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Dye biosorption sites in *Aspergillus niger*

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Abstract

Aspergillus niger is capable of removing dyes from an aqueous solution. In the study, the roles played by three major functional groups: carboxyl, amino and phosphate, and the lipid fraction in the biomass of *A. niger* in biosorption of four dyes, Basic Blue 9, Acid Blue 29, Congo Red and Disperse Red 1, were investigated. These functional groups in *A. niger* were chemically modified individually to determine their contribution to the biosorption of dyes. It was found that biosorption of dyes was influenced by the functional groups in the fungal biomass and the chemical structure of the dyes.

Keywords: *Aspergillus niger*; Biosorption; Functional groups; Basic Blue 9; Acid Blue 29; Congo Red; Disperse Red 1

1. Introduction

Dye wastewaters discharged from textile and dyestuff industries have to be treated due to their impacts on water bodies and particularly growing public concern over their toxicity and carcinogenicity (Banat et al., 1996). It has been rather difficult to treat dye wastewaters by conventional biological and physical–chemical processes because of the complex molecular structure of the dyes. Therefore, innovative treatment technologies are being investigated.

Decolorization of dye wastewater by fungal biomass (live and dead) is the subject of many studies reviewed in a recent paper (Fu and Viraraghavan, 2001a). Only limited information is available on interactions between dead fungal biomass and a variety of dyes with complex molecular structures.

The contribution of various functional groups such as carboxyl, amino, phosphate and sulfonate in biosorption of heavy metals has been examined in various studies on biosorption sites of heavy metals in fungal biomass (Akthar et al., 1996; Tobin et al., 1990; Fourest and Volesky, 1996; Kapoor and Viraraghavan, 1997). The roles played by these functional groups in biosorption of dyes have not been studied to the best of our knowledge. The objective of this study was to investigate the role of carboxyl, amino and phosphate functional groups, and lipids present in the fungal biomass, *A. niger*, in biosorption of four different dyes, Basic Blue 9, Acid Blue 29, Congo Red and Disperse Red 1.

2. Methods

2.1. Dye solution preparation

The dyes used in this study are listed in Table 1. Their chemical structures are shown in Fig. 1. The dyes were supplied by Sigma Chemical, St Louis, MO, USA. Dye solutions were prepared by dissolving accurately weighed dyes in distilled water at a concentration of 50 mg/l. The initial pH of each dye solution was adjusted to its effective pH obtained from the results of batch studies (Table 2). Details are available in an earlier paper (Fu and Viraraghavan, 2000).

2.2. Fungal biomass preparation

The *A. niger* strain used in this study was obtained from the American Type Culture Collection, Rockville, MD, USA (ATCC#11414). The culturing procedure, growth medium and different pretreatments have been described previously (Fu and Viraraghavan, 2000, 2001b). The fungal pellicles were separated by filtering the growth medium through a 150 μm sieve and washed with generous amounts of deionized water. They were pretreated by various methods found effective (Table 2) depending on the four dyes. The effectively pretreated biomasses were

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Table 1
Details of the four dyes

Name of the dye	Classification of the dye	C.I.	FW	λ_{max} (nm)
Basic Blue 9	Cationic thiazine	52015	373.9	660
Acid Blue 29	Anionic disazo	20460	616.5	600
Congo Red	Anionic direct disazo	22120	696.7	500
Disperse Red 1	Nonionic monoazo	11110	314.3	450

Note: C.I. – color index; FW – formula weight; λ_{max} – maximum absorbance wavelength.

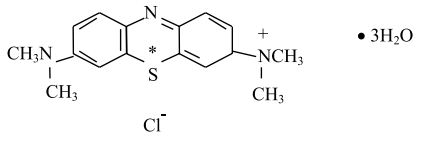
ground to powder using a mortar and a pestle. The powder with particles less than or equal to 300 μm was termed as the "raw biomass" for each of the four dyes.

2.3. Chemical modification of the biomass

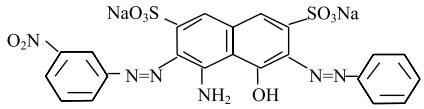
The modification to carboxyl functional groups was made by shaking at room temperature (22 ± 1 °C) 2 g (dry weight) of the raw biomass in 130 ml of anhydrous methanol (CH_3OH) and 1.2 ml of concentrated hydrochloric acid (HCl) for 6 h at 125 rpm. This treatment resulted in esterification of the carboxylic acids (Gardea-Torresdey et al., 1990; Drake et al., 1996). The general reaction scheme is

$$RCOOH + CH_3OH \xrightarrow{H^+} RCOOCH_3 + H_2O \quad (1)$$

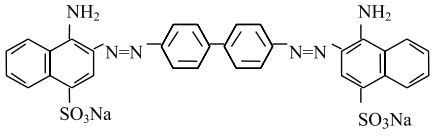
This biomass residue obtained was referred to as M1.



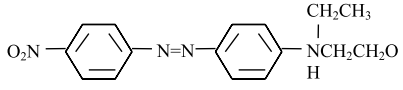
Basic Blue 9



Acid Blue 29



Congo Red



Disperse Red 1

Fig. 1. Chemical structures of the four dyes studied.

The modification to amino functional groups was made by shaking at room temperature 1 g (dry weight) of the raw biomass in 20 ml of formaldehyde (HCHO) and 40 ml of formic acid (HCOOH) for 6 h at 125 rpm. This treatment resulted in methylation of amine (Loudon, 1984). The general reaction scheme is

$$RCH_2NH_2 \xrightarrow{HCHO,HCOOH} RCH_2N(CH_3)_2 + CO_2 + H_2O \quad (2)$$

This biomass residue obtained was referred to as M2.

The modification to phosphate functional groups was made by heating 1 g (dry weight) of the raw biomass under reflux with 40 ml of triethyl phosphite ($(C_2H_5O)_3$ P) and 30 ml of nitromethane (CH_3NO_2) for 6 h. This treatment was reported to result in esterification of the phosphate group of orthophosphoric acid (Markowska et al., 1975). This biomass residue obtained was referred to as M3.

The extraction of the lipid fraction was carried out under two conditions:

1. by heating 1 g (dry weight) of the raw biomass under reflux with 75 ml acetone for 6 h;
2. by heating 1 g (dry weight) of the raw biomass under reflux with 75 ml benzene for 6 h (Tobin et al., 1990).

The biomass residues obtained after acetone treatment was referred to as M4 and the residue after benzene treatment was referred to as M5.

2.4. Dye biosorption experiments

Dye biosorption experiments were conducted by shaking 0.2 g of the raw biomass or chemically modified biomass in 75 ml of the dye solution (dye concentration 50 mg/l) at the effective initial pH and for a period equal to the equilibrium time for the dye adsorption (see Table 2). The mixture was shaken in 125 ml conical flasks closed with PARAFILM "M" to prevent evaporative losses on a LAB-LINE® rotary shaker operating at 125 rpm. Before measurement of the dye concentration, the mixture of dye solution and fungal biomass was vacuum filtered through a 0.45 μm AcetatePlus (supported, plain) membrane filter (47 mm diameter), supplied by MSI, Westboro, MA 01581, USA. The filtrate was analyzed for dye concentration and the dye adsorbed by the biomass was calculated by material balance. Dye concentration was measured at a pH of 7.6 by a spectrophotometer (Baush and Lomb-Spectromic 21).

Table 2
Results of batch studies for the four dyes

Name of the dye	Effective pretreatment of fungal biomass	Effective initial pH	Equilibrium time (h)
Basic Blue 9	Autoclaving	6.0	30
Acid Blue 29	H_2SO_4 + autoclaving	4.0	24
Congo Red	$NaHCO_3$ + autoclaving	6.0	42
Disperse Red 1	NaOH + autoclaving	4.0	48

In biosorption experiments, blanks were run simultaneously without any adsorbent to determine the extent of dye removal by filter and glass flasks.

3. Results and discussion

3.1. Basic Blue 9

The results of biosorption of Basic Blue 9 on the raw and the chemically modified biomasses are shown in Fig. 2. None of the chemical treatments increased the biosorption capacity for Basic Blue 9 and reductions ranging from 15% to 98% were observed.

M2 caused the highest reduction (98%) in biosorption. M1 resulted in a 78% reduction in biosorption. These results indicated that the amino and carboxyl groups played important roles in the biosorption of Basic Blue 9. M3 reduced biosorption 19%, while M4 and M5 decreased biosorption by 15% and 26%, respectively.

The final pH values decreased in all cases from an initial pH of 6.0 to final pH values in the range 4.0–5.4, while the final pH increased for the raw biomass to 6.6. M2 caused the highest pH reduction (final pH = 4.0). This could be due to the release of the residual formic acid in the modified biomass.

Basic Blue 9 is a salt and dissociates in an aqueous solution to the cation which is the colored part of the salt, and the anion, Cl^-. The dye molecule can be represented by Dye^+Cl^-. The cation, Dye^+, is associated with the atoms, nitrogen or sulfur, which are the two alternate locations in the dye structure. The alternate location is shown with asterisk (*) in Fig. 1 (Aspland, 1997). Therefore, the biosorption of Basic Blue 9 on fungal biomass is due to the interaction between the Dye^+ and the fungal biomass.

In this study, the results of Basic Blue 9 biosorption on fungal biomass of *A. niger* are similar to those observed by Kapoor and Viraraghavan (1997) on heavy metal biosorption on the same strain of fungal biomass. This could suggest that the mechanisms involved in Basic Blue 9 biosorption were similar to heavy metal biosorption.

In the case of M2, formaldehyde can reversibly replace labile H atoms on –COOH and –SH groups of proteins (Davis et al., 1973). Therefore, the reduction of biosorption of Basic Blue 9 on amino modified biomass

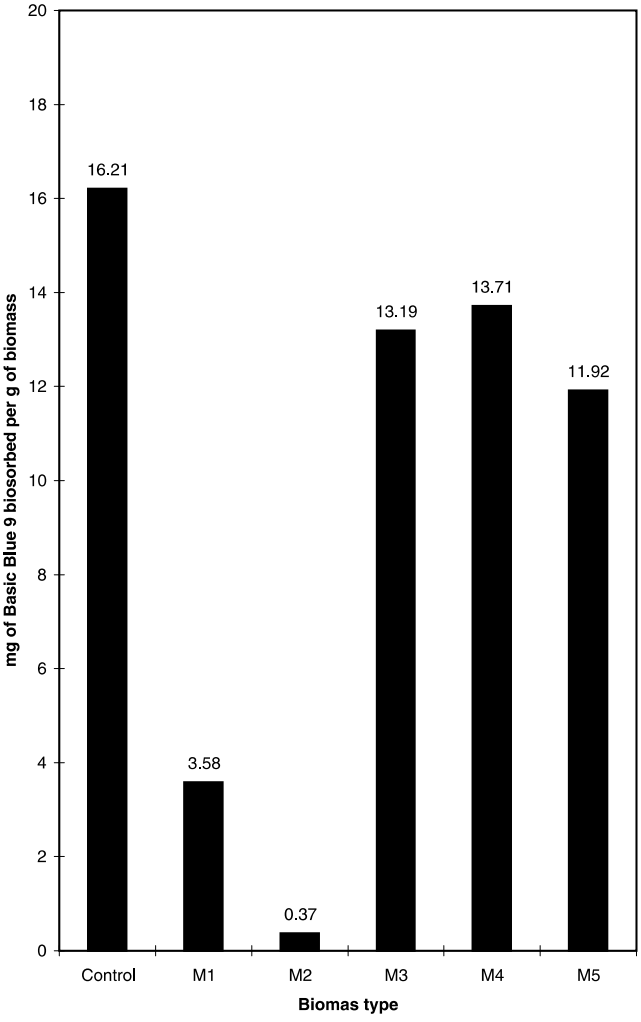


Fig. 2. Amount of Basic Blue 9 biosorbed by autoclaved biomass (control) and chemically modified biomasses of *A. niger* after contacting with 50 mg/l of dye solution.

(M2) could be attributed to amino and/or carboxyl groups.

The carboxylate anion in the fungal biomass could be a proper binding site for attracting Dye^+. The result of the study confirmed this and was consistent with the results of El-Hilw (1999).

In this study, the drastic reduction of Basic Blue 9 biosorption capacity after amino groups modification also indicated that the cation Dye^+ could prefer binding to the amine groups which could be similar to Cu^{2+}

biosorption on fungal biomass, and the interaction between the cation, Dye^+, and $R–NH_3^+$ was not electrostatic attraction but other mechanisms may be involved in Basic Blue 9 biosorption.

Because phosphate groups also exhibit a negative charge, it is expected that they should be the major binding sites for Dye^+. But the reduction of biosorption capacity for Basic Blue 9 (19%) was not significant after esterification of phosphate groups which indicated that the phosphate groups were not the major binding sites for Dye^+ of Basic Blue 9 and the mechanisms may not be electrostatic attraction only. This is not consistent with the results of Kamel (1993).

Therefore, carboxyl and amino groups could be the main binding sites on fungal biomass surface for Basic Blue 9 biosorption, while the phosphate groups and lipid fraction were less likely to be the major binding sites. The electrostatic attraction may not be the primary mechanism.

3.2. Acid Blue 29

The results of biosorption of Acid Blue 29 on the raw and the chemically modified biomasses are shown in Fig. 3. All of the chemically modified biomasses slightly increased bisorption of Acid Blue 29 and the enhancements ranging from 0.2% to 0.9% were observed.

The final pH values all slightly increased from an initial pH of 4.0 to final pH values in the range 4.2–4.5 except with the acetone treatment. The final pH decreased to 3.7 for the acetone treatment.

Acid Blue 29 has its vital substituents, sodium sulfonate groups ($–SO_3Na$), attached to the naphthalene ring which gives it the property of water solubility. In an aqueous solution, it ionizes into two sodium cations and two colored sulfonate anions. Acid Blue 29 can be written $Dye(acid)^{2-} \cdot 2Na^+$ (Aspland, 1997). Therefore, the functional groups which are charged positively on the fungal biomass surface can attract $Dye(acid)^{2-}$ and remove it from an aqueous solution.

Fu and Viraraghavan (2001b) reported that the effective pretreatment of the biomass in the case of Acid Blue 29 adsorption was H_2SO_4 plus autoclaving and suggested that H_2SO_4 pretreatment changed the negatively charged surface of the biomass to a positively charged one. It was observed that the biosorption process of Acid Blue 29 was rapid. These indicated that the mechanism of Acid Blue 29 biosorption could be electrostatic attraction.

In this study, all the modifications of different functional groups slightly increased biosorption capacity, which could indicate that modifications had no effects on Acid Blue 29 biosorption, or other functional groups could be involved in biosorption. The carboxylic acids dissociate into carboxylate anions to a small extent at the effective initial pH of 4.0. These may not be the

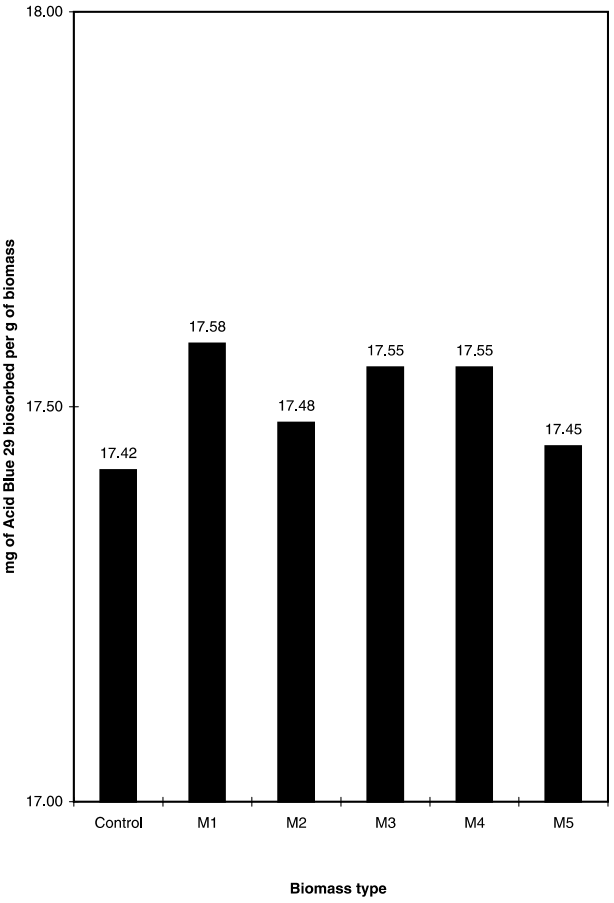


Fig. 3. Amount of dye biosorbed by H_2SO_4 pretreated biomass (control) and chemically modified biomasses of *A. niger* after contacting with dye solution containing 45.96 mg/l of Acid Blue 29.

binding sites for the $Dye(acid)^{2-}$. The phosphate groups also could not be the binding sites for $Dye(acid)^{2-}$ due to their negative charge. On the other hand, in the molecule of Acid Blue 29, there is a primary amine ($–NH_2$) attached to the naphthalene ring. The primary amine will be positively charged (NH_3^+) in the solution at the initial pH of 4.0, which would be expected to bind with negatively charged functional groups, such as carboxyl and phosphate groups. Therefore, the modifications of these groups could reduce the biosorption capacity of Acid Blue 29. However, the results did not show the reduction, but a slight increase which indicated that carboxylic acid and phosphate groups were not the binding sites for Acid Blue 29. This could be due to the position of amine in the molecule of Acid Blue 29 which might cause the weak basicity of primary amine (Loudon, 1984). The results of acetone and benzene treatment indicated that any biomass components extracted did not contribute to the biosorption of Acid Blue 29 on fungal biomass.

The amino groups in the fungal biomass will be protonated at the effective initial pH of 4.0. Meanwhile, in the process of H_2SO_4 pretreatment, the amino groups

in the fungal biomass are also protonated. Therefore, the amino groups could be the major binding sites for Acid Blue 29. In the fomadehyde–formic acid (HCHO–HCOOH) treatment for methylation of amino groups, the primary amine (R–NH2) is methylated to tertiary amine ($R–N(CH_3)_2$). The tertiary amine can be completely protonated in dilute mineral acids as follows (Loudon, 1984)

$$R–N(CH_3)_2 + H^+ \Longleftrightarrow R–N(CH_3)_2H^+ \quad (3)$$

Therefore, the tertiary amine groups would still be available as binding sites for Acid Blue 29 and could result in the slight change in biosorption capacity. This indicated that amino groups could be the binding sites in biosorption of Acid Blue 29.

3.3. Congo Red

The results of biosorption of Congo Red on the raw and the chemically modified biomasses are shown in Fig. 4. None of the chemical treatments increased the biosorption capacity and reductions ranging from 19% to 56% were observed. The lipid extraction with benzene (M5) significantly inhibited biosorption of Congo Red and caused the highest reduction (56%) in biosorption. The carboxyl groups esterification (M1) also markedly inhibited the biosorption of Congo Red and the reduction of biosorption was 50% next to the lipid extraction by benzene (M5). The phosphate groups esterification (M3) resulted in similar reduction of biosorption (50%) as the carboxyl groups modification (M1). Lipid extraction with acetone (M4) decreased biosorption to 45% less than that with benzene (M5), while the amino groups methylation (M2) reduced biosorption the least (19%).

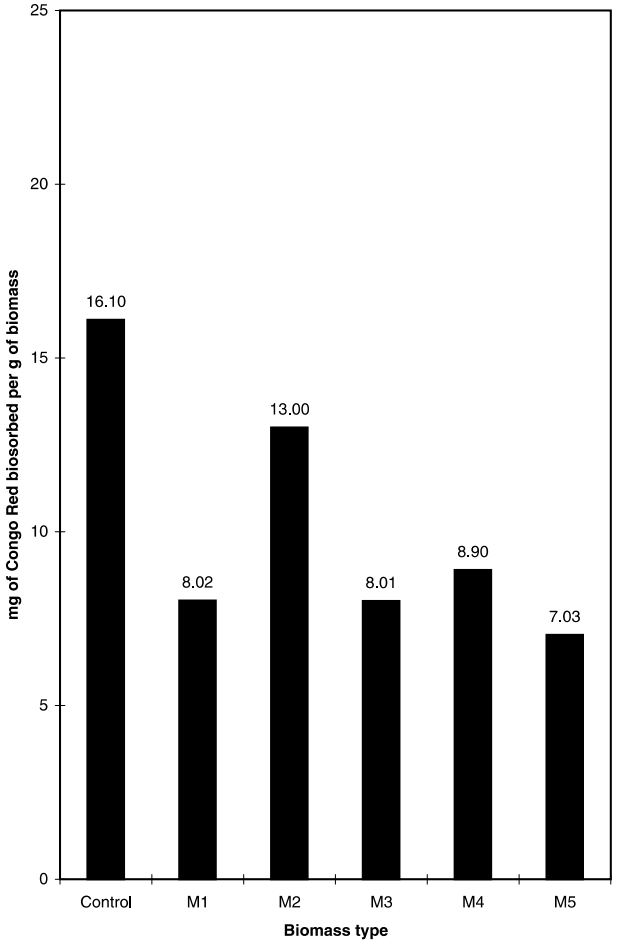


Fig. 4. Amount of dye biosorbed by $NaHCO_3$ pretreated biomass (control) and chemically modified biomasses of *A. niger* after contacting with dye solution containing 48.69 mg/l of Congo Red.

The final pH values all increased. The final pH value increased to the highest extent from an initial pH of 6.0 to a final pH of 7.1 for the carboxyl groups esterification (M1), while other modifications all enhanced pH to a range 6.6–6.7.

Congo Red is a direct dye and classified as an anionic dye. The important substituents are the two sodium sulfonate groups, $–SO_3Na$, each of which attaches to one of the two naphthalene rings and makes Congo Red water soluble. In an aqueous solution, Congo Red ionizes into two sodium cations and two colored sulfonate anions. Congo Red can be written as $Dye(direct)^{2-} \cdot 2Na^+$ (Aspland, 1997).

In this study, the results were totally different from that of Acid Blue 29 even though they are both classified as anionic dyes and have two sodium sulfonate groups. The amino groups modification resulted in the least reduction (19%) in biosorption of Congo Red. The reason would be similar to that of Acid Blue 29. After methylation, the tertiary amine groups ($–N(CH_3)_2$) are still positively charged at the initial pH of 6.0 for Congo Red. 81% of the biosorption capacity was retained after amino groups modification; this indicated that the amino groups could be the major ones contributing to Congo Red biosorption. It was observed that Congo Red biosorption capacities were all reduced by 50% after the modifications of carboxyl and phosphate groups, which indicated that these two functional groups could participate in the biosorption of Congo Red. Compared with the molecular structure of Acid Blue 29, the molecule of Congo Red has two primary amines ($–NH_2$) attached to the two naphthalene rings located at the two ends of the molecule, respectively. The two primary amines can be protonated ($–NH_3^+$) at the initial pH of 6.0 and could have stronger basicity, which could result in the attraction between the protonated amine ($–NH_3^+$) and the negatively charged functional groups, such as carboxylic acid and phosphate groups.

The treatment by acetone and benzene also decreased the Congo Red biosorption by 45–56% which indicated that lipid fraction could also participate in the biosorption of Congo Red and the mechanism could be different from electrostatic attraction. The lipid fraction

extracted by benzene seemed to affect biosorption of Congo Red more (56%) than that extracted by acetone (45%).

Therefore, in the biosorption of Congo Red, the amino group could be the major binding site, while carboxylic acid and phosphate groups as well as lipid fraction could also be important binding sites. Electrostatic attraction was the primary mechanism, while other mechanisms could also be involved in the biosorption of Congo Red.

3.4. Disperse Red 1

The results of biosorption of Disperse Red 1 on the raw and the chemically modified biomasses are shown in Fig. 5. The results were quite different from those of the other three dyes. Two kinds of chemical modifications significantly increased bisorption, while the other three decreased biosorption. The esterification of phosphate groups (M3) enhanced biosorption to the highest extent (109%), while the lipid extraction by benzene (M5) significantly increased biosorption (52%). The methylation of amino groups (M2) most severely inhibited biosorption (91%), while lipid extraction with acetone (M4) and esterification of carboxyl groups (M1) reduced biosorption by 55% and 28%, respectively.

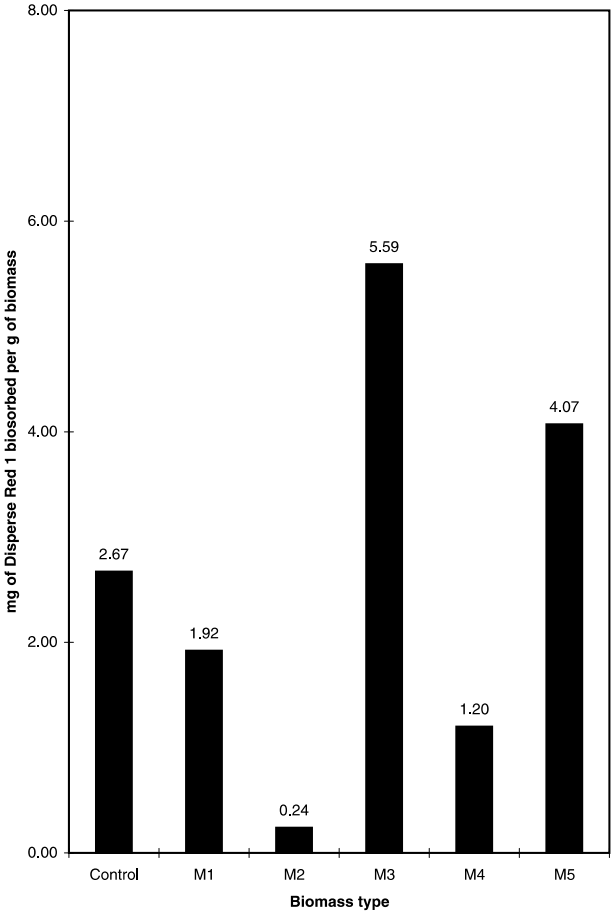


Fig. 5. Amount of dye biosorbed by NaOH pretreated biomass (control) and chemically modified biomasses of *A. niger* after contacting with dye solution containing 13.50 mg/l of Disperse Red 1.

The final pH increased in all cases. The final pH after biosorption on the raw biomass increased to the highest extent from an initial pH of 4.0 to a final pH of 5.8; while the modifications increased the pH to 4.2–4.6.

Disperse Red 1 is a nonionic dye. Due to nonionic solubilizing groups, it has a very low solubility in water at room temperature (Aspland, 1997). The left side of the molecule of Disperse Red 1 contains a nitro ($-NO_2$) group in which nitrogen is positively charged (Loudon, 1984) tending to attract electrons. The right side contains tertiary amine $-N(C_2H_5)(C_2H_4OH)$ tending to donate electrons (Aspland, 1997).

In the previous studies conducted by the authors, the effective pretreatment for biosorption of Disperse Red 1 was NaOH plus autoclaving and the fungal biomass pretreated by NaOH was negatively charged. The effective initial pH was 4.0.

In this study, the modification of phosphate groups increased (110%) biosorption of Disperse Red 1 showing that esterification of phosphate groups might form new binding sites for Disperse Red 1. So phosphate groups could be the potential binding sites. The removal of the lipid fraction through extraction by benzene also increased (52%) biosorption of Disperse Red 1 that showed that this lipid fraction inhibited the biosorption of Disperse Red 1 on unmodified fungal biomass (raw biomass). The modification of amino groups drastically reduced (91%) the biosorption capacity of Disperse Red 1 that indicated that these groups could be major binding sites for Disperse Red 1. Before and after modification, amino groups are all protonated amine in the effective initial pH of 4.0 (Loudon, 1984). In the molecule of Disperse Red 1, the tertiary amine was also in the protonated state in the initial pH of 4.0. Therefore, the amino groups in fungal biomass could not attach to the positions of $-NO_2$ and $-N(C_2H_5)(C_2H_4OH)$ in the dye molecule of Disperse Red 1 and should be other positions, which indicated that the mechanisms for the biosorption of Disperse Red 1 might not be the physical adsorption only and may involve chemical adsorption. The modification of carboxyl acid groups reduced biosorption capacity of Disperse Red 1 to a relative low extent (28%). This could be because the $-NO_2$ and $-N(C_2H_5)(C_2H_4OH)$ in the molecules are all positively charged. So the carboxylate anions in the fungal biomass can attract these groups in the dye molecules. So the carboxyl groups could be the binding sites but not the main sites. Lipid fraction extracted by acetone also significantly reduced (55%) biosorption of Disperse Red 1 which indicated that this lipid fraction could be also the binding sites.

Therefore, in the biosorption of Disperse Red 1, amino groups and lipid fraction extracted by acetone could be the major binding sites; carboxylic acid groups could be the binding sites, but not the major one. The phosphate groups could be the potential binding sites which were effective after esterfication. The lipid fraction extracted by benzene inhibited the biosorption significantly. The mechanism of Disperse Red 1 biosorption could include both physical and chemical adsorption.

4. Conclusions

The mechanisms of the biosorption of the dyes are dependent upon the chemical structure of the dyes and the functional groups in the dye molecules. The different functional groups in the fungal biomass of *A. niger* play different roles in biosorption of different dyes.

In biosorption of Basic Blue 9 on fungal biomass, *A. niger*, the mechanisms are similar to the heavy metal biosorption. The carboxyl and amino groups are the main binding sites, while the phosphate group and the lipid fraction are not the major binding sites.

In biosorption of Acid Blue 29 on the fungal biomass, electrostatic attraction could be the primary mechanism. The amino groups could be the major biosorption sites while the carboxylic acid, phosphate groups and the lipid fraction may not form the binding sites.

In biosorption of Congo Red on the fungal biomass, the mechanism is different from that of Acid Blue 29. The amino, carboxylic acid, phosphate groups and the lipid fraction could all be important binding sites. In addition to the electrostatic attraction, other mechanisms may be involved in biosorption of Congo Red.

In biosorption of Disperse Red 1 on fungal biomass, the mechanism is different from the other three ionic dyes. The electrostatic attraction could be only part of the whole mechanism. Physical and chemical adsorption could occur at the same time. The amino groups and the lipid fraction extracted by acetone are the major binding sites, while the carboxylic acid groups could be minor binding sites. The phosphate groups could be potential binding sites.

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