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ORIGINAL PAPER

Enhancement of nickel biosorption on fungal biomass by enzymatic and alkali pretreatments

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Abstract Enzymatic and alkali pretreatments were employed to improve nickel biosorption capacity of *Rhizomucor pusillus* biomass. Pretreatment with 0.002–80 g l^{-1} NaOH and 0.0001–0.1 Anson Unit (AU) g^{-1} protease enhanced the biosorption capacity of fungal biomass. Increasing the concentration of NaOH from 0.002 to 5 g l^{-1} improved nickel removal from 93.2 to 100.0 % while untreated biomass showed 64.6 % Ni(II) removal. Pretreatment with higher concentrations of NaOH, 5–80 g l^{-1} resulted in nearly complete removal of nickel ions. Pretreatment of the biomass with 0.0001 AU g^{-1} protease improved the nickel removal to over 91 %, while increasing the enzyme loading to 0.1 AU g^{-1} improved the removal to 93 %. Untreated biomass removed 78.4, 63.0, and 96.3 % of chromium, copper, and lead ions, respectively, from a mixture solution of the ions. Respective metal removals were increased to 100, 98.9, and 100 % after pretreatment with 0.2 g l^{-1} NaOH solution and to 87.8, 86.7, and 100 % after the enzymatic pretreatment with 0.1 AU g^{-1} protease. Scanning electron microscopy analysis indicated that alkali and enzymatic pretreatments enhanced the porosity of the biomass. Furthermore, compositional analysis showed that both of the pretreatments removed a major part of fungal proteins (2.1–95.8 % removal). Glucosamine, *N*-acetyl glucosamine, and phosphates were the major ingredients of the pretreated biomass.

Keywords Alkali pretreatment · Biosorption · Enzymatic pretreatment · Heavy metal removal · *Rhizomucor pusillus*

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Introduction

Heavy metals at concentrations higher than the limits defined by World Health Organizations may create serious problems for human health and the environment. Nickel (Ni) is one of the heavy metals which is present in wastewater of different industries and has been reported to cause lung, larynx, and prostate cancers even at very low concentrations (Ahlaya et al. 2003). Nickel concentration in plating industry may vary between 2 and 900 mg l^{-1}. Effluents of other industries such as paint and ink formulation and copper sulfate production contain 0–40 mg l^{-1} Ni (Padmavathy 2008). Different methods such as ion-exchange, reverse osmosis, chemical precipitation, and membrane filtration are only effective for removal of heavy metals at high concentrations (Fu and Wang 2011). However, at low levels of heavy metals, these methods do not perform well and are not economically feasible (Wang and Chen 2009). In contrast, biological treatments are promising alternatives for treatment of such dilute wastewaters (Ahlaya et al. 2003).

Living and dead biomass of different microorganisms, e.g., algal, fungal, and bacterial biomass, are among the biological adsorbents for heavy metal removal (Verma et al. 2008; Wang and Chen 2006, 2009). Although living cells usually show higher biosorption capacity than dead cells, maintaining of the cells viability during the

removal process dictates some major limitations such as the need to conduct the process at cell-favored living conditions (Bai and Abraham 2002). In contrast, dead cells, especially when they pretreated by physical or chemical methods, are utilized more easily in the process (Butter et al. 1998). Chemical treatments with alkali, acids, and detergents are among the most effective pretreatments of biomass for improvement of the biosorption (Ashraf 2010; Kapoor and Viraraghavan 1995; Kapoor et al. 1999).

Fungal biomass has been effectively used for removal of heavy metals with low concentrations. Among various fungal strains, those who contain chitin and chitosan typically show higher biosorption properties. Although chitin is one of the major ingredients of the cell wall of many fungal strains, between different members of fungal kingdom, zygomycetes fungi are the only group having chitosan in their cell wall. *Rhizomucor pusillus*, which is available in some traditional Asian food such as Tempe, is safe for human, grows faster, and produce higher biomass compared with other genera of zygomycetes fungi. Furthermore, its cell wall contains high concentration of chitosan (Lang 2013; Zamani 2010). Although performance of a number of zygomycetes fungi has been evaluated in some studies, to our knowledge, there is no report investigating the heavy metal removal by this fungus (Lo et al. 1999; Yan and Viraraghavan 2000; Yan 2001; Yan and Viraraghavan 2003; Zamani et al. 2010; Javanbakht et al. 2011). Recently, zygomycetes fungi are receiving growing attentions because of their ability for growing on various low-cost feedstocks such as lignocellulosic hydrolysates and production of different value-added biological products such as ethanol (Karimi et al. 2006). Additionally, though the impacts of different chemical pretreatments on metal removal by different fungi have been previously studied, no report is available studying the effect of enzymatic pretreatment. Moreover, limited reports have been connected compositional and structural modifications created during the alkali treatments to metal adsorption capacities of fungal biomasses.

The objective of the current study was improvement of heavy metal adsorption capacity of the biomass of *R. pusillus* by enzymatic and alkali pretreatments. Furthermore, studying the morphological and chemical changes of biomass during the course of pretreatments was among the goals of this work. This work was performed at Department of Chemical Engineering, Isfahan University of Technology during 2012–2013.

Materials and methods

Microorganism and cultivation

Rhizomucor pusillus CCUG 11292 (Culture Collection of University of Göteborg, Sweden) was cultivated on a xylose-rich spent sulfite liquor (from Domsjo AB paper pulp plant, Sweden) in a 60-l air-lift fermenter for 48 h. Twice a day 85 % of the working volume was replaced by the fresh liquor. The fungal mycelium was harvested on a metal screen, washed with water, and freeze-dried. The dried biomass was stored at room temperature until use.

Alkali pretreatment of the fungal biomass

Freeze-dried fungal biomass (1 g) was mixed with 30 ml NaOH solution with different concentrations (0.002–80 g l^{-1}) and autoclaved at 120 °C for 20 min. Subsequently, solid residue of the pretreated biomass, referred to as alkali insoluble material (AIM), was separated by centrifugation (3,400×*g* for 10 min). AIM was washed with water until neutral pH and freeze-dried.

Enzymatic pretreatment of the fungal biomass

Freeze-dried biomass (1 g) was mixed with 100 ml deionized water, and pH was adjusted to 6.5 (optimal pH for neutrase activity). Then, 0.1–100 µl of Neutrase 0.8 l (Novozyme, Denmark) was added to the mixture. As reported by the supplier, the enzyme is a neutral protease produced by *Bacillus amyloliquefaciens* that hydrolyzes internal peptide bonds with activity of 0.8 Anson Unit (AU) per gram of the enzyme. Enzymatic hydrolysis was performed at 50 °C and 50 rpm for 24 h. The solid residue of the pretreated biomass was separated by centrifugation (3,400×*g* for 10 min), washed twice with water, and freeze-dried.

Heavy metal removal by (pretreated) biomass

Freeze-dried biomass and its pretreated derivatives were added to 20 ml of a 12.5 ppm Ni (II) solution and mixed

for 20 h at 31 ± 2 °C and 130 rpm to reach the adsorption equilibrium. The mixtures were then centrifuged (3,400×*g* for 15 min), and concentration of Ni(II) in the supernatant was analyzed with an atomic absorption spectrophotometer (Rayleigh, Model AA68, HCL Lamp, China).

The best pretreated biomasses, in terms of nickel removal, as well as untreated biomass, were subjected to similar experiments for removal of chromium, Cr(III), copper, Cu (I), and lead, Pb(II), and concentrations of these heavy metals in the solutions were measured after 20 h.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used for investigation of changes in the morphology of biomass after the pretreatments. Untreated and treated biomasses were coated with gold and subjected to scanning electron microscopy (XL30, Philips, Netherlands). The images were taken by a secondary electron detector in high vacuum mode at 15–20 kV.

Determination of proteins

Biuret method was used for measurement of protein contents of the fungal biomass and its derivatives. An amount of 0.01 g of different biomass derivatives was mixed with 3 ml of NaOH 1 M, boiled for 10 min, and immediately cooled in an ice bath. Then, 1 ml of 2.5 % $CuSO_4 \cdot 5H_2O$ was added and mixed for 5 min. The mixtures were finally centrifuged (3,400×*g* for 4 min), and the absorbance at 555 nm was recorded after 15 min (Verduyn et al. 1990).

Determination of glucosamine and *N*-acetyl glucosamine

Glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) were measured according to a method presented by Mohammadi et al. (2012). Different biomass preparations were subjected to a two-step sulfuric acid followed by a one-step nitrous acid hydrolyses to convert chitin and chitosan to anhydromannose and acetic acid. Afterward, these products were analyzed by HPLC. Moles of *N*-acetyl glucosamine were considered to be equal to the moles of acetic acid where moles of glucosamine were calculated by subtraction of moles of anhydromannose and acetic acid.

Determination of phosphates

Ammonium molybdate spectrometric method, according to European standard ISO 6878 (2005), was used for measuring the phosphate contents of pretreated biomass. A dark blue color complex was created by mixing of sulfuric acid hydrolyzed pretreated biomasses with an acid molybdate reagent and ascorbic acid, and the absorbance at 880 nm was measured (Shimadzu spectrophotometer, Model 240, Japan).

Determination of heavy metals

Atomic absorption spectrometer (Rayleigh, Model AA68, HCL Lamp, China) was used for measuring the metal ions concentration.

The amount of absorbed metal ions was calculated according to the following equation:

$$q_e = \frac{(C_0 - C_e)V}{W} \quad (1)$$

where W is the weight of (pretreated) biomass, V is the volume of the metal solution, and C_0 and C_e are initial and final concentration of metal ions (mg l^{-1}), respectively.

All experiments were performed at least in duplicate, and standard deviations of the replications were <4 %.

Results and discussion

Effect of alkali pretreatment on composition and morphology of the fungal biomass

Biomass of the fungus *R. pusillus* was pretreated with 0.002–80 g l^{-1} NaOH solution. The yield of alkali insoluble material of biomass (AIM) was significantly decreased by increasing NaOH concentration (from 0.52 to 0.09 g/g) (Table 1). As reported in Table 1, proteins made 47 % of the untreated biomass. The major ingredient of AIM prepared using 0.002–2 g l^{-1} NaOH was

Table 1 Yield and composition of pretreated biomass (AIM) after alkali treatment

NaOH (g l^{-1})	AIM (g g^{-1} biomass)	AIM composition (g g^{-1} AIM)			
		GlcN	GlcNAc	Phosphate	Protein[a]
0.002	0.52 ± 0.00	0.19 ± 0.01	0.04 ± 0.00	0.06 ± 0.00	0.38 ± 0.01
0.02	0.42 ± 0.00	0.2 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.41 ± 0.01
0.2	0.40 ± 0.00	0.2 ± 0.09	0.02 ± 0.02	0.06 ± 0.01	0.47 ± 0.07
2	0.30 ± 0.00	0.2 ± 0.00	0.07 ± 0.00	0.09 ± 0.01	0.30 ± 0.01
5	0.14 ± 0.00	0.31 ± 0.01	0.16 ± 0.01	0.09 ± 0.01	0.06 ± 0.01
10	0.13 ± 0.01	0.38 ± 0.01	0.18 ± 0.01	0.07 ± 0.01	0.04 ± 0.00
12.5	0.12 ± 0.00	0.41 ± 0.00	0.19 ± 0.00	0.06 ± 0.01	0.04 ± 0.00
15	0.10 ± 0.00	0.29 ± 0.04	0.18 ± 0.04	0.05 ± 0.01	0.02 ± 0.02
17.5	0.11 ± 0.00	0.37 ± 0.00	0.18 ± 0.00	0.05 ± 0.00	0.03 ± 0.00
80	0.09 ± 0.01	0.37 ± 0.01	0.22 ± 0.01	0.05 ± 0.01	0.02 ± 0.00

[a] Protein content of untreated biomass was 0.48 ± 0.04 g g^{-1}

protein (30.0–37.8 %), while GlcN and GlcNAc, respectively, made an average of 19.6 and 4.5 % of AIMs prepared under these conditions.

At NaOH concentrations above 2 g l^{-1}, however, proteins were the minor constituents of the AIM (<6.5 %), while GlcN and GlcNAc became dominant components with contribution of 31.3–40.6 % and 16.3–18.9 %, respectively. This is in the line of previous reports about cell wall of zygomycetes fungi (Mohammadi et al. 2012; Zamani 2010; Zamani et al. 2008).

By increasing the NaOH concentration from 5 to 17.5 g l^{-1}, the yield of AIM was continuously decreased from 13.6 to 10.7 %. In contrast, at these conditions, significant changes in composition of AIM did not occur. The AIM prepared using 80 g l^{-1} NaOH had a yield of 8.9 %, and a comparable composition to that of AIMs prepared using 5.0–17.5 % g l^{-1} NaOH.

At all alkali concentrations, phosphates were another important ingredient which appeared at lower concentrations (5.0–9.2 %). The highest phosphate contents (7.3–9.2 %) were belonged to AIMs prepared using 2–10 g l^{-1} NaOH.

Scanning electron microscopy was used to investigate the effect of alkali treatment on morphology of the fungal biomass. As shown in Fig. 2a, freeze-dried biomass had a porous sponge-like structure. Alkali treatment using 0.002 g l^{-1} NaOH created a more swollen structure (Fig. 2b). Higher concentration (10 g l^{-1}), however, considerably changed the morphology of the biomass and formed a sheet-like structure (Fig. 2c).

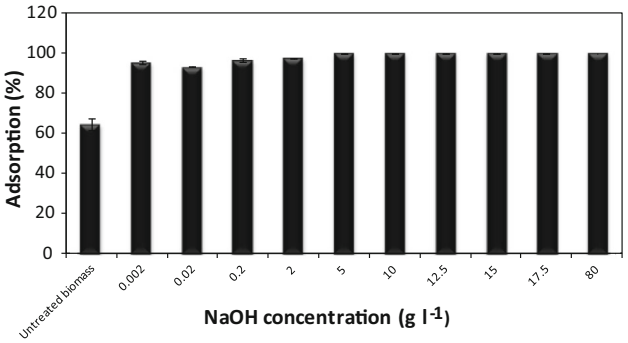
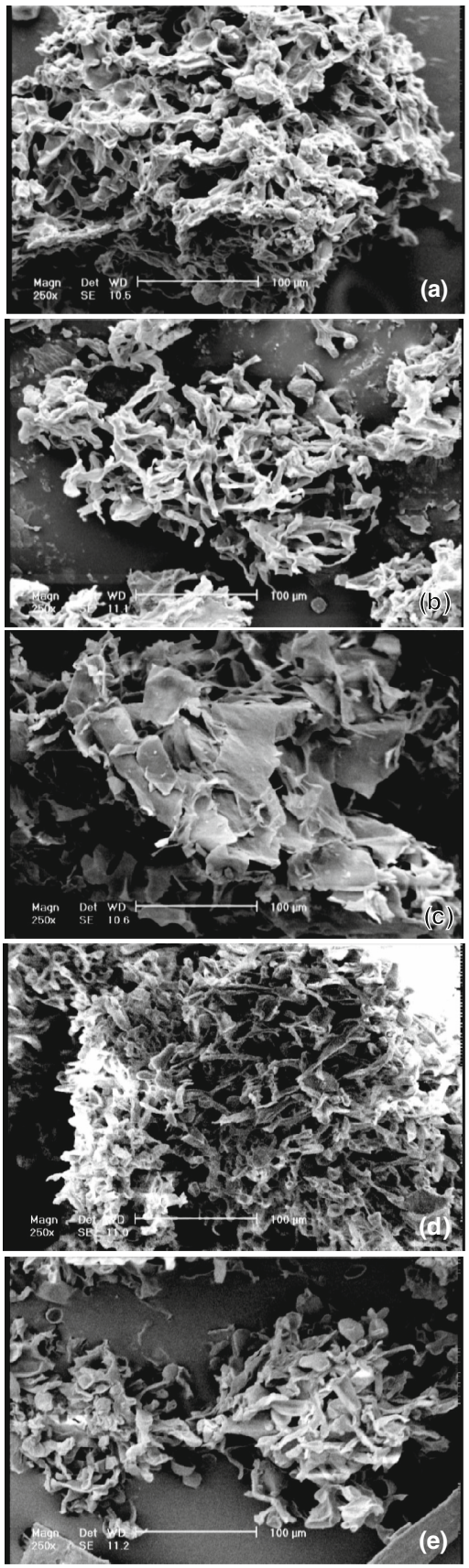


Fig. 1 Ni(II) removal by the fungal biomass chemically pretreated at 120 °C for 20 min at different sodium hydroxide concentrations

Effect of the alkali pretreatment on biosorption of Ni(II) by the fungal biomass

Untreated and alkali pretreated freeze-dried fungal biomasses were used to remove Ni(II) from aqueous solutions. Freeze-dried biomass, without alkali pretreatment, removed 64.6 % of Ni(II) after 20 h. Pretreatment of biomass with very dilute alkali solution (0.002 g l^{-1}) enhanced the removal of this ion to 95.4 % (Fig. 1). Gradual increase in concentration of NaOH up to 80 g l^{-1} was accompanied with slight continuous enhancement of the metal removal. At NaOH concentrations higher than 5 g l^{-1}, however, the pretreated biomass removed nearly all Ni(II) from the solution. Considering the yield of pretreated biomass and protein concentrations (Table 1), obviously, 58–81 % of the



◀**Fig. 2** SEM images of untreated biomass (**a**), biomass pretreated with 0.002 (**b**) and 10 (**c**) g l^{-1} NaOH, and biomass pretreated with 0.0001 (**d**) and 0.01 (**e**) AU g^{-1} protease

initial fungal proteins were removed through treatment with 0.002–2 g l^{-1} NaOH. Expectedly, higher protein removals were achieved at higher NaOH concentrations. Since the adsorption capacity of biomass pretreated with 0.002 g l^{-1} NaOH was significantly higher than that of untreated biomass (Fig. 1), probably, protein removal has been accompanied with creation of free binding sites on the surface of the biomass for metal biosorption. Additionally, as proved by SEM images, enhancement of metal removal maybe related to increase of porosity of biomass and as a result enlargement of accessible surface area for the metal binding. Moreover, alkalization of chitin and chitosan (polymers of glucosamine and *N*-acetyl glucosamine) during the course of pretreatment maybe is another factor enhancing the metal removal. Alkalization of these biopolymers usually results in activation of some functional groups and enhancement of their reactivity (Zamani and Taherzadeh 2012). Alkali pretreatment of biomass of other zygomycetes fungi for improving metal removal has been reported in some studies. Javanbakht et al. (2011), Bai and Abraham (2002), and Yan and Viraraghavan (2000, 2003) improved metal adsorption capacity of biomass of *Mucor indicus* and *Rhizopus oryzae* by employing alkali pretreatments (Table 2).

Effect of the enzymatic pretreatment on composition and morphology of the fungal biomass

Enzymatic pretreatment of biomass using 0.0001–0.1 AU g^{-1} protease eliminated 53.8–68.4 % of biomass as soluble materials, and the amount of dissolved materials was enhanced by increasing the dose of enzyme. SEM

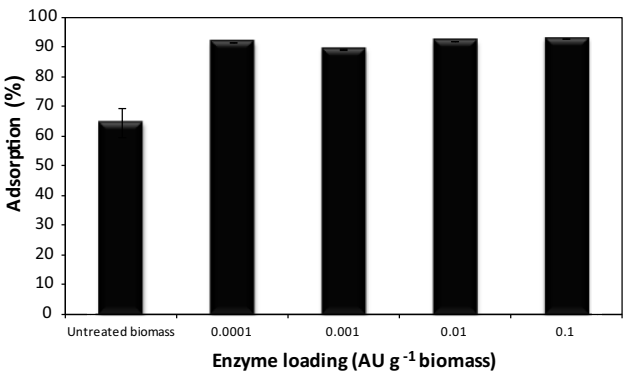


Fig. 3 Ni(II) removal by the fungal biomass enzymatically pretreated at 50 °C for 24 h at different protease loadings

Table 2 Yield and composition of pretreated biomass (AIM) after enzymatic pretreatment

Enzyme loading (AU g^{-1})	AIM (g g^{-1} biomass)	Pretreated biomass composition (g g^{-1} Biomass)			
		GlcN	GlcNAc	Phosphate	Protein
0.0001	0.46	0.14 ± 0.04	0.11 ± 0.00	0.07 ± 0.00	0.29 ± 0.03
0.001	0.33	0.08 ± 0.02	0.21 ± 0.00	0.08 ± 0.00	0.25 ± 0.03
0.01	0.32	0.08 ± 0.00	0.18 ± 0.00	0.09 ± 0.00	0.25 ± 0.03
0.1	0.32	0.09 ± 0.03	0.19 ± 0.2	0.09 ± 0.0	0.23 ± 0.01

analysis (Fig. 2d, c) indicated that enzymatic pretreatment enhanced the porosity of biomass, though it did not change the type of morphology, i.e., sponge-like structure. The yields of pretreated biomasses were 31.6–46.2 % of the initial fungal biomass, and their main ingredients were proteins (23.4–29.1 %), GlcN (7.6–14.5 %), GlcNAc (11.5–21.5 %), and phosphates (7.1–8.6 %).

Effect of the enzymatic pretreatment on biosorption of Ni(II) by the fungal biomass

Although metal binding capacity of the biomass was enhanced upon pretreatment with dilute NaOH solutions, enzymatic pretreatment was more interested because of milder process conditions and, therefore, lower energy consumptions. In this study, for the first time metal binding capacity of the fungal biomass was enhanced by an enzymatic pretreatment, and the results were compared with that of a more conventional pretreatment, i.e., alkali pretreatment. To investigate the effect of enzyme loading on the nickel adsorption, various amounts of protease were used for pretreatment of biomass. Fig. 3 illustrates the effect of enzymatic pretreatment on biosorption of nickel. Untreated biomass removed 64.6 % of Ni(II) from aqueous solution. The metal removal was enhanced to 91.8, 89.4, 92.1, and 92.9 % after pretreatment with 0.0001, 0.001, 0.01, and 0.1 AU g^{-1} protease, respectively. Maximum nickel removal was belonged to the biomass pretreated with 0.1 AU g^{-1} protease. Pretreatment with an enzyme loading as low as 0.0001 AU g^{-1} biomass resulted in removal of 72 % of the initial fungal proteins and considerably enhanced the metal removal. Similar to alkali pretreatment at low concentrations, enzymatic pretreatment increased the porosity of biomass. Removal of unwanted proteins and consequently creation of free sites for metal binding as well as enhanced porosity of biomass are probably among the most important factors facilitating the metal removal process.

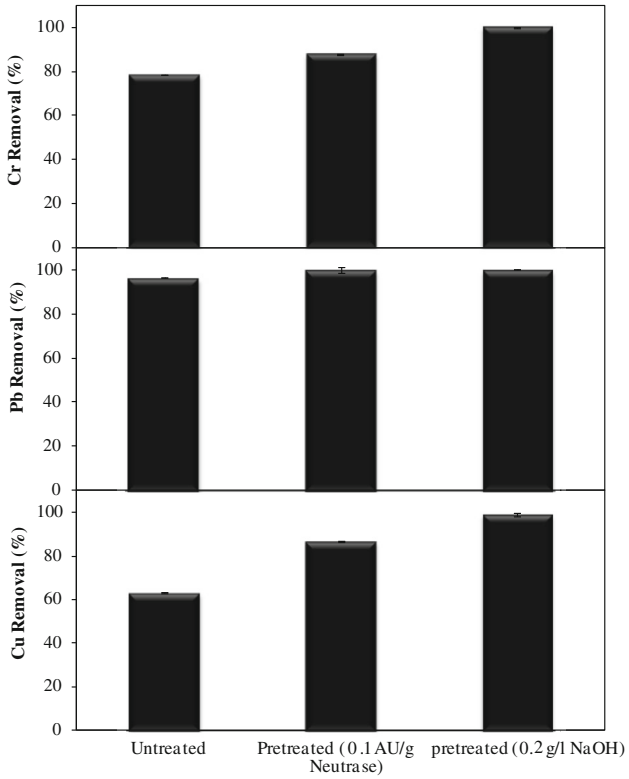


Fig. 4 Cr (**a**), Cu (**b**), and Pb (**c**) removal by the untreated biomass and the biomass treated using 0.2 g l^{-1} NaOH and 0.1 AU g^{-1} protease

Adsorption of different heavy metals by untreated and pretreated biomass

As shown in Figs. 1 and 3, the lowest enzyme loading and NaOH concentration which led to the highest nickel removal were 0.1 AU g^{-1} protease and 0.2 g l^{-1} NaOH, respectively. Therefore, removal of some other heavy metals by the pretreated biomasses was also investigated. As shown in Fig. 4, the fungal biomass exhibited 78.4 % Cr(II) removal. Enzymatic (0.1 AU g^{-1}) and alkali

($0.2\ g\ l^{-1}$) pretreatments improved the Cr(II) removal to 87.8 and over 99.9 %, respectively. Although the fungal biomass removed a high amount of Pb ion (96.3 %), its alkali and enzymatic pretreated biomass showed more than 99.9 % Pb removal. On the other hand, untreated biomass had a relatively low Cu removal (63.0 %) (Fig. 4), and enzymatic and alkali pretreatments enhanced the removal of this ion to 86.7 and 98.9 %, respectively (Fig. 4). The difference between the effects of the pretreatments on the biosorption of Ni compared to other ions may be related to difference in the mechanism of metal binding for different ions. Thus, further investigation is necessary to find the optimum conditions for higher removal of other ions.

Conclusion

Enzymatic and alkali pretreatments with very low enzyme loadings and NaOH concentrations significantly enhanced the Ni removal by the biomass of *R. pusillus.* The biomass of this fungus is a promising bio-sorbent for treatment of effluents containing Ni as well as other heavy metals.

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