Bioprocess Biosyst Eng (2013) 36:1601–1611
DOI 10.1007/s00449-013-0934-2

ORIGINAL PAPER

Enzyme-based glucose delivery: a possible tool for biosorbent preparation for heavy metal removal from polluted environments

Mihaela Palela · Gabriela Elena Bahrim · Julia Glazyrina · Eva Brand · Peter Neubauer

Received: 9 November 2012 / Accepted: 17 February 2013 / Published online: 3 March 2013

Abstract This study was performed to examine the influence of the controlled glucose supply technology, EnBase® Flo, on growth and heavy metals uptake capacity of two *Bacillus* strains isolated from food industry wastewater. *Bacillus* sp. growth on EnBase Flo (mineral salt complex medium containing starch-derived polymer as substrate) was examined in 24 deep well plates, controlling the glucose amount release by adding two amyloglucosidase concentrations (3 and 6 UL^{-1}). Adsorption of the heavy metals Zn^{2+}, Cd^{2+} and Pb^{2+} was assessed in a single component system using synthetic metal solutions and as a function of the initial concentration of adsorbate, equilibrium time and removal efficiency. The Langmuir and Freundlich adsorption models were used for the mathematical description of the biosorption equilibrium and isotherm constants. A pseudo second-order model was applied to describe the uptake rate for two isolates. The EnBase® Flo technology improved the cells growth over ten times after 24 h of fed-batch cultivation. The EnBase® Flo technology improved the Cd^{2+} and Pb^{2+} uptake capacity of the bacterial strains by approximately 55 and 44 %, respectively. The biosorption of each metal was fairly rapid (within 30 min), which could be an advantage for large scale treatment of contaminated sites. This initial study may be a basis for future developments to apply EnBase Flo for the biomass production used further as biosorbent for heavy metal removal from aqueous solutions.

Keywords *Bacillus* sp. · EnBase® Flo system · Growth · Metal biosorption · Wastewater

M. Palela (✉) · G. E. Bahrim
Faculty of Food Science and Engineering,
"Dunarea de Jos" University, 111 Domneasca Street,
800201 Galaţi, Romania
e-mail: palela_mihaela@yahoo.com

G. E. Bahrim
e-mail: Gabriela.Bahrim@ugal.ro

J. Glazyrina · E. Brand · P. Neubauer
Department of Biotechnology, Chair of Bioprocess Engineering,
Technische Universität Berlin, Ackerstraße 71-76, ACK 24,
13355 Berlin, Germany
e-mail: julia.glazyrina@tu-berlin.de

E. Brand
e-mail: eva.brand@tu-berlin.de

P. Neubauer
e-mail: peter.neubauer@tu-berlin.de

Introduction

Metal ions from aqueous solutions (e.g., wastewater) may be removed by physical, chemical or biological means. Conventionally, metal ions are removed from aqueous solution by chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon or evaporation. However, chemical precipitation and electrochemical treatment are ineffective, especially when the metal ion concentration in the aqueous solution is between 1 and 100 mg L^{-1}; this high amount producing a large quantity of sludge is very difficult to treat. Methods which would be feasible, such as ion exchange, membrane and activated carbon adsorption processes, are too expensive for treating the large amounts of wastewaters that contain heavy metals in low concentration and thus cannot be applied at the final scale [1].

A feasible cheaper and more efficient alternative for the removal of metallic elements, especially for heavy metal removal from aqueous solutions, may be represented by various types of microbial biomass that can serve as a basis

for producing new, very potent metal-sequestering biosorbents. They would have a potential as "low cost" alternatives if their generation requires little processing, if they are abundant in nature, or especially, if they are an industrial byproduct or waste material [2]. A special source may be the indigenous microbial flora from wastewaters which forms a diverse microbiota adapted to the specific physicochemical conditions and has an essential role in the biodegradation of pollutants. The composition of the distinct microbiota depends on the origin of the wastewater and, thus, locally available polluted wastewater-derived biomass may have a large potential for application. Both living and dead cells are capable of metal uptake, but the use of dead biomass seems to be a preferred alternative for the majority of metal uptake studies reported. The wide acceptability of dead cells is due to a series of advantages: the absence of toxicity limitations, the absence of requirements of growth media and nutrients in the feed solution, the easy recovering of the biosorbed metals, the regenerated biomass can be re-used and the metal uptake reactors can be easily modelled mathematically [3].

An interesting area of research is the findings of the effects of pretreatments to enhance the biosorption process and, along with the variation of other parameters, to enhance specificity [4]. The methods used to change the cell surface characteristics to increase the number of the available uptake sites are physical methods, such as vacuum and freeze drying, boiling, autoclaving and mechanical disruption, or chemical methods including contacting the biomass with various organic and inorganic compounds. One of the most frequent microorganisms Genera identified in wastewaters and used in biosorption studies is *Bacillus.* Different species have a very high metal uptake potential and they were used in various biosorbent formulation studies [5–7]. Methods such as caustic treatment and granulated *Bacillus* product enhanced the uptake capacity of the bacterial biomass and were used by biosorbent producing companies such as Vista Tech Partnership, Ltd., Salt Lake City Utah, U.S. [8].

The biosorption capability also depends on the culture conditions, growth media composition and growth phase [9]. Many complex microbiological media contain extracts (e.g. yeast extract and beef extract) that vary in their precise chemical composition. Therefore, minimal media with a known chemical composition are often used to study the uptake capacity of the microbial biomass applied as biosorbent [10]. The new cultivation method EnBase Flo is a mineral salt medium containing vitamins and trace elements. Optionally, also complex components (booster) can be added. This buffered medium contains a soluble starch-derived polymer acting as substrate source, after it is degraded to glucose by the addition of an amyloglucosidase enzyme [11, 12]. The enzyme concentration determines the glucose release rate and, thus, the growth rate of the culture, in the same way as the pump speed controls the growth rate in a glucose-limited fed-batch bioreactor [14]. The fed-batch system, EnBase Flo, proved to be a valuable cultivation strategy, which improved the cell growth for *E. coli* that has been exploited for improved recombinant protein production and growth, and biotransformation capacity of a variety of different yeasts [13].

In this work, we showed that the cultivation media can influence both the cells growth and the biosorbent capacity to remove the heavy metals from the liquid environment. Knowledge about the growth conditions can be later implemented in the production of the biomass used for heavy metals removal.

Materials and methods

Isolation of microbial strains

Wastewater samples were collected from a food industry wastewater effluent stream in Galati city, Romania. The sampling was performed in the outlet canal, before the wastewater overflowed in the municipal collecting system. One milliliter of wastewater was serially diluted and spread over nutrient agar plates. The bacterial colonies were observed after aerobic incubation for 48 h at 37 °C. After the incubation periods, colonies were picked up and purified by repeated streaking on plate count agar medium till pure cultures were obtained. The pure cultures were coded according to the Microorganisms Collection of the "Dunarea de Jos" University (Table 1), which is affiliated to the ICCF national collection and kept as pure stock cultures for molecular characterization. The bacteria strains were preserved as 10 % glycerol stock in LB (Luria–Bertani) medium at −80 °C.

Molecular characterization of the isolates

For the identification of the isolates, the colony polymerase chain reaction PCR technique was applied and the bacterial genes were amplified using specific 16S rRNA primers. For PCR, the following parameters were applied: an initial denaturation step at 95 °C for 6 min followed by 30 cycles

Table 1 The isolated strains and the corresponding MIUG collection code

Category of microorganisms	Genera. Species	MIUG collection code	BLAST accession
Bacteria	*Bacillus subtilis*	MIUG 6.160	GQ280027.1
	Bacillus sp.	MIUG 6.161	FJ960508.1

at 95 °C for 30 s, annealing at 49 °C for 30 s and extension at 72 °C for 3 min, with a final extension step at 72 °C for 10 min. For the identification of the isolates, the colony polymerase chain reaction technique was applied and the bacteria genes were amplified using the specific primers 16S rRNA (27F) 5′-AGAGTTTGATCCTGGCTCAG-3′; (1492R) 5′-GGTTACCTTGTTACGACTT-3′), respectively.

The PCR products of 1,500 bp were purified using Hi-Yield PCR clean-up kit (QIAGEN). Sequencing was carried out by LGC Genomics Company (Berlin, Germany). The sequences were aligned with close matches using the Tuebingen multiple sequence alignment program (http://toolkit.tuebingen.mpg.de). Nucleotide sequence similarities were determined using BLAST (NCBI database; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Batch and fed-batch cultivation system

A novel fed-batch type cultivation called EnBase® Flo, provided by BioSilta (Oulu, Finland) was used to obtain high amount of biomass for the biosorption studies. It was used for the first time for the cultivation of microoganisms isolated from wastewater. 10 mL of pre-cultures in 100 mL Erlenmeyer shake flasks were inoculated from glycerol stock on Luria–Bertani broth medium (yeast extract 5.0 g L^{-1}, bacto tryptone 10.0 g L^{-1}, sodium chloride 10.0 g L^{-1}) and cultivated for 14 h. The main cultures were inoculated with cells from the pre-cultures with a starting optical density (OD_{600}) of 0.15 AU. The growth of the *Bacillus* strains on EnBase Flo was tested by supplying the medium with different concentrations of EnzI′m (3 and 6 UL^{-1}, BioSilta, Oulu, Finland). The cultivations were performed at 37 °C, in 24 deep well plates with 2 mL of medium. A shake flask incubator (Kühner Shaker LT-X, Switzerland) with a 5 cm shaking amplitude and a shaking frequency of 200 rpm was used. In order to monitor the microbial growth, the optical density was measured at a wave length of 600 nm using a spectrophotometer (Ultraspec 3300). All cultivations were performed in triplicates. The batch cultivation system on LB broth has been considered as control.

Biomass preparation

Both LB and EnBase main cultures were inoculated with pre-cultures obtained in LB medium, with the same composition and in the same conditions as described above. The EnBase Flo medium and technology have been described extensively in recent publications [11–13]. For both growth media, the initial pH was adjusted to 7.0. The main LB and EnBase cultures were prepared in 200 mL and 100 mL of broth, respectively, in 500 mL Erlenmeyer flasks, with a starting optical density (OD_{600}) of 0.15 AU. Cells were incubated at 37 °C and at 200 rpm in a shake flask incubator (Kühner Shaker LT-X, Switzerland), with 2.5 cm shaking amplitude. The cultures were harvested after 10 h, in the late exponential phase, in the case of LB medium, and after 30 h, during glucose-limited growth, from EnBase Flo medium, and centrifuged at 5,000×*g*. The wet biomass was washed several times with bidistilled water until the conductivity of the supernatant was below 20 µS. The inactive biomass used as biosorbent for the experiments was obtained by drying the bacterial cells at 60 °C to constant weight for 24 h. After pulverization to a uniform maximum particle size of 0.18 mm, using an electric grinding apparatus (A10, IKA, Staufen), mortar, pestle and standardized sieves, the biomass was stored at 4 °C.

Biosorption experiments

Sterilized stocks of 1 g L^{-1} of Zn $(NO_3)_2$, Cd $(NO_3)_2 \times 4H_2O$ and Pb $(NO_3)_2$ were aseptically prepared. For all experiments, 8 mL of metal solution of a known initial concentration (50, 100 and 200 mg L^{-1} of Zn^{2+}, Cd^{2+} and Pb^{2+} ions) came in contact with 8.0 mg of adsorbent at room temperature (final biomass dosage of 1 g L^{-1}). The experiments were performed in 10 mL propylene tubes treated overnight with 5 % nitric acid and kept for 24 h in pure water before use. The batch equilibrium technique was carried out using an overhead orbital shaker (Heidolph Reax 20) with an agitation rate of 15 rpm for 24 h, at room temperature (22 ± 2 °C). All biosorption studies were started at pH values of approximately 5 and the pH was not adjusted during the experiments.

At the end of the experiments, the solutions were centrifuged at 5,000×*g* for 10 min. The supernatant was filtered through Rotilabo® Nylon membranes with a diameter of 0.20 µm (Carl-Roth), and the metal concentration was determined by Absorption Atomic Spectroscopy (AAS) with flame atomization in a NOVA 300/400 spectrometer (Analytik Jena AG, Jena, Germany). The results were recorded as milligrams of metal per gram dry-weight biomass. The uptake capacity at equilibrium was calculated using the mass balance Eq. (1) for the biosorbent [14].

$$q = \left[V\left(C_i - C_f\right)\right]/M \quad (1)$$

where q is the metal ion uptake capacity (mg g^{-1}), V is solution volume (L), C_i is initial concentration of the metal in the solution (mg L^{-1}), C_f is final concentration of the metal in the solution (mg L^{-1}) and M is the mass of biosorbent (g).

The removal efficiency was calculated with the Eq. (2).

$$R_e = \left(C_i - C_f\right)/C_i \times 100 \quad (2)$$

Sorption isotherm models

The empirical Langmuir and Freundlich isotherm models cited in different scientific works [15] are the earliest, most common and simplest known relationships describing the ad/bio-sorption phenomenon. These two models were used for interpreting the lead, cadmium and zinc biosorption equilibrium.

The linearized Langmuir isotherm model is given by the Eq. (3).

$$\frac{C_f}{q_e} = \frac{1}{q_m b} + \frac{C_f}{q_m} \quad (3)$$

where q_e is the metal uptake at equilibrium (mg g^{-1}), q_m (q_{max}) the maximum achievable uptake by a system (g mg^{-1}) and b is the affinity between the sorbate and the sorbent (l mg^{-1}). The adsorption constants q_m and b were evaluated from the intercept and the slope of the linear plot of C_f/q_e versus C_f based on experimental data.

The linearized Freundlich equation is given by the Eq. (4).

$$\log q_e = \log k_F + {}^{1}/_{n_F} \log C_f \quad (4)$$

where k_F and $1/n_F$ are the Freundlich's constants related to the adsorption capacity and adsorption intensity. The two values were evaluated from the intercept and the slope of the linear plot of $log\ q_e$ versus $log\ C_f$ based on experimental data.

Adsorption kinetics

The bacterial sorption kinetics was investigated at room temperature, using a constant adsorbent concentration of 1 g L^{-1} and an initial metal concentration of 100 mg L^{-1}. Samples were taken at different time intervals up to 120 min. The adsorption kinetics of cadmium, lead and zinc were analyzed using pseudo first-order [16] and pseudo second-order kinetic models [12].

The pseudo first-order kinetic model is expressed by

$$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303} t \quad (5)$$

where k_1 is the first-order rate constant (min^{-1}). The plot of $log\ (q_e - q_t)$ versus t shows a linear relationship from which k_1 and q_e could be determined from the slope and intercept of the plot, respectively. The equation for a pseudo second-order kinetic models is expressed by:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t \quad (6)$$

where q_t is the metal uptake at any time t (mg g^{-1}) and k_2 is the second-order rate constant (g mg^{-1} min^{-1}). The plot of t/q_t versus t of Eq. (6) shows a linear relationship from which q_e and k_2 could be determined from the slope and intercept of the plot, respectively.

Results

Molecular characterization of the isolates

In order to identify the two strains isolated from the food industry wastewater, they were subjected to 16S rRNA gene sequence analysis. The nucleotide sequences of 1,500 pb PCR fragment of the 16S rRNA gene matched 100 % with those of *B. subtilis* and with those of *Bacillus* sp. (Table 1).

Growth in batch and fed-batch cultivation systems

A comparison between the novel EnBase Flo cultivation method and the standard growth media Luria–Bertani was performed. Cultivation with standard LB medium resulted in a final OD_{600} of 2.4 AU for *Bacillus* sp. strain and in

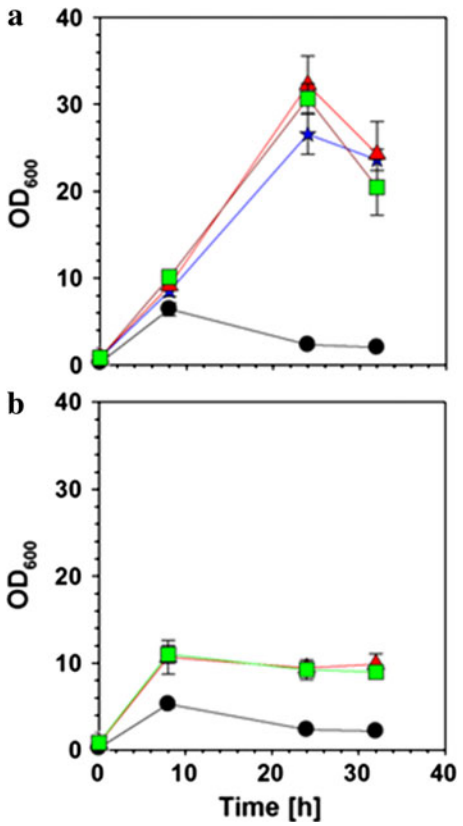

Fig. 1 Growth dynamic of *Bacillus* sp. wild strains cultivated on EnBase-Flo (EnB), supplimented with different concentrations of amyloglucosidase (AG): **a** *Bacillus* sp. MIUG 6.161; **b** *Bacillus subtilis* MIUG 6.160; *filled circle* LB medium, *asterisk* EnB without AG, *filled triangle* EnB with 3 U L^{-1} AG, *filled square* EnB with 6 U L^{-1} AG

OD_{600} of 2.8 AU for *Bacillus subtilis*. In contrast, the use of the mineral salt medium EnBase Flo supplied with 3 and 6 UL^{-1} EnzI′m improved the bacterial growth by 30 and 11 times after only 24 h for *Bacillus* sp. and *B. subtilis*, respectively (Fig. 1). Both strains showed a higher preference for EnBase medium supplied with enzyme.

Effect of the culture media on metal removal processes

A comparison between the metal uptake capacities of bacterial biomass grown in standard LB medium or in EnBase Flo medium was performed for both *Bacillus* strains. The Langmuir model was applied to predict the maximum uptake capacity (q_{max}). The biomass of *Bacillus* sp. and *B. subtilis* showed higher Cd^{2+} maximum uptake capacities for the EnBase Flo grown cells than for the cells grown in LB medium. The maximum uptake values obtained for the cells grown in EnBase Flo medium indicate an improvement of 18 and 44 %, respectively. *Bacillus* sp. and *B. subtilis* showed a better maximum uptake capacity for Pb^{2+} if grown in EnBase Flo medium compared to LB, with an improvement of 7.5 and 55 %, respectively. For Zn^{2+}, no enhancement of the uptake capacity for EnBase Flo grown cells was detected (Fig. 2), but the sorption affinity expressed by the Langmuir constant b increased 2.5 and 1.8 times for *Bacillus* sp. and *B. subtilis,* respectively (Table 2). The affinity regarding the Cd^{2+} ions uptake by the biomass grown on EnBase Flo has been improved only with 1.6 times to the *B. subtilis* strain, and with three times in *Bacillus sp.* No significant difference regarding the Pb^{2+} ions affinity of the two strains grown on both growth media could be observed (Table 2).

Biosorption profile

The Langmuir and Freundlich isotherms of heavy metals biosorption by the biomass of strains *Bacillus* sp. and *B. subtilis* are presented in Tables 2 and 3, while the best fit of curves generated in accordance with these models is depicted in the Fig. 3a and b. The regression coefficients obtained for heavy metals from the Langmuir and Freundlich models applied for the biomass grown on EnBase medium were between 0.83 and 1, excepting the Pb ions uptake by the *B. subtilis* biomass, which was best described by the Freundlich model with a correlation coefficient over 0.91. There is a direct correlation between the *b* value and the biosorbent affinity. According to the Langmuir constant *b* values calculated for the *B. subtilis* biomass obtained from the culture grown on EnBase medium, the strain could absorb the heavy metal ions in the order: Zn > Cd > Pb. The *Bacillus* sp. strain showed the highest sorption affinity for Zn^{2+} ions and the lowest for the Cd^{2+} ions. A different situation could be observed to the biomass grown on Luria–Bertani medium where the *Bacillus* sp. exhibited the following sorption order: Cd > Pb > Zn, while the *B. subtilis* biomass showed similar sorption capacity for Cd^{2+} and Zn^{2+} ions and the lowest for Pb^{2+} ions.

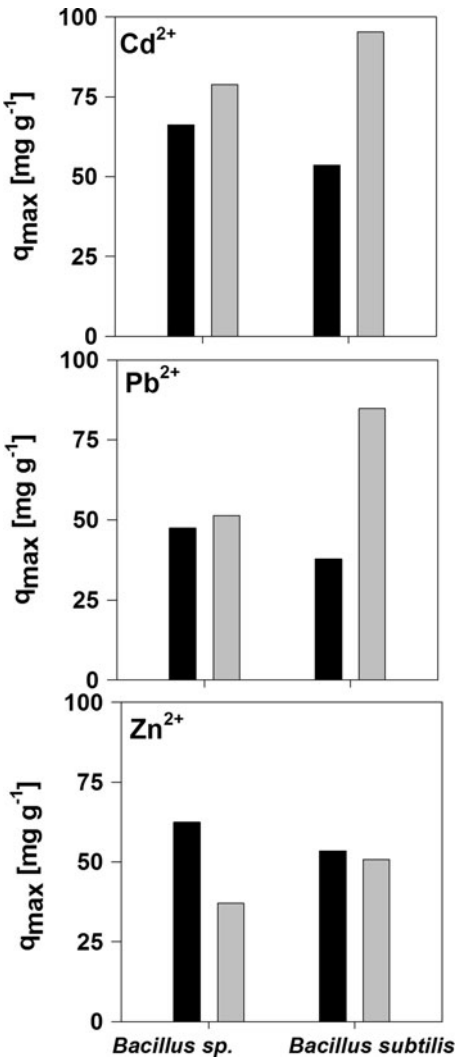

Fig. 2 Influence of the culture medium on heavy metal biosorption (Zn^{2+}, Cd^{2+} and Pb^{2+}) by free inactivated biomass of *Bacillus* strains (*black bar* Luria–Bertani; *gray bar* EnBase)

The adsorption partition-constant for metals was further determined by the Freundlich isotherm, when *k* and *1/n* were evaluated from the intercept and the slope of the linear plot of *log* q_e versus *log* C_e based on the experimental data. The degree of adsorption is directly correlated with the *k* value. In our study, both *Bacillus* strains showed the highest *k* value for Cd^{2+} and the lowest for Pb^{2+} (Table 3).

Table 2 Langmuir isotherm parameters for biosorption of heavy metal ions with nonviable *Bacillus* sp. grown on EnBase Flo and Luria–Bertani media

Growth medium	Strain	Zn^{+2}			Cd^{+2}			Pb^{+2}		
		q_{max} (mg g^{-1})	b (L mg^{-1})	R^2	q_{max} (mg g^{-1})	b (L mg^{-1})	R^2	q_{max} (mg g^{-1})	b (L mg^{-1})	R^2
EnBase Flo	*Bacillus* sp.	38	0.061	0.99	93.34	0.024	0.90	55.25	0.041	1
	B. subtilis	50.76	0.156	0.99	93.24	0.130	0.98	55.87	0.015	0.58
Luria–Bertani	*Bacillus* sp.	62.50	0.024	0.88	66.23	0.076	0.99	47.39	0.044	0.88
	B. subtilis	53.48	0.085	0.99	53.48	0.085	0.99	37.88	0.018	0.55

Table 3 Freundlich adsorption isotherm parameters for the biosorption of Zn^{2+}, Cd^{2+} and Pb^{2+} ions with nonviable wastewater isolated microorganisms grown on EnBase Flo medium

Strain	Zn^{2+}			Cd^{2+}			Pb^{2+}		
	k	$1/n$	R^2	k	$1/n$	R^2	k	$1/n$	R^2
Bacillus sp.	5.89	0.37	0.98	7.33	0.48	0.91	4.85	0.49	0.99
B. subtilis	6.34	0.47	0.92	9.98	0.55	0.83	2.43	0.63	0.91

Sorption kinetics

In order to predict the rate of biosorption operation by the two bacterial strains isolated from the food industry wastewater, the Lagergren pseudo first-order model as well as the pseudo second-order model were used to fit the experimental data. The kinetic parameters of the sorption are presented in the Tables 4 and 5. Unlike the pseudo first-order model, the pseudo second-order model fitted very well the experimental data for the biosorption rate of all three metals, the correlation coefficients (R^2) being higher than 0.94. Therefore, only the pseudo second-order model will be taken into discussion to describe the sorption rate of the heavy metals to the *Bacillus* strains used in this study. The *B. subtilis* biomass achieved a higher uptake rate k_2 than the *Bacillus* sp. strain for all three metals, in the following order: Zn > Pb > Cd. The biomass of *Bacillus* sp. showed an uptake rate k_2 in opposition with the other strain, the highest value being recorded for the Pb^{2+} ions and the lowest for Zn^{2+}.

Contact time is one of the important factors that influence the biosorption process. In our study, both bacterial biomasses achieved the system equilibrium within almost 30 min for all three metal ions (Fig. 4).

Effect of the initial metal concentration

The metal uptake capacity of the inactive biomass of the isolated strains was investigated at equilibrium conditions, as a function of the initial metal ion concentration, with solutions that contained each of the metal ions. Both bacteria strains showed a similar behavior regarding the effect of the initial metal concentration on the uptake capacity. An indirect correlation between the removal efficiency and the initial metal concentration was observed in the biomass and metal ions used in this work (Fig. 5). As a consequence of the asymptotic tendency to saturation of the biosorbants obtained on LB and EnBase media, the removal efficiency decreases with increasing metal concentrations for Zn^{2+} and Cd^{2+} ions. On the contrary, the uptake capacity at equilibrium increased with an increasing initial metal ion concentration till the biomass saturation occurred (Fig. 5a, b). The bacterial biomass obtained on both LB and EnBase media showed a different behavior regarding the Pb^{2+} ions uptake capacity. Therefore, *B. subtilis* reached the maximum saturation at the initial Pb^{2+} concentration of 100 mg L^{-1} on both types of growth media. The same statement can be made for *Bacillus* sp., but only for the EnBase medium.

At low initial metal concentrations, a slight increase in the final pH values from 5.0 to 6.0 occurred through the added bacterial biomass, but generally, the final pH was decreasing with increasing initial metal concentration. The difference between the initial and the final pH values varied from 1.0 for Zn^{2+} and Cd^{2+} to 2.0 units for Pb^{2+}. The lowest final pH values of 4.0 and 3.0 were recorded in the case of Pb^{2+} ions (data not showed).

Discussion

The EnBase Flo cultivation is a typical fed-batch like process strategy characterized by a high initial glucose concentration, which steadily decreases over 32 h of cultivation. Unlike the LB medium, the EnBase Flo is a fed-batch technology where the amyloglucosidase enzyme controls the amount of glucose released through the dextrin degradation; the growth phase is extended as long as the cells find the carbon and energy sources necessary for their metabolism. Here we cultivated successfully wastewater isolated bacteria cells. The two strains identified as *B. subtilis* and *Bacillus* sp. exhibited 11 and 13 times higher final cell densities in EnBase Flo medium, compared to the LB standard cultivation medium, which is a batch cultivation system. These approaches have shown that an

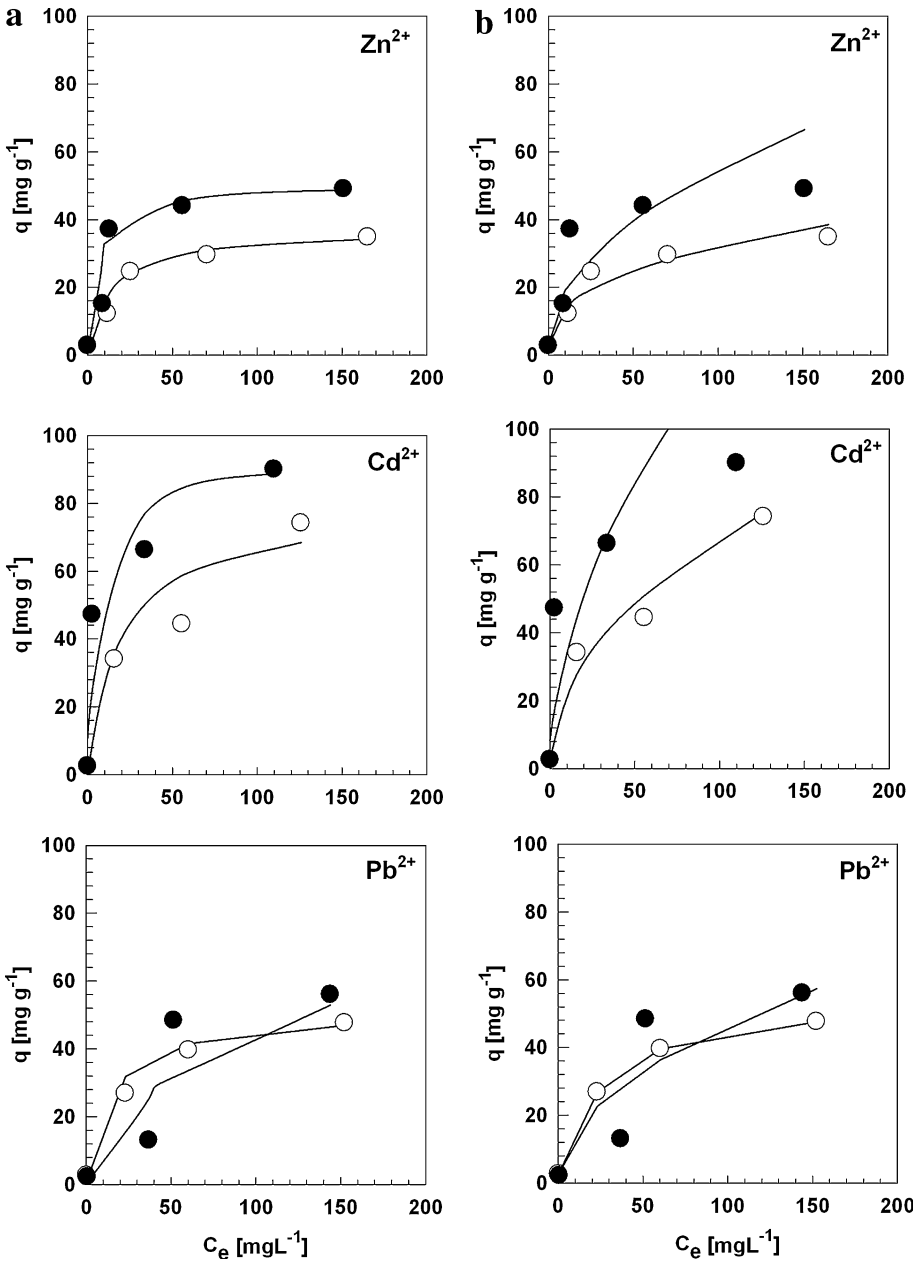

Fig. 3 Langmuir (**a**) and Freundlich (**b**) plots for Zn^{2+}, Cd^{2+} and Pb^{2+} ions uptake by free inactive biomass: *open circle Bacillus* sp. *filled circle Bacillus subtilis*

optimization of the EnBase medium in terms of the enzyme amount for glucose release for the cultivation of bacteria was beneficial.

The growth state and medium composition affect the metal removal processes. Here we have applied the EnBase Flo cultivation system that, aside from being a well-defined mineral salt medium, also has the advantage that the growth rate of the strain can be controlled by the release of glucose from a soluble polymer which is contained in the liquid. Due to the relation between volumetric growth rate and oxygen consumption, at lower growth rates much higher cell densities can be obtained compared to standard cultivation media. Surprisingly, there are not many studies concerning the impact of the growth medium on the heavy metal uptake capacity. The majority of scientific data present results with biomass obtained from fermentation processes or strains isolated from different environments, and maintained in standard laboratory growth media or grown in mineral salt media. To accurately characterize metal adsorption kinetics, the chemical composition of the medium and the physiological state of the cells must be well-defined. In this view, chemically defined media have the advantage that all components capable of interacting with metals can be taken into consideration [16].

In our case, using EnBase Flo helped in a faster screening by obtaining large cell amounts in shake flasks.

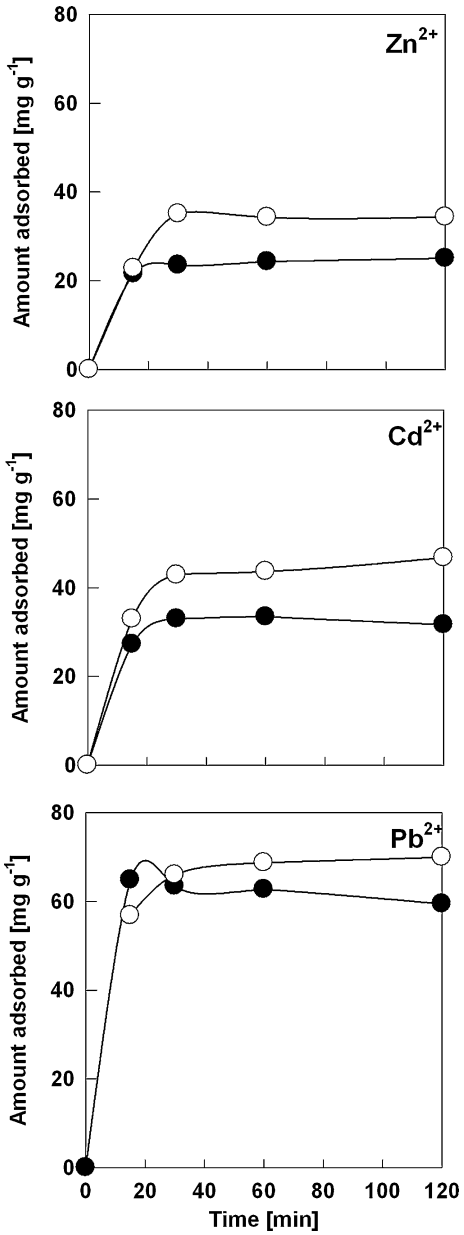

Fig. 4 Biosorption of heavy metals by strains of *Bacillus subtilis* (*open circle*) and *Bacillus* sp. (*filled circle*) over the reaction time at an initial concentration of 100 mg L^{-1}, pH 7 and 22 ± 2 °C temperature

In this study, we showed that cells grown under glucose limitation by a fed-batch like technology may have improved characteristics in connection to their metal sorption ability. The EnBase technology may help to perform parallel screening studies, without increasing the amount of cultivation work significantly. Furthermore, as the EnBase Flo system is a fed-batch cultivation technology, a scale-up from laboratory to industrial production would be straight forward. This is different if batch-type cultivation strategies are applied for laboratory experiments.

Biosorption is a metabolically passive process carried out by the non-living biomasses which doesn't undergo metabolic functions. Therefore, the process is much easier and cheaper, and the potential toxic effects of the heavy metals can be avoided [17].

Different studies confirmed that the removal capacity of resting cells from the *Bacillus* strain was markedly higher than that of growing cells [18]. Thus, to get better performance, the biomass submitted to biosorption studies was harvested during the glucose-limited growth in the EnBase Flo medium and the late exponential phase in the LB medium. The inactive bacterial biomass has been obtained by drying the cells at 60 °C till the constant weight was achieved.

The biosorption results showed that there is a strong dependence of the initial metal concentration on the removal efficiency. At high metal concentrations, the number of sites available for sorption becomes lower compared to the number of moles of solute [19–23]. Langmuir models characterized successfully the metal biosorption by the isolated strains, the correlation coefficients (R^2) being between 0.83 and 1 for 80 % of the calculated data. The validity of the Langmuir model implies a monolayer adsorption mechanism and a homogeneous distribution of the active sites onto the adsorbents surface. The experimental data obtained for the uptake capacity of Pb^{2+} by *B. subtilis* biomass are better described by the Freundlich model, which assumes that the sorbent surface is heterogeneous and a multilayer adsorption takes place.

Other studies on the uptake capacity of *B. subtilis* revealed maximum values of 63 mg g^{-1} for Zn^{2+} [24], 22 mg g^{-1} for Cd^{2+} and 124 mg g^{-1} for Pb^{2+} [25]. The maximum uptake capacities for our *Bacillus* strains were in the same order of magnitude, and even higher for Cd^{2+} (93 mg g^{-1}). This encourages us to use this strain in future studies. Similar results were obtained by Kim [5] for their *Bacillus* sp. CPB4 strain. All these comparisons with other

Table 4 Pseudo first-order model rate constants for adsorption of Zn^{2+}, Cd^{2+} and Pb^{2+} on wastewater isolates biomass

Strain	Zn^{2+}			Cd^{2+}			Pb^{2+}		
	q_1 (mg g^{-1})	k_1 (min^{-1})	R^2	q_1 (mg g^{-1})	k_1 (min^{-1})	R^2	q_1 (mg g^{-1})	k_1 (min^{-1})	R^2
Bacillus sp.	13.96	0.002	0.78	16.58	0.001	0.12	12.98	0.003	0.97
B. subtilis	3.38	0.012	0.15	15	0.023	0.91	12.95	0.023	0.88

Table 5 Pseudo second-order model rate constants for adsorption of Zn^{2+}, Cd^{2+} and Pb^{2+} on wastewater isolates biomass

Strain	Zn^{2+}			Cd^{2+}			Pb^{2+}		
	q_2 (mg g^{-1})	k_2 (g/mg min^{-1})	R^2	q_2 (mg g^{-1})	k_2 (g/mg min^{-1})	R^2	q_2 (mg g^{-1})	k_2 (g/mg min^{-1})	R^2
Bacillus sp.	19.34	3.14×10^{-3}	0.96	48.07	8.62×10^{-4}	0.94	78.74	9.76×10^{-4}	0.97
B. subtilis	36.23	5.58×10^{-3}	1.00	49.02	3.28×10^{-3}	1.00	71.94	4.28×10^{-3}	1.00

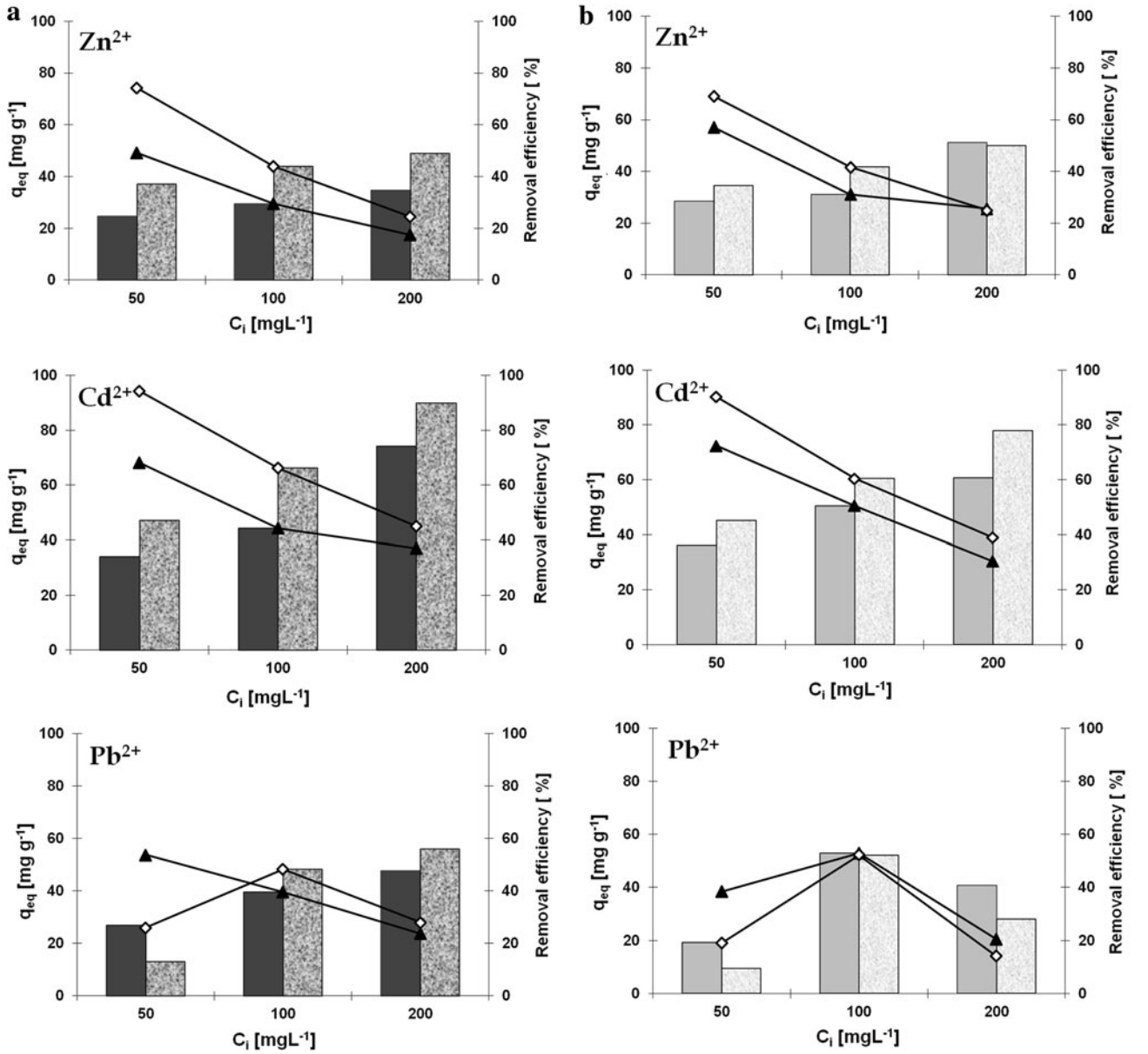

Fig. 5 Effect of increasing concentration of Zn^{+2}, Cd^{+2} and Pb^{+2} ions on the removal efficiency of inactive *Bacillus* sp. dry biomass obtained by cultivation on EnBase (**a**) and Luria–Bertani (**b**) media. *Dark* and *texture gray bars* indicate the metal uptake at equilibrium (q_{eq}) of *Bacillus* sp. and *B. subtilis* strains, respectively; *up triangle* and *opened diamonds* represent the removal efficiency (%) of *Bacillus* sp. and *B. subtilis* strains, respectively

published results indicate that our isolated bacteria are interesting candidates for further adsorption studies. However, it may be remarked that the specific numbers from different studies must be compared with caution, due to the different experimental conditions employed (pH control, temperature, equilibrium time and biomass dosage) in the different research works. In our study, the two isolated strains grown on EnBase and LB media

showed a preference for Zn^{2+} and Cd^{2+}, respectively. The sorption order Zn > Cd > Pb exhibited by the *B. subtilis* biomass on the EnBase medium could be in agreement with Tobin [26], cited by Chatterjee [27], who demonstrated that there is an indirect correlation between the metal ionic radius and the sorption rate onto the fixed area of biosorbent. The ionic radius of Zn (88 pm) is followed by Cd (109 pm) and Pb (133 pm). But the statement cannot be maintained also for the sorption capacity revealed by the bacterial strains grown on LB medium where the following retention order was identified at the *B. subtilis* strain: Cd > Zn > Pb. The *Bacillus* sp. strains showed similar retention capacity for Cd^{2+} and Zn^{2+} ions and the lowest for the Pb^{2+} ions.

A strong difference between strains could be also remarked on the uptake rate predicted by the second-order model. The *B. subtilis* biomass achieved higher uptake rate k_2 than the *Bacillus* sp. strain, for all three metals, in the following order: Zn > Pb > Cd. The biomass of *Bacillus* sp. showed an uptake rate k_2 in opposition with the other strain, the highest value being recorded for the Pb^{2+} ions. The results showed that the biosorption isotherms may exhibit an irregular pattern due to the complex nature of both the biosorbents and their varied multiple active sites, as well as the complex solution chemistry of some metallic compounds [3, 28] or growth media.

As sorption takes place on the inactive biomass surface, increasing/activating the binding sites on the surface would be an effective approach for enhancing the biosorption capacity. The comparison between two growth media showed that the cultivation strategy can influence the biosorption phenomenon. The fed-batch cultivation system EnBase Flo improved the uptake capacity of Cd^{2+} and Pb^{2+} ions by 55 and 44 %, respectively. Both *Bacillus* strains exhibited lower uptake capacity regarding the sorption of Zn^{2+} ions on EnBase Flo medium, compared to LB medium, but a twofold higher affinity was detected. The EnBase content in Zn^{2+} ions (80 μg L^{-1}) increases the possibility that a high number of active sites are already occupied during the cells growth on this medium. A more stringent washing procedure of the cells may provide an improvement. Metal biosorption by the microbial biomass depends on the protonation or deprotonation of the functional groups on the cell wall: at low pH, i.e., a high concentration of protons, metal binding sites become positively charged and metal cations and protons compete for binding sites, which results in lower uptake rate of the metal ion [28, 29]. The same phenomenon could be observed in this study, when the difference between initial and final pH values varied from 1.0 unit for Zn^{2+} and Cd^{2+} to 2.0 units for Pb^{2+} ions. This fact proves that ion-exchange mechanisms take place during metal uptake. Moreover, the pH is decreased with an increasing initial metal ion concentration (data not shown). The highest affinity recorded on the EnBase media, as well as the strong change in the solution pH can be due to the variety of active groups offered by the high polysaccharides concentration produced by the high cell density achieved on the EnBase.

Both bacterial strains isolated from food industry wastewaters removed Zn^{2+}, Cd^{2+} and Pb^{2+} from the aqueous environment and may provide an interesting "low cost" pool for heavy metals removal from the industrial wastewater.

Conclusions

In this paper, we showed that the EnBase Flo medium improved not only the growth of bacteria, which have been isolated from wastewater, but also the quality of the cells in respect to the metal uptake capacity. Nevertheless, further research is needed to show whether similar results can also be obtained for other bacterial species or microorganism categories. Clearly, the cultivation conditions are an important target for future improvement.

Acknowledgments This work was supported by the Deutsche Bundestiftung Umwelt (DBU) and it was coordinated by the Institute of Biotechnology from the Technical University in Berlin, Germany.

Conflict of interest The authors have declared no conflict of interest.

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