from Fig. S2, there were hydroxyl, amine and phosphate groups on LB–EPS and TB–EPS. The optimal pH value for KN–R, CR, RBR and MG was 2.0, 6.2, 2.0 and 3.8, respectively. For KN–R and RBR, the functional groups on EPS were protonated at pH 2.0, and the dyes were adsorbed through the electrostatic interaction between SO$_3^-$ and protonated functional groups (such as amine group). For CR, the functional groups of EPS became progressively negative-charged under pH 6.2 lower electrostatic attraction produced lower $q_e$. MG, a kind of cationic dye, can be represented as Dye$^+$/C$_1$Cl$^-$ in the aqueous solution. The Dye$^+$ would attract the materials with negative charge. Therefore, at pH 3.8, the biosorption of MG may be owing to the electrostatic attraction between the Dye$^+$ and negative functional groups on EPS. Compared with TB–EPS, the protein and polysaccharides content of LB–EPS was very low, however the humic acids of LB–EPS was high. So, the MG biosorption by EPS may be due to chemical reaction between humic acids and Dye$^+$. Under low pH conditions, EPS of Bacillus subtilis biosorption of Reactive Blue 4 (an anionic dye, anthraquinone dye) was very low, however the humic acids of LB–EPS was high. So, the MG biosorption by EPS may be due to chemical reaction between humic acids and Dye$^+$. Under low pH conditions, EPS of Bacillus subtilis biosorption of Reactive Blue 4 (an anionic dye, anthraquinone dye) was very low, however the humic acids of LB–EPS was high. So, the MG biosorption by EPS may be due to chemical reaction between humic acids and Dye$^+$. Under low pH conditions, EPS of Bacillus subtilis biosorption of Reactive Blue 4 (an anionic dye, anthraquinone dye) was very low, however the humic acids of LB–EPS was high. So, the MG biosorption by EPS may be due to chemical reaction between humic acids and Dye$^+$. Under low pH conditions, EPS of Bacillus subtilis biosorption of Reactive Blue 4 (an anionic dye, anthraquinone dye) was very low, however the humic acids of LB–EPS was high. So, the MG biosorption by EPS may be due to chemical reaction between humic acids and Dye$^+$.
3.4. Biosorption test before and after chemical treatment on the residual sludge

3.4.1. KN–R

Results obtained from the biosorption of KN–R on all kinds of treated biosorbents are shown in Fig. 5a. None of the treatments increased the biosorption capacity and the reductions ranged from 1.83% to 29.48%. The lipid extraction with acetone (T5) obviously restrained the biosorption and caused the highest reduction (29.48%). The esterification of phosphate groups (T3) and the lipid extraction with benzene (T4) also inhibited the biosorption and the reduction of biosorption capacity was 17.58% and 21.26%, respectively. However, reductions caused by T1 (methylation of amine) and T2 (esterification of carboxyl groups) were negligible (3.84% and 1.83%). All results demonstrated that the amine and carboxyl groups contributed a little on the biosorption of KN–R, while the phosphate functional groups and lipid fractions played important roles.

As seen from the chemical structure of KN–R, there was a primary amino group (–NH₂) attached to the anthraquinone ring. This amino group would be positively charged (–NH₃⁺) in acid solution, which would be probable to bind with negatively charged functional groups on aerobic granules, such as carboxyl and phosphate groups. However, the biosorption capacity reduction caused by modification of carboxyl (T2) was negligible (only 1.83%), indicating that carboxyl groups could contribute to the biosorption of KN–R but not the major one. 17.58% of the biosorption capacity was reduced after phosphate groups modification (T3), demonstrating that the phosphate groups could be the major ones contributing to KN–R biosorption because they exhibited a negative charge but not the only one. In the presence of H⁺, the amino groups (–NH₂) of aerobic granules were protonated, and –NH₃⁺ was produced. Electrostatic attraction then proceeded between the protonated –NH₃⁺ and sulfonate groups (–SO₃⁻) of KN–R. However, the amine group modification (T1) resulted in small reduction (3.84%) in the biosorption KN–R, illustrating that the amine groups were involved in biosorption of KN–R and did not play an important role. The treatment of biosorbent with benzene (T4) and acetone (T5) extracted the lipid fractions and hence largely reduced biosorption capacity (21.16% and 29.48%, respectively) indicating that lipids gave the most contribution to the removal of KN–R and mechanisms other than electrostatic attraction were involved.

3.4.2. CR

Results obtained from the biosorption of CR on all kinds of treated biosorbent are shown in Fig. 5b. All chemical treatments decreased the biosorption capacity and the observed reduction ranged from 39.33% to 75.86%. As seen from Fig. 5b, the lipid extraction with acetone (T5) obviously restrained the biosorption and caused the biggest reduction of 75.86%. The methylation of amines (T1) also significantly inhibited the biosorption and the reduction was 75.60% next to lipid extraction with acetone (T5). The esterification of carboxyl groups (T2) and phosphate groups (T3) resulted in similar reduction of biosorption (about 51%). The lipid extraction by benzene (T4) resulted in the least reduction (39.33%).

CR is disazo dye. In aqueous solutions CR ionizes into two sodium cations and two colored sulfate anions, and it can be written as Dye²⁻–2Na⁺ (Aspland, 1997). In this study, all the treatments significantly decreased the biosorption capacity. In the case of T1, formaldehyde can reversibly replace labile H atoms on –COOH and –SH groups of proteins (Davis et al., 1990). Therefore, the reduction caused by amine modified biosorbent maybe the results of the mask of amino and/or carboxyl groups (Fu and Viraraghavan, 2002). In acid solution, the carboxylic acids would dissociate into carboxylate anions to a small extent. In view of this theory, the carboxyl groups may not be the binding sites for the Dye²⁻. For their negative charge, the phosphate groups couldn’t be the binding sites for Dye²⁻, too. However, these weren’t identical with the experimental results, demonstrating that there were other mechanisms in CR biosorption. There are two primary amino groups (–NH₂) attached to the two naphthene rings located at the two ends of the CR molecule, respectively, which would be protonated (–NH₃⁺) in the acid solution. Hence, the attraction would exist between –NH₃⁺ of the dye and anions such as carboxyl and phosphate groups of residual sludge. Similarly, the amino groups on residual sludge also may result in the attraction between –NH₃⁺ and Dye²⁻. The biosorption capacity reductions were 75.60%, 51.34% and 51.58% after modification of amine (T1), carboxyl (T2) and phosphate groups (T3), suggesting that the three groups maybe the major binding sites and the electrostatic attraction maybe the mechanism in biosorption of CR. The extraction of lipid by benzene (T4) and acetone (T5) also inhibited the biosorption of CR, illustrating that the lipid fractions could also play important roles in the biosorption and the mechanism must be different from electrostatic attraction.

3.4.3. RBR

Results obtained from the biosorption of RBR on all kinds of treated biosorbent are shown in Fig. 5c. As seen from Fig. 5c, both T1 and T2 increased the biosorption capacity and the additions were 9.10% and 6.23%, respectively. The other treatments (T3, T4 and T5) all decreased the capacity. The reduction caused by lipid fractions extraction by acetone (T5) was 25.73%, which was lower than that by benzene (T4, 39.08%). The modification of phosphate groups (T3) caused a reduction of 20.96% next to the lipid extraction with acetone (T5). RBR is single–azo dye and will produce anionic ion (–SO₃⁻) as the colored dye ions in acidic solutions and RBR could be written as Dye⁺–4Na⁺ (Aspland, 1997). Due to the negative charge, the phosphate and carboxyl groups may not be the binding sites for RBR for their repulsion with Dye⁺–. In this study, modification of carboxyl (T2) slightly increased the biosorption, which indicated that carboxyl group had little effect on RBR biosorption. However, the modification of phosphate groups (T3) inhibited the biosorption and the reduction was 20.96%, which demonstrated that phosphate groups interacted with some groups other than –SO₃⁻ of the dye, for the electrostatic repulsion of –SO₃⁻ with phosphate groups. The initial pH value of solution was 2.0 and the –NH₃⁺ on residual sludge would be protonated as –NH₄⁺, leading to the attractions between –NH₄⁺ and anions, such as –SO₃⁻. Then, the modification of amine (T1) would restrain RBR biosorption. However, the biosorption capacity of T1 was slightly increased, indicating that the amines had little effect on the biosorption of RBR. From these results, it could be deduced that not only the electrostatic attraction but also other mechanisms may be involved in biosorption process. The removal of lipid fractions through extraction by benzene (T4) and acetone (T5) reduced the biosorption of RBR (39.08% and 25.31%, respectively), which indicated that the lipid fractions maybe the main binding sites for RBR.

3.4.4. MG

As seen from Fig. 5d, the results of MG biosorption were different from the other three dyes. The modification of amine (T1), carboxyl (T2) and phosphate (T3) groups all inhibited the biosorption capacity and the reduction was 58.01%, 56.47% and 69.47%, respectively. The lipid fractions extracted by benzene (T4) and acetone (T5) all slightly enhanced the biosorption capacity (2.52% and 1.83%, respectively). The esterification of phosphate groups (T3) caused the biggest reduction (69.47%), followed by the methylation of amine (T1). Compared with the results obtained from biosorption on T1, the modification of carboxyl groups (T2) had a similar reduction (56.47%). All these results might indicate that the
phosphate groups played the most important roles in the biosorption of MG.

MG is a triphenylmethane dye. Different from KN-R, CR and RBR, it is a cationic dye and can be represented as Dye"+Cl− in aqueous solution. In this study, the drastic reduction of MG biosorption capacity was obtained after the modification of phosphate groups (T3), which indicated that the Dye" preferred to bind with the phosphate groups owing to the electrostatic attraction. The reduction caused by modification of carboxyl (T2) groups may be due to the similar mechanism. The carboxyl groups on aerobic granules may be the proper binding sites for MG, which was consistent with the results obtained from El–Hilw's research (El-Hilw, 1999). Furthermore, after amine group modification (T1), the biosorption was sharply decreased, which demonstrated that the amine groups also could interact with the Dye"+. However, the interaction between Dye" and −NH3 was not electrostatic attraction and there may be other mechanisms in MG biosorption. After removal of the lipid fractions by benzene (T5) and acetone (T6), the biosorption capacity was slightly enhanced, indicating that the lipid played little roles in MG biosorption on aerobic granules.

4. Conclusions

The contribution of residual sludge to dyes biosorption by aerobic granules was always greater than that of EPS. The biosorption mechanisms were dependent on the dyes' chemical structures and functional groups on biosorbent. The lipid and phosphate groups may be the major binding sites for KN−R biosorption. The functional groups studied could all be important binding sites for CR biosorption. However, the carboxyl and amine groups may not play roles in RBR biosorption, the lipid fractions could be the major binding sites. For MG, the phosphate groups gave the largest contribution, followed by amine and carboxyl groups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.08.119.

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