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A comparative study of the adsorption of humic acid, fulvic acid and phenol onto *Bacillus subtilis* and activated sludge

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Abstract

The adsorption of humic acid and fulvic acid onto *Bacillus subtilis* cells and activated sludge biomass was studied as a function of pH and incubation time. The adsorption of humic and fulvic acids was strongly pH-dependent and followed the same trend on both surfaces, increasing in a sigmoidal way with decreasing pH over the 2–10 pH range. This behaviour is explained in terms of hydrophobic interactions between the uncharged biomass and the uncharged humic and fulvic acids. In contrast, the adsorption of phenol onto *B. subtilis* cells and activated sludge biomass showed in both cases an optimum pH at around 7.0. This optimum value may be interpreted in terms of a combination of hydrophobic interactions and hydrogen bonds between undissociated phenol and polar groups on the cell walls. Kinetic studies on the adsorption of humic acid, fulvic acid and phenol onto *B. subtilis* cells and sludge biomass pointed to a rapid uptake of the substances, with an equilibrium time of about 30 min. In all cases, the kinetic curves were acceptably fitted by non-linear regression to an exponential function, suggesting a first-order kinetic phenomenon.

The specific adsorption values collected at optimum pH revealed that with the materials used in this work both *B. subtilis* and activated sludge follow the same adsorption trend: humic > fulvic > phenol. The lower adsorption of fulvic acid as compared with humic acid may be explained in terms of its lower hydrophobicity rather than its lower molecular size. On comparing the specific adsorption values of activated sludge versus *B. subtilis*, similar but lower figures were found for the three organic compounds studied. This similar behaviour suggests that both types of biomass base their adsorption capacity on the general characteristics of the bacterial cell wall, and the lower adsorption by the sludge would be due to a lower specific area due to clustering of the cells. This is remarkable, since sludge is a heterogeneous and cheap material in comparison with cultured bacterial cells.

Keywords: Adsorption/biosorption; Humic acid; Fulvic acid; Phenol; Biomass; *Bacillus subtilis*; Activated sludge

1. Introduction

Aquatic humic substances are typical naturally occurring compounds that account for 30–80% of the dissolved organic matter in natural water [1,2]. They are straw-coloured, hydrophobic organic acids that are mainly derived from soil humus and plants. It has recently been reported [3] that 3–28% of the dissolved organic matter in effluents from wastewater treatment plants are also humic substances, which, after hydrophilic acids, appear to be the second most prevalent fraction in such effluents. Thus, humic substances represent an important recalcitrant dissolved organic matter and their removal is an important environmental issue since they react with chlorine to produce carcinogenic by-products derived from disinfection protocols [4]. Humic substances are mainly made up of humic acid and fulvic acid, and their adsorption onto microbial surfaces is in itself important and also because they may affect the transport of contaminants and their removal [5].

The biosorption of humic and fulvic acids on different microorganisms under different conditions has been studied by several authors. Thus, the adsorption of humic acid by *Bacillus subtilis* was investigated by Fein et al. [5] who found that adsorption was strongly pH-dependent, increasing with decreasing pH. Fein's group [6] also studied the above adsorption system in the presence and absence of Cd^{2+}, observing that the presence of Cd^{2+} does not affect the extent of humic acid adsorption onto the surface of *B. subtilis* cells. Frost et al. [7] studied the adsorption of fulvic acid onto *B. subtilis* and the effect

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of Cd^{2+} on such adsorption, and they found that fulvic acid adsorption decreased with increasing pH, both in the absence and presence of Cd^{2+}, exhibiting a similar behaviour to that observed for humic acid on the same bacterial surface [5,6]. In contrast, the adsorption of Cd^{2+} onto *B. subtilis* increases with increasing pH and there is no effect of fulvic acid on such adsorption of Cd^{2+} by *B. subtilis* [7]. Later, the same group [8] analysed fulvic acid fractionation upon adsorption to *B. subtilis* by high-pressure size exclusion chromatography (HPSEC) and reported a preferential adsorption of higher-molecular weight fulvic acid components, especially at high pH and low surface coverage.

The biosorption of humic and fulvic acids on activated sludge has been studied by Esparza-Soto and Westerhoff [9], who reported that humic acid was removed more efficiently than fulvic acid, and that pH and ionic strength are important parameters affecting the removal of humic substances from water. Furthermore, activated sludges have been investigated as adsorbents for other compounds such as phenol [10–13], chlorinated phenols [14,15], and dyes [16,17] for removing these hazardous compounds from water by means of a natural and available adsorbent.

Although biosorption of humic substances onto *B. subtilis* and activated sludge has been addressed previously, there is a lack of studies that quantitatively compare the adsorption of cheap activated sludge biomass and the more expensive biomass of isolated cells of some particular microorganism. Thus, the aim of this work was to carry out such a comparative study of the capacity of *B. subtilis* cells and activated sludge biomass to adsorb humic and fulvic acid under similar experimental conditions. We also studied the adsorption of phenol, as a reference compound, onto the same adsorbents.

2. Materials and methods

2.1. Chemicals

Humic acid, sodium salt, was purchased from Aldrich Chemical. It was extracted from waters draining from an open pit mine in Oberhessen, Germany, and it has been characterized chemically by Ochs et al. [18]. The fulvic acid used was Standard Suwannee River Fulvic Acid from the International Humic Substances Society (IHSS). Phenol was from Merck as a 99.5% pure analysis product. Three stock solutions were used: 200 mg/L humic acid, 25 mg/L fulvic acid and 30 mg/L phenol. All solutions were prepared in 0.1 M NaCl, which was the background electrolyte used in the experiments.

2.2. Biomass preparation

B. subtilis cells (strain CECT 4522 from the Microbiological Department at Salamanca University, Spain) were initially cultured in 3 L of LB medium (bactotryptone 1%, yeast extract 0.5% and NaCl 1%) at 37 °C for 24 h in an orbital shaker at 200 rpm. Cells were removed from the nutrient medium by centrifugation (6.000 rpm, 10 min), soaked in HNO_3 (pH 1.8) during 30 min and then centrifuged (6.000 rpm, 10 min). Following this, cells were rinsed five times in 0.1 M NaCl (the electrolyte used in the experiments) and centrifuged (6.000 rpm, 10 min).

The activated sludge biomass was collected from the Water Treatment Plant of the City of Salamanca at the effluent from the sludge reactor. At the laboratory, the biomass was washed following the same procedure described above for *B. subtilis* cells.

The washing protocol included HNO_3 soaking, in accordance with Fein et al. [6], to remove contaminating cations from the bacterial surfaces without significantly altering the cell wall structure. In all cases, the biomass concentration was expressed as grams of wet weight per litre.

2.3. Batch adsorption experiments

Experiments were conducted using a batch procedure in which a known concentration of humic acid, fulvic acid, or phenol was placed in contact with a known concentration of *B. subtilis* or activated sludge, all compounds being suspended in 0.1 M NaCl medium as background electrolyte. The concentrations chosen for the compounds were 100 mg/L for humic acid, 12.5 mg/L for fulvic acid and 15.0 mg/L for phenol. The higher value for humic acid was used to take into account the precipitation of humic acid against the full solubility of fulvic acid and the different origins and qualities of both materials. In all assays, the concentrations of *B. subtilis* cells and activated sludge biomass were 10 and 25 g/L, respectively. The higher value for sludges was chosen to compensate the expected lower adsorption of sludges. The pH of each suspension was adjusted to the desired value using HCl or NaOH at the appropriate concentration. Pyrex flasks were placed in an orbital shaker at 100 rpm over the desired incubation time. In each case, a blank was prepared using the same experimental conditions and biomass concentration, but in the absence of the compound being assayed. These blanks were used to evaluate the absorption background to be subtracted in the UV–vis spectrophotometric measurements of the substances. An incubation time between 120 and 240 min was considered sufficient for adsorption equilibrium to be reached in the pH studies. In the case of humic acid, special care was taken to measure, on one hand, the fraction of precipitated substance due to a simple pH effect and, on the other, the fraction of substance really adsorbed. For kinetic experiments, samples were taken at different times between 0 and 240 min in order to follow the kinetic adsorption curves.

2.4. Analytic methods

After the selected incubation time, solutions were centrifuged for 3 min at 4000 rpm to separate the cells and then filtered through 0.45 μm Nylon membrane filters. Finally, the pH value of the solution was measured again and taken as the true value. After filtration, 27 mL of sample was rendered basic with 3 mL of 1 M NaOH to ensure that the humic acid was completely soluble and to attain the alkaline pH used in the calibration curves.

For *B. subtilis* adsorption studies, both humic and fulvic acids were measured at 450 nm [5,7], while phenol was measured at

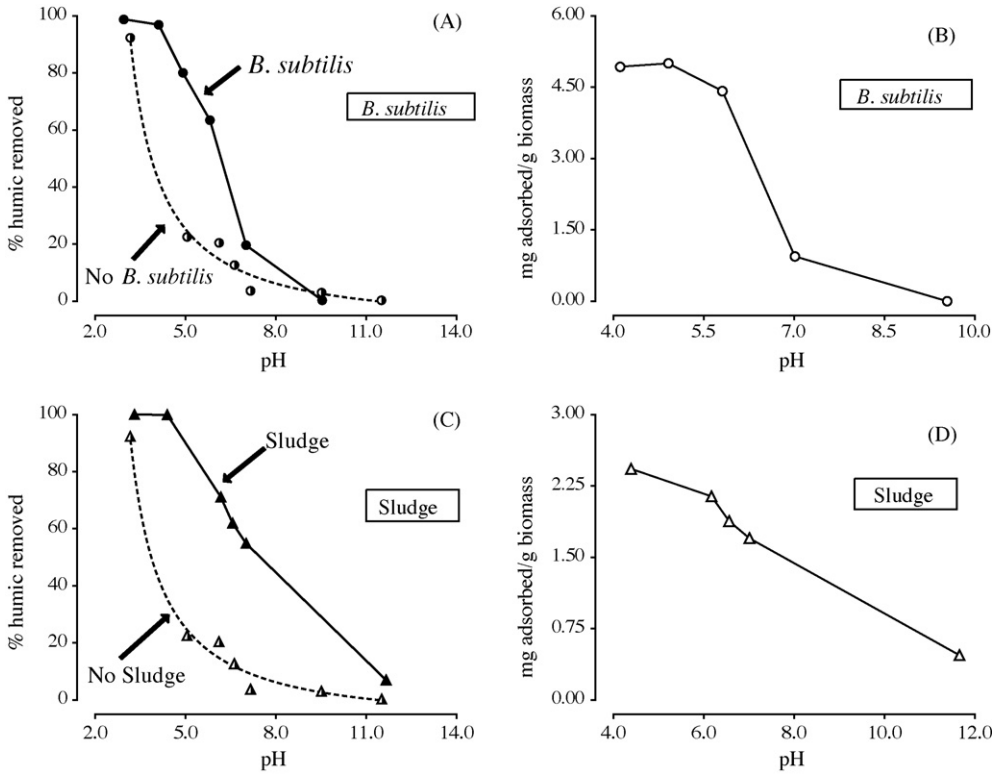

Fig. 1. Adsorption of humic acid onto *B. subtilis* and activated sludge as a function of pH. (A) Humic acid onto *B. subtilis*: (●, ------)% of humic acid precipitated in the absence of *B. subtilis*, (—●—) % of humic acid removed (precipitated + adsorbed) in the presence of *B. subtilis*. Conditions were: [humic acid] = 100 mg/L, [*B. subtilis*] = 9.6 g/L (where required), T = 20 °C, incubation time = 120 min. (B) True specific adsorption of humic acid onto *B. subtilis* expressed in mg/g. (C) Humic acid onto activated sludge: (▲, ------)% of humic acid precipitated in the absence of activated sludge, (—▲—) % of humic acid removed (precipitated + adsorbed) in the presence of activated sludge. Conditions were: [humic acid] = 100 mg/L, [activated sludge] = 25 g/L (where required), T = 20 °C, incubation time = 180 min. (D) True specific adsorption of humic acid onto activated sludge expressed in mg/g.

288 nm from our own spectroscopic studies. In the activated sludge experiments, the wavelength used to measure fulvic acid was 330 nm instead of 450 nm in order to avoid the greater interference generated by the activated sludge background. The blank samples were subjected to the same procedure described above in all cases. Absorbance measurements were carried out in a 1 cm quartz cell on a Beckman DU-7 spectrophotometer. Calibration curves were obtained under the same experimental conditions as the samples and blanks.

2.5. Curve fitting

The SIMFIT statistical package [19] was used to perform curve fitting and predictions by non-linear regression techniques.

3. Results and discussion

3.1. Adsorption of humic acid

As is known, the solubility of humic acid varies considerably as a function of pH. Thus, to study the adsorption of this compound it is necessary to distinguish between the precipitated fraction and that actually adsorbed at each pH. This can be achieved by measuring the humic acid precipitated in the absence of the adsorbent in one experiment and the total substance removed in the presence of the adsorbent in another. Thus, the difference between both values will reflect the substance really adsorbed. The variation between the precipitated and removed humic acid with pH is shown in Fig. 1(A) for *B. subtilis*. This figure shows two curves, decreasing with pH. The lower curve refers to the study of the precipitation of humic acid in the absence of *B. subtilis*, and the upper one shows the total amount of humic acid removed in the presence of *B. subtilis* as measured in a different study, since it is almost impossible to duplicate this kind of biological sample with exactly the same pH. The lower precipitation curve was fitted by non-linear regression to an empirical function for prediction purposes when the pH was known and the percentage of the precipitated substance was to be evaluated. Fig. 1(B) plots the humic acid really adsorbed at different pH values, calculated from Fig. 1(A) as the difference between the total amount removed at a given pH and the amount precipitated at that pH, as predicted from the precipitation curve. In Fig. 1(B) it may be seen that adsorption increases with decreasing pH above the 3.8–10.0 pH range. This kind of behaviour is not different to that reported by Fein's group in similar experiments [5,6]. It is known that the cell wall of *B. subtilis* is composed of peptidoglycans and teichoic acids, and that it exhibits carboxyl, phosphoryl and hydroxyl groups at the surface [20]. Furthermore, in titration experiments, the deprotonation constants of these three groups have been reported to

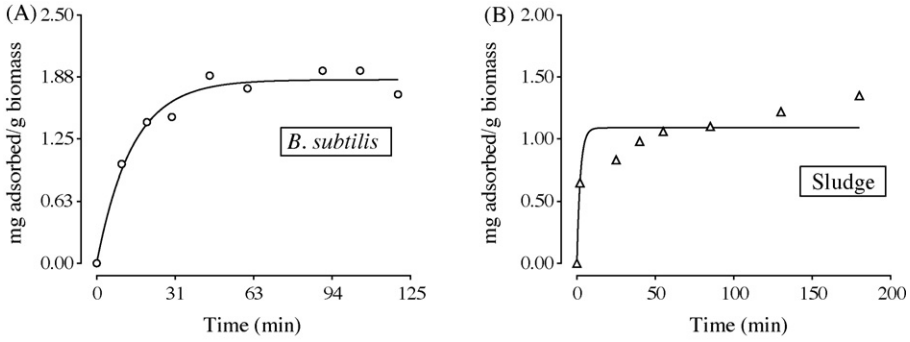

Fig. 2. Adsorption of humic acid onto *B. subtilis* and activated sludge as a function of time. (A) Humic acid onto *B. subtilis*., [humic acid] = 100 mg/L, [*B. subtilis*] = 10.5 g/L, pH 6.02, $T = 20\,^{\circ}C$. (B) Humic acid onto activated sludge, [humic acid] = 100 mg/L, [activated sludge] = 25 g/L, pH 6.2, $T = 20\,^{\circ}C$, incubation time = 180 min. In both cases the solid line is the non-linear regression fit to the exponential function $y = A(1 - \exp(-kt))$.

have pK values of 4.8, 6.9 and 9.4, respectively [21]. Thus, in agreement with Fein et al. [5], it may be concluded that since the adsorption of humic acid onto *B. subtilis* is stronger at lower pH, where the humic acid and the bacterial surface are uncharged, the interaction is mainly hydrophobic.

The same experiments were performed using activated sludge instead of *B. subtilis* in order to investigate possible differences between both biomasses. Fig. 1(C) shows again in the lower curve the influence of pH on humic acid precipitation in the absence of sludge, while in upper curve shows the effect of pH on humic acid removal in the presence of sludge. Fig. 1(D) shows the real adsorbed humic acid, calculated by the difference between the total amount removed and that precipitated at each pH value. On comparing Fig. 1(D) with its counterpart Fig. 1(B), it may be seen that the same pH trend is followed, and in both cases adsorption increases with decreasing pH. To interpret this, it must be recalled that activated sludge from wastewater treatment plants contains bacteria and protozoa. The cell walls of bacteria consist mainly of polysaccharides, lipids and proteins, all of which are capable of adsorbing substances. Protozoa are unicellular eukaryotic cells that lack cell walls but do have outer membranes with lipids and proteins that can also adsorb several different compounds [12,22–24]. The pH value affects the surface properties of the activated sludge biomass and as pH is lowered the overall surface becomes uncharged and the greater degree of adsorption observed at lower pH again suggests hydrophobic interactions.

Fig. 2A and B shows kinetic adsorption studies for humic acid on *B. subtilis* cells and activated sludge biomass, respectively. Both have fast adsorption curves, which were acceptably fitted by non-linear regression to the exponential function $y = A(1 - \exp(-kt))$, which would mean a first-order kinetic phenomenon. From these curves it can be seen that an equilibrium time of more than 30 min would be sufficient for these adsorption studies.

3.2. Adsorption of fulvic acid

Fulvic acid remains soluble throughout the full pH range, which simplifies adsorption studies. The influence of pH on the adsorption of fulvic acid onto *B. subtilis* is shown in Fig. 3(A). In this case too, adsorption is strongly pH-dependent, increasing with decreasing pH. On comparing Fig. 3(A) with Fig. 1(B) it may be observed that the effect of pH on the adsorption of fulvic and humic acids onto *B. subtilis* follows the same trend. As in the case of humic acid, the higher adsorption at lower pH can be explained in terms of the notion that hydrophobic interactions must exist between the cell wall and the non-polar regions of fulvic acid.

A similar study was conducted for the adsorption of fulvic acid onto activated sludge biomass; the results are shown in Fig. 3(B). The pH behaviour is equal to that observed with *B. subtilis* cells (Fig. 3(A)), and adsorption decreases when pH increases.

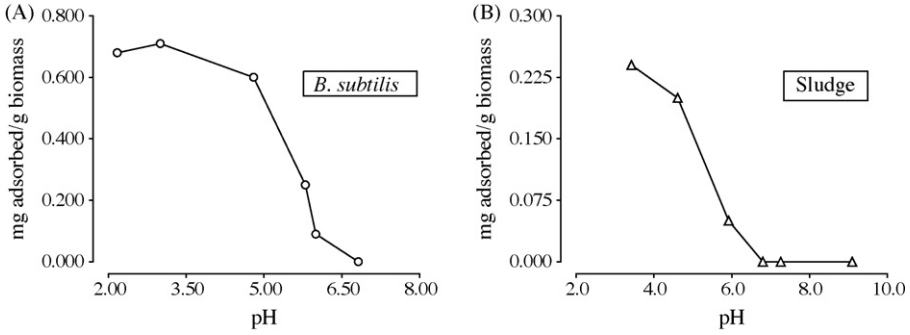

Fig. 3. Adsorption of fulvic acid onto *B. subtilis* and activated sludge as a function of pH. (A) Fulvic acid onto *B. subtilis*: [fulvic acid] = 12.5 mg/L, [*B. subtilis*] = 9.8 g/L, $T = 20\,^{\circ}C$, incubation time = 180 min. (B) Fulvic acid onto activated sludge: [fulvic acid] = 12.5 mg/L, [activated sludge] = 25 g/L, $T = 20\,^{\circ}C$, incubation time = 180 min.

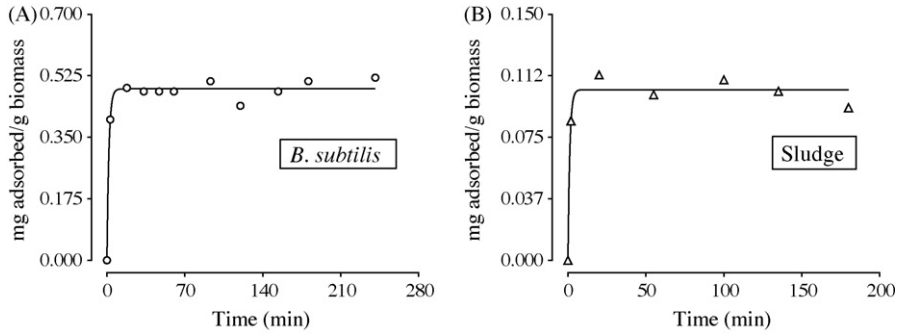

Fig. 4. Adsorption of fulvic acid onto *B. subtilis* and activated sludge as a function of time. (A) Fulvic acid onto *B. subtilis*: [fulvic acid] = 12.5 mg/L, [*B. subtilis*] = 9.8 g/L, pH 4.07, $T = 20\,^{\circ}C$. (B) Fulvic acid onto activated sludge: [fulvic acid] = 12.5 mg/L, [activated sludge] = 25 g/L, pH 5.92, $T = 20\,^{\circ}C$. In both cases the solid line is the non-linear regression fit to the exponential function $y = A(1 - \exp(-kt))$.

Fig. 4(A) and (B) show the results of a study on the kinetics of the adsorption of fulvic acid onto *B. subtilis* and activated sludge, respectively. The data are well fitted to the exponential function $y = A(1 - \exp(-kt))$, which would imply a first-order kinetic phenomenon. From these curves, it can be seen that the equilibrium time is around 30 min, and hence the incubation times for adsorption studies must be always higher than this value.

3.3. Adsorption of phenol

The adsorption of phenol onto *B. subtilis* and activated sludge was also included in this study since this compound is considered a standard adsorbate and because phenol and phenolic compounds are among the most common organic pollutants of wastewaters.

Fig. 5(A) and (B) show the adsorption behaviour of phenol with changes in the pH for *B. subtilis* and activated sludge, respectively. Here, an optimum pH is observed for both systems at around pH 7.0, in contrast with the monotonic decreasing curves upon increasing pH seen with humic acid (Fig. 1(B) and (D)) and fulvic acid (Fig. 3(A) and (B)). This optimum pH is three units distant from the 9.9 p*K* value of phenol, which means that the protonated phenol is better adsorbed than the phenolate ion. Nevertheless, adsorption does not increase indefinitely with decreasing pH, as might be expected. The optimum pH of about 7.0 may be due to a combination of hydrophobic interactions and hydrogen bonds between the protonated phenol and the cell walls. Such hydrophobic interactions would take place between the aromatic ring of the phenol and the hydrocarbonated regions on the cell wall, while the hydrogen bonds could arise from the hydroxyl group of the phenol and deprotonated carboxyl and phosphate groups existing on the cell wall, with p*K* values of 4.8 and 6.9, respectively [21]. When the cell groups gradually become protonated at lower pH, the hydrophobic attractions would still remain active but the hydrogen bonds would decrease and the whole adsorption phenomenon would decrease from its optimum value.

Fig. 6(A) and (B) show the kinetic behaviour observed for the adsorption of phenol on *B. subtilis* and activated sludge, respectively. As in the previous studies, the data were fitted to the exponential function under the assumption of a first-order kinetic phenomenon. Nevertheless, experimental error was greater in this case.

In order to analyse the above studies from a comparative point of view, Table 1 summarizes some values collected at the optimum pH. From this table it may be seen that in the case of *B. subtilis* cells the sequence of the specific adsorption values would be humic acid > fulvic acid > phenol, although the absolute values must be taken with caution, considering the different origin of the humic and fulvic acids. The lower adsorption of fulvic acid as compared with that of humic acid may be explained in terms of differences in their hydrophobicity rather than in their molecular size. Humic acid has been shown to be more hydrophobic than fulvic acid [1], and since the surface of *B. subtilis* is quite hydrophobic, more efficient hydrophobic inter-

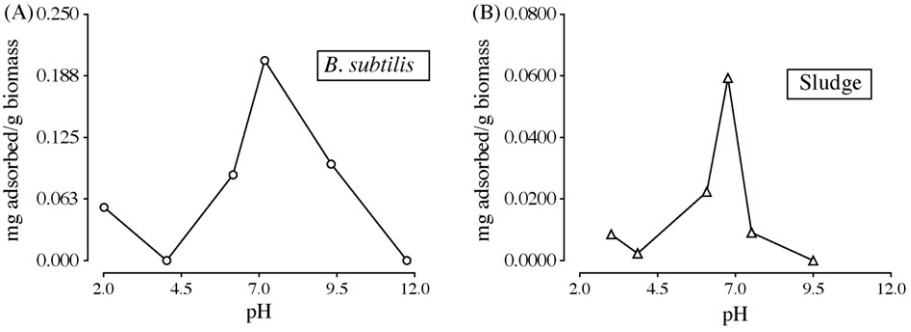

Fig. 5. Adsorption of phenol onto *B. subtilis* and activated sludge as a function of pH. (A) Phenol onto *B. subtilis*: [phenol] = 15 mg/L, [*B. subtilis*] = 10 g/L, $T = 20\,^{\circ}C$, incubation time = 240 min. (B) Phenol onto activated sludge: [phenol] = 15 mg/L, [activated sludge] = 25 g/L, $T = 20\,^{\circ}C$, incubation time = 180 min.

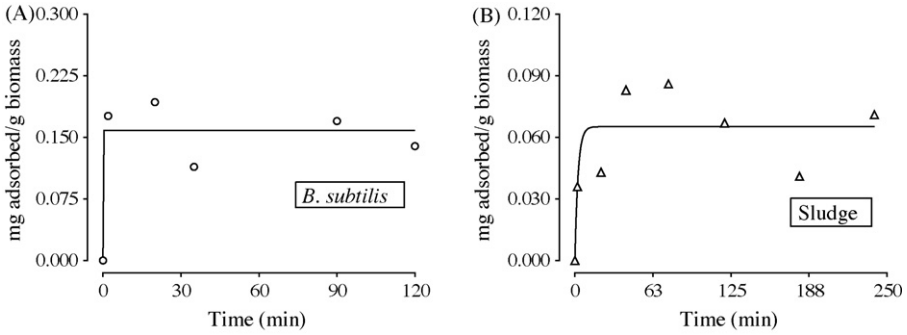

Fig. 6. Adsorption of phenol onto *B. subtilis* and activated sludge as a function of time. (A) Phenol onto *B. subtilis*: [phenol] = 15 mg/L, [*B. subtilis*] = 10 g/L, pH 6.45, $T = 20\,^{\circ}C$. (B) Phenol onto activated sludge: [phenol] = 15 mg/L, [activated sludge] = 25 g/L, pH 6.60, $T = 20\,^{\circ}C$. In both cases the solid line is the non-linear regression fit to the exponential function $y = A(1 - \exp(-kt))$.

Table 1
Comparison of the specific adsorption values of humic acid, fulvic acid and phenol onto *B. subtilis* and activated sludge at the optimum pH

Adsorbate (C_0)	Adsorbent (C_0)	pH	Adsorption (mg/g)
Humic acid[a] (100 mg/L)	*B. Subtilis* (10 g/L)	4.1	4.9
	Act. sludge (25 g/L)	4.4	2.4
Fulvic acid[b] (12.5 mg/L)	*B. Subtilis* (10 g/L)	3.0	0.71
	Act. sludge (25 g/L)	3.4	0.24
Phenol (15 mg/L)	*B. Subtilis* (10 g/L)	7.2	0.20
	Act. sludge (25 g/L)	6.8	0.06

[a] Technical grade from Aldrich (50–60% humic acid content).
[b] Standard grade from IHSS (assumed of great purity).

actions would be expected for humic acid than for fulvic acid. The lower specific adsorption of phenol as compared with that of humic and fulvic acid would mean that the interactions between phenol and *B. subtilis* cells are weaker than for humic substances. When activated sludge biomass is used as an adsorbent instead of *B. subtilis*, the same adsorption trend – humic > fulvic > phenol – is seen (Table 1). More important would be to compare the adsorption values for each organic substance between the activated sludge and *B. subtilis*. In this sense, Table 1 shows that adsorption is similar with both biomasses, although it is always lower in the case of sludge. The reason for the lower value would be the lower specific area of sludge, since – as seen by electron microscopy (micrographs not shown) – sludge is a cluster of cells as compared with the individual cells of *B. subtilis* biomass. Nevertheless, the similar adsorption behaviour suggests that both types of biomass base their adsorption capacities on the general characteristics of the bacterial cell wall. This is remarkable, since the sludge is a heterogeneous and cheap material in comparison with homogenous cultured bacterial cells. Therefore, activated sludge seems to be a candidate for further consideration in adsorption studies since it is an abundant residue from wastewater treatment plants.

4. Conclusions

The adsorption of humic and fulvic acids onto *B. subtilis* cells and activated sludge biomass follows the same trend under the influence of pH: when pH decreases adsorption increases. This kind of behaviour can be interpreted in terms of hydrophobic interactions between the uncharged biomass surface and the uncharged humic and fulvic acids. This is in contrast with the optimum pH of around 7.0 observed for phenol on the same adsorbents.

Kinetic studies on the adsorption of humic acid, fulvic acid and phenol onto *B. subtilis* cells and activated sludge revealed a rapid uptake of the substances, with equilibrium times of about 30 min. In all cases, the kinetic curves were well fitted by the exponential function $y = A(1 - \exp(kt))$, suggesting a first-order kinetic phenomenon.

Specific adsorption values collected at optimum pH revealed that with the materials used in this work both *B. subtilis* and activated sludge follow the same adsorption trend: humic > fulvic > phenol. The poorer adsorption of fulvic acid as compared with humic acid could be interpreted in terms of its lower hydrophobicity rather than its lower molecular size. When specific adsorption onto *B. subtilis* and onto activated sludge was compared, similar but lower values were found for all three organic compounds studied. This is remarkable since sludge is a heterogeneous and cheap material in comparison with cultured bacterial cells. This kind of behaviour suggests that both type of biomass base their adsorption capacity on the general characteristics of the bacterial cell wall, and hence the much cheaper activated sludge seem to be a good candidate as a biomass for consideration in further adsorption studies.

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