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Impact of substrates and cell immobilization on siderophore activity by Pseudomonads in a Fe and/or Cr, Hg, Pb containing-medium

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Abstract

To increase the amount of bioavailable metals in phytoextraction purposes, soil bioaugmentation with Pseudomonads, as siderophore producers with high metal complexation levels, could be relevant. Unfortunately, siderophore synthesis may be inhibited by soluble iron in soil and bacteria can suffer at the same time from the toxicity of some other metals, predation and oligotrophy. To overcome these drawbacks, we attempted to co-locate a carbon substrate and *Pseudomonas aeruginosa* or *P. fluorescens* in Ca-alginate beads. First, free-cell cultures showed that glycerol, fructose, mannitol and skim milk enhanced the siderophore activity which was the highest in the medium with neither Fe or TM (toxic metal) (Cr, Hg and Pb) and the lowest in the Fe-containing medium without TM. The negative effect of iron was partly offset when TM was added to the medium. In a second part, co-location of microorganisms and substrates was only feasible with skim milk. By comparison with free cells, siderophore activity by immobilized cells was higher in culture media containing Fe with or without TM (up to a ratio of 9), and varied in a narrow margin, according to the medium composition.

Keywords: Cell immobilization; Iron; *Pseudomonas aeruginosa*; *Pseudomonas fluorescens*; Siderophore activity; Toxic metals; Carbon substrates

1. Introduction

In order to enhance the amount of bioavailable toxic metals (TM) in phytoremediation processes, various synthetic compounds (e.g., DTPA, EDGA, EDTA, etc.) have already been used but their biodegradability is rather low by comparison with microbial chelates [1] and they can be phytotoxic at high concentrations [2]. As an alternative, several studies have shown the ability of microbial siderophores to increase metal bioavailability in soil [3,4–8] at a higher rate than synthetic chelates [9,10]. Among several siderophore producers, we showed recently that Pseudomonads were serious candidates [11,12]. Thus, soil bioaugmentation combined with phytoextraction is a promising technology for the soil clean-up [6].

Two major requirements must be fulfilled to optimize bioaugmentation processes and to guarantee a reliable process irrespective of the soil physico-chemical characteristics. On one hand, microbial survival must be ensured. This can be achieved by immobilizing microorganisms into carriers (e.g. alginate, clay, peat, etc.) which protect them against the natural competition of the soil microflora [13–15] and protozoa grazing [16]. On the other hand, the soil conditions must favour the siderophore synthesis which is all the more high since the environment is low in Fe [17–19]. In spite of the high insolubility of iron (Fe(III)), soluble forms are found in some environmental conditions, e.g. anoxic soil conditions [20]. Consequently the siderophore biosynthesis can be inhibited. In a previous study [11], we demonstrated that immobilization of *Pseudomonas aeruginosa* and *P. fluorescens* contributed to increase the siderophore activity by creating a deficiency in minerals, in particular Fe, inside Ca-alginate beads. This deficiency in minerals was due to their adsorption onto the bead surface [21]. Microbial survival was improved towards Cr, Hg and Pb toxicity, as already observed with several metals, e.g., Cd, Co, Cu, Hg, Mn, Zn [22–25].

Substrate supply to free microbial cells (e.g., glucose, glycerol, lactose, and mannitol) can increase both the cell growth and the siderophore activity [26–28]. At the same time, immobilized cells survival in soil was shown to be enhanced thanks to the co-location of the carbon substrate and the inoculant strain [29]. This co-location can selectively promote the growth of inoculated cells and not the indigenous microbial population of the soil.

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The aim of our study was to compare the ability of various substrates (fructose, glucose, glycerol, lactose, mannitol, skim milk, starch, sucrose) to enhance the siderophore activity by *P. aeruginosa* and *P. fluorescens*. Free and immobilized cells in Ca-alginate beads were cultivated in a liquid culture medium containing Fe and/or TM (mixture of Cr, Hg and Pb) in concentrations which corresponded to the bioavailable fraction of a contaminated agricultural soil.

2. Materials and methods

2.1. Microbial strains and preculture conditions

P. aeruginosa ATCC 9027 and *P. fluorescens* DSM 50090 were precultivated at 28 °C for 24 h in shaken Erlenmeyer flasks containing LB medium. Cultures were centrifuged and washed twice with a solution of KCl (9 g l^{-1}) [11]. Cell concentration of bacterial suspensions was determined by measuring the optical density (OD) of the samples at 600 nm and by relating to cells ml^{-1} from appropriate standard curves.

2.2. Immobilization procedure

A 100 ml sterile solution of Na-alginate was mixed thoroughly with the cell suspension to reach a final 3% (w/v) Na-alginate. Ca-alginate beads were obtained by dropping the Na-alginate cell mixture into a 750 ml stirred-solution of $CaCl_2$ (30 g l^{-1}) by using a peristaltic pump supplied with a calibrated needle [11]. Ca-alginate beads (5 g) were introduced per Erlenmeyer flask.

2.3. Microbial cell cultures

Erlenmeyer flasks were filled with 75 ml of modified minimal medium with or without 4 mg Fe l^{-1} [11]. Iron in the culture medium supplied or not with substrates did not exceed 10 ppb.

The medium was sterilized at 120 °C during 20 min. Toxic metals (TM) (Cr, 5 mg l^{-1}; Hg, 0.2 mg l^{-1}; Pb, 13 mg l^{-1} in the following forms: $CrCl_3$. $6H_2O$; $HgCl_2$, $Pb(NO_3)_2$) were added or not to the medium after filtration through a 0.45 μm pore size filter (cellulose-acetate membrane, Dutscher Distribution).

A solution of either fructose, glucose, glycerol, lactose, mannitol, skim milk, starch or sucrose was sterilized at 115 °C during 30 min. The same amount of substrate was added to the medium for free and immobilized cell cultures. For free cells, 0.67 g l^{-1} of medium (low concentration, C1) or 6.7 g l^{-1} (high concentration, C2) was added. For immobilized cells, 100 g l^{-1} Na-alginate (high concentration, C2) was added.

Each Erlenmeyer flask was inoculated with free or immobilized cells: low inoculum size (LIS) was 6.7×10^4 cells ml^{-1} of medium or 1×10^6 cells ml^{-1} of alginate; high inoculum size (HIS) was 6.7×10^7 cells ml^{-1} of medium or 1×10^9 cells ml^{-1} of alginate. Control with non-inoculated beads was used.

All Erlenmeyer flasks were incubated at 28 °C and shaked at 200 rpm.

Experiments were done in triplicate.

2.4. Analytical methods

2.4.1. Siderophore measurement

Siderophore activity was determined according to the universal liquid CAS test [30]. Bacterial supernatant was mixed with CAS-reagent 1:1 (v/v). The reaction was accelerated by addition of 5-sulfosalycilic acid 4 mM. Disappearance of the complex CAS–iron was measured at the wavelength 630 nm after 2 h with a Beckman-Coulter DU530 spectrophotometer. Appropriate dilutions of bacterial supernatants were made in minimal medium. To avoid bias caused by interference of metals contained in medium with CAS reactive, a correction previously described [11] was applied to the values.

2.4.2. Microbial biomass determination

Cell growth in Erlenmeyer flasks was determined from both liquid medium at 600 nm, as mentioned above and from the beads at the same wavelength after dissolution with 50 mM of trisodium citrate. Cell concentrations were determined by measuring OD of samples and by relating to cells ml^{-1} from an appropriate standard curve. Because of the opaqueness of skim milk-containing medium, bacterial growth was determined in this case by plating 100 μl of the culture medium on LB-agar plates after decimal dilutions in KCl 9 g l^{-1}.

2.5. Statistical analysis

Analysis of variance (ANOVA) were performed from a randomised multifactorial design with a mean comparison according to the Newman–Keuls test, by using Statbox Pro software (GrimmerSoft, version 5, Paris, France). Statistical significance was determined at $P = 0.05$. Homogeneous groups were designated by letters.

3. Results and discussion

3.1. Effect of substrates on the growth and siderophore activity of free cells

Eight substrates were tested, i.e., fructose, glucose, glycerol, lactose, mannitol, skim milk, starch and sucrose, for their ability to enhance the siderophore activity [26–28,31]. Indeed the chemical composition of media (e.g. carbohydrate substrates) influences greatly the siderophore activity [32] although siderophores were often produced in minimal media but only in the aim to facilitate their purification and chemical characterization. For example, glucose strongly influenced the siderophore activity by *P. fluorescens* CHAO [26]. Fructose [26], mannitol [26,31], and glucose [27,31] improved pyochelin and pyoverdine production, two main siderophores produced by fluorescent Pseudomonads. Glycerol can also enhance the siderophore production in the presence of zinc sulfate [26]. Carbon substrates are known also to influence the variability of biocontrol agents along with the metabolite production in soils and in host-plant rhizosphere whose root exudates composition change [33]. Carbohydrate substrates tested in this study aimed to determine the effect of some substrates known to be exudated by maize (our

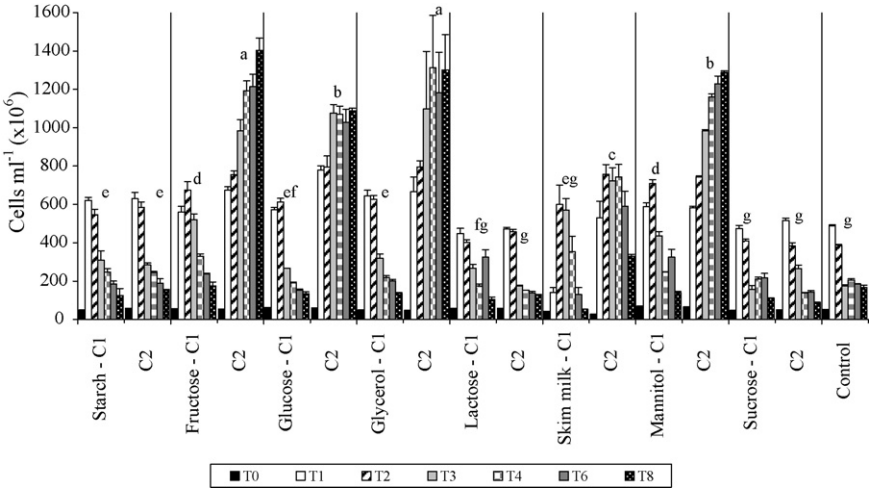

Fig. 1. Free-cell growth of *Pseudomonas aeruginosa* in the presence of different substrates at low (C1) and high (C2) concentrations. High inoculum size was used in this experiment. Time of incubation (T0, T1, etc.) was expressed in days and results were shown as mean ± S.D. Letters corresponded to homogenous groups from ANOVA irrespective of the time of incubation.

model for phytoremediation), i.e., glucose, sucrose and fructose [34], on the siderophore activity. As it is explained afterwards, skim milk was also tested because it is the only one substrate that was not released out of alginate beads when bacterial cells were immobilized. Consequently we also tested the effect of lactose, the main carbohydrate source in skim milk.

Effects on the cell growth are shown in Fig. 1. By comparison with the control (minimal medium without substrate), fructose, glucose, glycerol or mannitol increased significantly ($P < 0.05$) the growth of both *P. aeruginosa* (Fig. 1) and *P. fluorescens* (data not shown). At concentration C1, maximal cell concentration occurred at 1 up to 2 days after the beginning of incubation and later for C2, i.e., from 2 up to 8 days, because of the higher amount of substrate available for bacteria. Substrates increased the growth of both bacteria by more than one log unit at the maximum. Maximal concentration was observed with the concentration C2 and reached 1.4×10^9 cells ml^{-1} for *P. aeruginosa* cultivated with fructose as compared with *P. fluorescens* with mannitol (8.3×10^8 cells ml^{-1}). Skim milk and starch had also a positive effect on the growth of *P. aeruginosa* (expect in the case of skim milk at the concentration C1), as did sucrose at the concentration C2 for *P. fluorescens* (data not shown). However, there was not any effect of lactose for the two microorganisms and starch for *P. fluorescens*. We also observed a slightly adverse effect of skim milk towards *P. fluorescens*. With substrates such as fructose, glucose, glycerol and mannitol at the high concentration (C2) the cell growth was higher most probably because of the optimal C/N ratio. Indeed, C/N was about 25 which is close to that of bacteria against only 2.5 for C1.

The content in available nutrients is usually low in soils [14] and homeostasy prevails. Consequently the survival along with the colonization of the exogenous microorganisms added to soils most often failed [35,36]. Therefore, well suited substrates supplied to exogenous microorganisms give a competitive advantage to the inoculated microorganisms. At the same time, Thompson et al. [35] suggested selecting microorganisms for bioremediation purposes on the basis of their growth rate as a fundamental parameter to be considered for the bioaugmentation success. In our case, *P. aeruginosa* was a good candidate for soil colonization as observed in current experiments with GFP-tagged *P. aeruginosa* (data not shown).

The same substrates as for cell growth increased the siderophore activity of both *P. aeruginosa* (Fig. 2) and *P. fluorescens* (data not shown). The highest siderophore activities were observed with the high substrates concentration (C2), except glucose. Acidic pH of the medium – 4.3 and 5.2 for *P. fluorescens* and *P. fluorescens*, respectively – might explain this result as already shown with pyoverdine siderophores [37]. In general, the maximum siderophore activity was higher with *P. aeruginosa* which might have been related to its chemical characteristics rather than its amount [38]. It was also observed that the nature of the siderophores changed with the medium [39]. For example in iron-limiting conditions, percentage of pyoverdine was higher in *P. aeruginosa* over pyochelin because of its efficiency to bind iron.

In the present study, the coupling between growth and siderophore activity was shown to be dependent on the substrate. In a previous study, siderophore production was shown to be coupled with growth for glucose while it started only when the stationary stage of growth was reached for succinate [27]. Siderophore activity increased significantly ($P < 0.05$) by using the substrates in the following order for *P. aeruginosa* (Fig. 2) at the concentration C2: control, lactose, starch, sucrose < glucose < fructose < mannitol, glycerol < skim milk, and for *P. fluorescens* (data not shown): control, lactose, skim milk, starch, sucrose < glucose < mannitol < glycerol < fructose. By comparison with the control, the siderophore activity of *P. aeruginosa* supplied with skim milk at C2 and that of *P. fluorescens* supplied with fructose at C2 increased by a factor of 18 and 84, respectively.

Moreover, we observed that skim milk stimulated the growth and siderophore activity contrary to lactose. We can suppose that another compound of skim milk, e.g. caseinogen which is the main proteic compound, was responsible in enhancing the

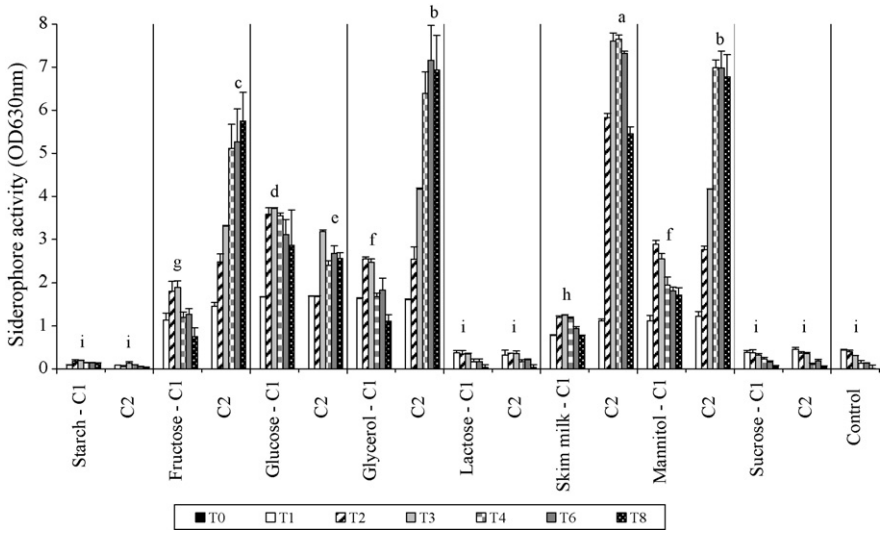

Fig. 2. Siderophore activity of free *P. aeruginosa* cells in the presence of different substrates at low (C1) and high (C2) concentrations. High inoculum size was used in this experiment. Time of incubation (T0, T1, etc.) was expressed in days and results were shown as mean ± S.D. Letters corresponded to homogenous groups from ANOVA irrespective of the time of incubation.

siderophore activity. During the manufacturing process of skim milk (steam-sterilization), a small amount of lactose was also hydrolyzated in glucose and galactose, which most probably enhanced the siderophore activity.

The specific siderophore activity, which is the siderophore activity to the biomass ratio, was shown in Fig. 3. This parameter gives an overall indication of the microbial metabolism towards the siderophore synthesis. Specific siderophore activity of *P. aeruginosa* (Fig. 3a) increased with fructose, glucose, glycerol, mannitol and skim milk – up to 15 times for glucose by comparison with the control – contrary to what was observed with lactose, starch and sucrose (no effect). With *P. fluorescens* (Fig. 3b), conclusions were the same except for glucose and skim milk at the concentration C1 and C2, respectively. Glycerol and mannitol increased both the growth and the siderophore activity of both bacteria along with glucose and skim milk for *P. aeruginosa*. Fructose stimulated the cell growth of *P. aeruginosa* but not the siderophore activity. The results were different with *P. fluorescens*: fructose stimulated the siderophore activity, skim milk had no effect on both growth and siderophore activity and glucose only stimulated the growth. Our results confirmed the positive effect of fructose, glucose, mannitol and to a lower extent glycerol shown by Duffy and Defago [26] and Abd-Alla [31] with the same species.

3.2. Effect of Fe and/or TM on siderophore activity by free cell cultures supplied with either fructose, glycerol, mannitol or skim milk

Quite the same positive effect of substrates were shown on the cell growth rates (μ_{max}) of *P. aeruginosa* and *P. fluorescens* (Fig. 4a and b), except skim milk which showed a proportionally higher effect on μ_{max} than the other substrates. Cultures with low inoculum sizes (LIS) exhibited higher μ_{max} as compared with their high inoculum (HIS) counterparts, irrespective of the treatment. Addition of metals in medium showed opposite effects depending on the metal added to the culture medium, i.e., toxic metals (TM) and/or Fe. By comparison with the control (LIS and HIS), iron slightly stimulated the growth, as already observed by Abd-Alla [31] while TM associated or not with iron lowered μ_{max}, especially that of *P. fluorescens*. In fact when iron is the limiting factor in culture media, requirement of *P. fluorescens* in nitrogen is higher than in carbon [40]. This phenomenon might explained why no significant difference in μ_{max} was observed with Fe-free media (with or without TM) irrespective of the three substrates (fructose, glycerol, mannitol) (Fig. 4a). Only skim milk allowed a higher cell growth irrespective of the treatment maybe because of nitrogen supplied by this substrate.

Cell cultures supplied with substrates exhibited a higher maximum biomass (1400×10^6 cells ml^{-1} for *P. fluorescens* and 5400×10^6 cells ml^{-1} for *P. aeruginosa* with the LIS-Fe-glycerol and the LIS-Fe-skim milk, respectively) as compared with the substrate-free medium (Fig. 4a and b). The difference reached about 1 log unit depending on the treatment. As for μ_{max}, the maximum biomass was higher with Fe added to the culture medium while TM had an adverse effect, especially in iron-free medium. Maximum biomass of both bacteria was reached with skim milk. In the main the type of substrate (except skim milk) and the inoculum effect did not matter much on the maximal biomass contrary to what was observed with μ_{max}.

The siderophore activity was shown in Table 1a and b. Overall the siderophore activity of *P. aeruginosa* cultures was by far the highest, irrespective of the treatments. The siderophore activity was the highest in the medium without Fe and TM and the lowest in the Fe-containing medium irrespective of the inoculum size and the bacteria. Iron limitation is known to stimulate siderophore production [32]. The highest to the lowest siderophore activity ratio reached a factor of 66 and 308 at the maximum for *P. fluorescens* (Table 1b) and *P. aeruginosa* (Table 1a), respectively. The lowest siderophore activity was observed with Fe-containing medium as already described by several authors [17–19]. Indeed, it was shown [27,41] that

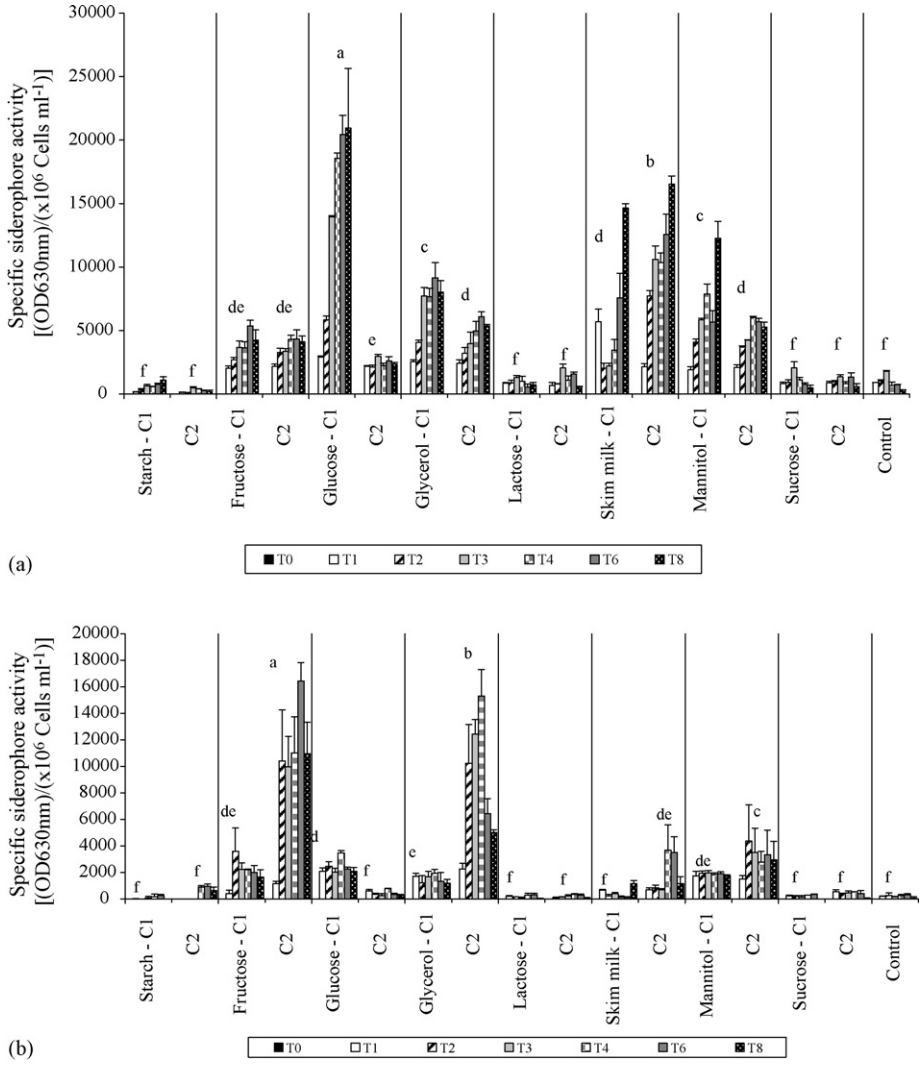

Fig. 3. Specific siderophore activity of free *P. aeruginosa* and *P. fluorescens* cells in the presence of different substrates at low (C1) and high (C2) concentrations. High inoculum size was used in this experiment. Time of incubation (T0, T1, etc.) was expressed in days and results were shown as mean ± S.D. Letters corresponded to homogenous groups from ANOVA irrespective of the time of incubation: (a) *P. aeruginosa* and (b) *P. fluorescens*.

pyoverdine production was inhibited when Fe(III) concentrations reached 10 μM and Visca et al. [42] recorded an inhibition of both pyoverdine and pyochelin at 0.1 μM. In our study, iron was added at a concentration of 70 μM. The repression of the siderophore synthesis was the result of a regulation cascade involving the protein Fur whose activity was regulated by iron [43,44]. The negative effect of iron was partly offset when TM was added to the medium (Table 1a and b), which confirmed our previous results [11], without reaching however the level of the control medium (LIS, HIS). TM might competed with Fe–siderophore complexes as it was shown for vanadium [45], Mn^{2+}, Co^{2+} and some other divalent cations [46]. One hypothesis suggests that divalent cations such as Cd could interact with thiols of the protein Fur, so that repression mechanism of siderophore synthesis was not activated [47]. Some metals were even shown to stimulate the siderophore production [26,48], depending on the metal and its interaction with carbon substrates. Höfte et al. [49] postulated that the stimulation of the siderophore production could be part of a general stress in response to metals observed with Cd, Ni and Zn while Dao et al. [50] showed the contrary with Hg. The only moderate adverse effect of TM on the siderophore activity might also have been the consequence of the low concentrations in Cr, Pb and Hg used in our study. Indeed, at higher concentrations, TM would most probably inhibit the siderophore synthesis as already shown by Abd-Alla [31] with the hydroxamate siderophore – its synthesis was only 26 and 16% of the control with 80 and 160 mg Hg l^{-1}, respectively – and by Hassen et al. [51] with pyoverdine with greatly higher Cr and Hg concentrations, i.e., 26 and 4 mg l^{-1}, respectively, than in our study.

The effect of substrates was contrasted. In the main they enhanced the siderophore activity by comparison with the control, irrespective of the treatment except for the Fe-containing medium where the siderophore activity was not enhanced (same level as the control). The difference in the siderophore activity was in the range of 2 up to 17 for *P. fluorescens*, depending on the treatment, and 10 up to 22 for *P. aeruginosa* which exhibited

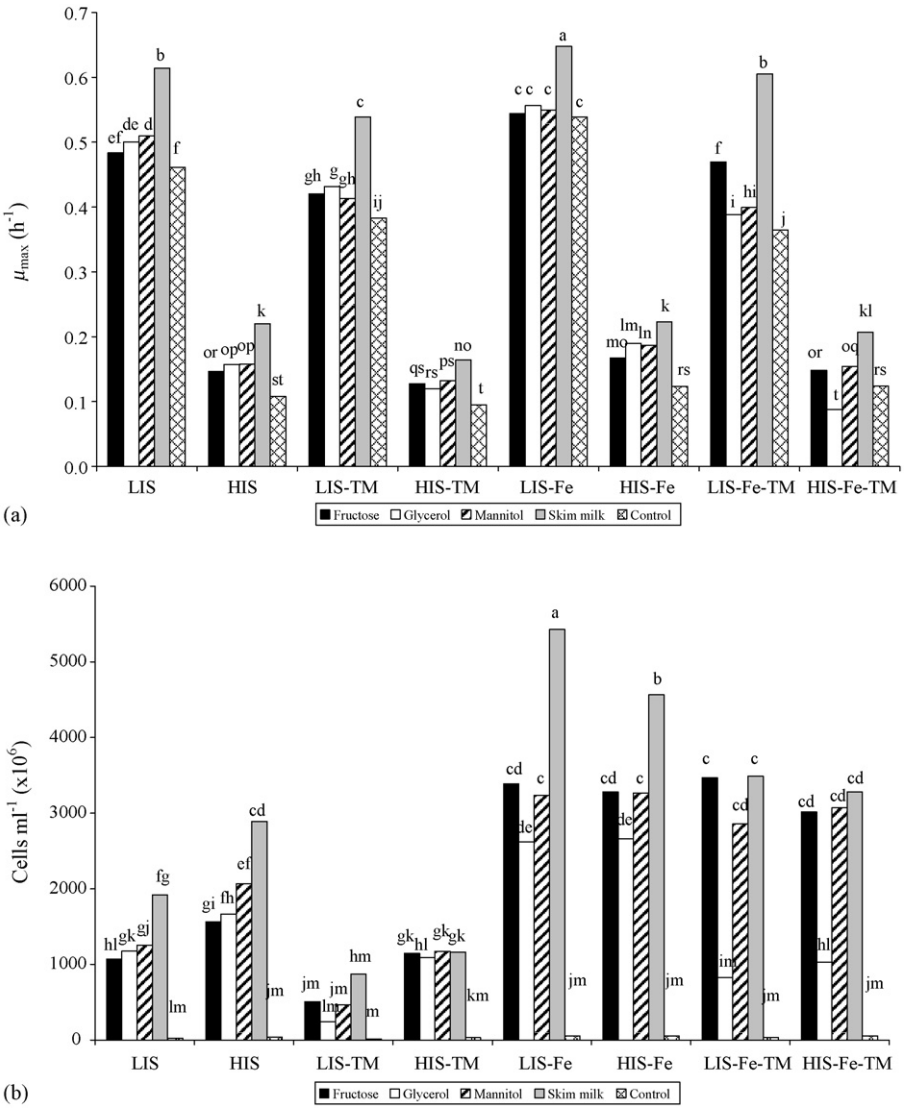

Fig. 4. Free-cell growth (μ_{max} and maximal biomass) of *P. aeruginosa* cultivated in the presence of Fe and/or TM and supplied with either fructose, glycerol, mannitol or skim milk (concentration C2). LIS, low inoculum size; HIS, high inoculum size. Results show means and letters correspond to homogenous groups from ANOVA irrespective of the time of incubation: (a)μ_{max}; (b) maximal biomass.

the highest siderophore activity (about 2 up to 94 times by comparison with *P. fluorescens*). Fructose clearly increased the most the siderophore activity in the Fe-containing medium with TM irrespective of the microorganism and the inoculum size. The same result was observed for skim milk in the TM-containing medium (except for the HIS for *P. aeruginosa*).

The specific siderophore activity (Table 1c and d) confirmed inhibition of the siderophore activity by Fe and the suppression of inhibition when Fe was combined with TM. In the main, TM showed only a moderate negative effect on the siderophore synthesis. By comparison with the control, substrates had contrasted effects, depending on the treatment, except skim milk which showed either no effect or a marked negative effect with *P. fluorescens* irrespective of the treatment and with *P. aeruginosa* cultivated in the medium enriched with Fe and Fe with TM. With this substrate, the level of the specific siderophore activity was close to that recorded with the Fe-containing medium.

3.3. Co-location of carbon substrates and bacteria in alginate beads

Bioaugmentation studies imply that exogenous microorganisms are competitive towards indigenous ones otherwise introduced cells do not always survive [36]. In this aim, immobilization of viable cells was shown by several authors as a convenient technique for improving their survival [13–15]. Also the location of substrate into the immobilization carrier can give a competitive advantage to the inoculated bacterium as already shown with glycerol and *P. fluorescens* immobilized cells in microgranules [29]. Likewise, we demonstrated in a previous work [11] the positive effect of cell immobilization in the maintenance of the siderophore activity in Fe-containing media. Thus, to both selectively favour the growth of immobilized *P. aeruginosa* and *P. fluorescens* and to improve the siderophore activity and the cell survival, we attempted to locate the substrates in Ca-alginate beads by mixing them with Na-alginate solution. Unfortunately

Table 1
Siderophore activity ((a) and (b)) and specific siderophore activity ((c) and (d)) of free *Pseudomonas aeruginosa* and *P. fluorescens* cells cultivated in the presence of Fe and/or TM and supplied with either fructose, glycerol, mannitol or skim milk (concentration C2)

	Control	Fructose	Glycerol	Mannitol	Skim milk
(a) *P. aeruginosa*					
LIS	0.872 (o)	3.255 (m)	6.824 (g)	8.952 (c)	8.005 (d)
HIS	0.796 (o)	5.258 (jk)	10.131 (b)	10.400 (a)	7.546 (e)
LIS-TM	0.394 (ps)	1.886 (n)	0.243 (qs)	0.805 (o)	5.583 (i)
HIS-TM	0.635 (op)	4.142 (l)	5.369 (ij)	8.120 (d)	6.205 (h)
LIS-Fe	0.236 (qs)	0.096 (s)	0.184 (rs)	0.146 (s)	0.026 (s)
HIS-Fe	0.211 (qs)	0.131 (s)	0.346 (ps)	0.169 (rs)	0.033 (s)
LIS-Fe-TM	0.34 (ps)	7.192 (f)	1.916 (n)	4.159 (l)	0.568 (l)
HIS-Fe-TM	0.354 (ps)	7.872 (d)	5.017 (k)	3.984 (l)	0.604 (l)
(b) *P. fluorescens*					
LIS	0.304 (hj)	0.564 (f)	0.451 (g)	0.296 (hj)	0.325 (h)
HIS	0.089 (os)	1.464 (b)	1.522 (b)	0.756 (d)	0.080 (ps)
LIS-TM	0.057 (os)	0.169 (lp)	0.143 (mr)	0.169 (lp)	0.470 (g)
HIS-TM	0.094 (op)	0.552 (f)	0.272 (hk)	0.216 (jm)	0.470 (g)
LIS-Fe	0.050 (qs)	0.042 (rs)	0.063 (ps)	0.047 (qs)	0.012 (s)
HIS-Fe	0.058 (ps)	0.022 (s)	0.045 (rs)	0.064 (ps)	0.016 (s)
LIS-Fe-TM	0.190 (ko)	0.635 (e)	0.201 (kn)	0.154 (mr)	0.098 (ns)
HIS-Fe-TM	0.258 (il)	2.287 (a)	0.939 (c)	0.357 (h)	0.160 (lq)
(c) *P. aeruginosa* ($\times 10^6$ cells ml^{-1})					
LIS	10830.3 (a)	4473 (g)	7548.5 (cd)	8486.5 (c)	9303 (b)
HIS	4443.6 (gh)	4219.4 (gh)	6552.3 (ef)	5751 (f)	8404.4 (c)
LIS-TM	4623.3 (g)	5853.9 (f)	2527.6 (j)	4920.2 (g)	7581.3 (cd)
HIS-TM	2995.5 (ij)	4375.1 (gb)	7211.1 (de)	7721.5 (cd)	7439.3 (cd)
LIS-Fe	886.1 (l)	72 (l)	136.7 (l)	106.4 (l)	12.2 (l)
HIS-Fe	681.3 (l)	63.2 (l)	159.7 (l)	93.1 (l)	14.7 (l)
LIS-Fe-TM	5965.8 (f)	4214.5 (gh)	9773.3 (b)	954 (gh)	289.7 (l)
HIS-Fe-TM	1147.5 (kl)	3635.6 (hi)	8223.3 (cd)	1755 (k)	275.2 (l)
(d) *P. fluorescens* ($\times 10^6$ cells ml^{-1})					
LIS	2728.8 (eh)	1604.8 (gk)	1511 (gk)	1248.6 (hk)	511.7 (jk)
HIS	351.5 (jk)	3741.4 (de)	2878.6 (eg)	1564.9 (gk)	56 (k)
LIS-TM	2561.7 (eh)	3149.5 (ef)	4440.3 (cd)	6306 (b)	764.8 (ik)
HIS-TM	886.5 (ik)	2019.2 (fi)	2116.6 (fi)	2551.8 (eh)	286.6 (jk)
LIS-Fe	220.8 (k)	101.7 (k)	59.8 (k)	68.9 (k)	17.7 (k)
HIS-Fe	158.3 (k)	35.2 (k)	52.5 (k)	71.7 (k)	21.9 (k)
LIS-Fe-TM	9835.4 (a)	5328.6 (c)	6915.6 (b)	6711.2 (b)	130.8 (k)
HIS-Fe-TM	1739.3 (fj)	2937.1 (eg)	2407.1 (eh)	1489.1 (gk)	86 (k)

LIS, low inoculum size; HIS, high inoculum size. Results show means. In parentheses, letters correspond to homogenous groups from ANOVA irrespective of the time of incubation: (a) and (c), *P. aeruginosa*; (b) and (d), *P. fluorescens*.

all the substrates, except skim milk, released from beads until their concentrations equilibrated with the bulk liquid medium (data not shown). Indeed diffusion coefficients of glucose, fructose, sucrose and lactose in Ca-alginate membrane liquid-core capsules and in Ca-alginate beads immobilizing microorganisms were shown to be only 5–20% lower than the corresponding diffusivity in water [52,53] which explained why the concentration of solute in the bulk liquid medium reached equilibrium after a few minutes. The same conclusion was drawn by Duquenne et al. [29] who suggested that diffusion of glycerol from microgranules to the soil limited the competitive advantage of such location effect. Conversely, skim milk remained in beads due to its chemical properties involving interactions with alginate. Several authors showed beneficial effects of this substrate in bioaugmentation purposes [54–56]. Although skim milk was not shown as the best substrate to stimulate the siderophore production, we subsequently used this substrate in the study.

3.4. Siderophore activity of bacteria co-located with skim milk in Ca-alginate beads

In the main, μ_{max} of free and immobilized cells were not very different (0.6 h^{-1} at the maximum) and varied irrespective of the medium (Fe and/or TM) and the bacteria (data not shown). However, μ_{max} of immobilized cells cultivated with skim milk was mainly higher than that of free cells. Due to both growth in beads and saturation of the porosity, cells released out of the beads and multiplied in the culture medium as shown by several authors [56–58]. μ_{max} of released cells was higher (about 1 h^{-1}) than that recorded in beads or for free cell cultures and did not depend on metals (TM, Fe). We concluded that

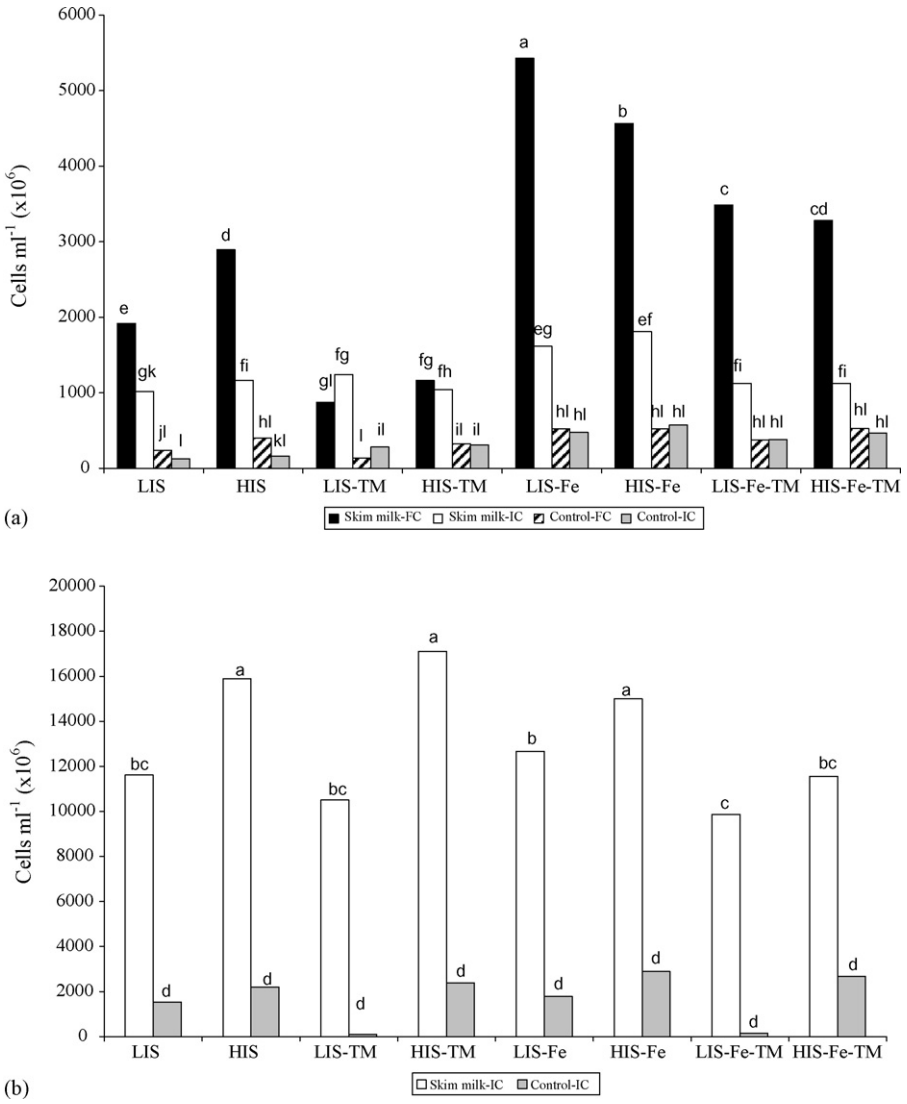

Fig. 5. Maximal biomass of free (FC) and immobilized (IC) *P. aeruginosa* cultivated in the presence of Fe and/or TM and supplied with skim milk (concentration C2). LIS, low inoculum size; HIS, high inoculum size. Results show means and letters correspond to homogenous groups from ANOVA irrespective of the time of incubation: (a) bacteria in the liquid medium; (b) bacteria immobilized in Ca-alginate beads.

metals were not available for cells, or to a lower extent, due to their adsorption onto beads. Indeed, we demonstrated in a previous study [11] that 99.1% of Cr, 57.4% of Hg and 99.6% of Pb were adsorbed onto beads. The maximal biomass of free *P. aeruginosa* cells and that of the cells released out of the beads were shown in Fig. 5a. The maximum biomass of the released cells was higher with skim milk as a consequence of the saturation of the porosity in beads as confirmed with the results shown in Fig. 5b. Indeed maximal biomass in beads supplemented with skim milk was 1 log unit higher than that of the control. As already observed with μ_{max}, the maximum biomass was nearly the same irrespective of the medium and the inoculum size (Fig. 5b). Conversely the maximum biomass varied a lot with free cells supplied with skim milk (the highest with Fe and the lowest with TM) (Fig. 5a). This result confirmed the maintenance in close limits of the environmental conditions in beads instead of external modifications of the environmental conditions.

P. aeruginosa exhibited higher siderophore activity than *P. fluorescens* up to a factor of 96, as previously observed, depending on the treatment (Table 2a and b). Skim milk increased the siderophore activity of the bacteria (up to a factor of 20 and 8 at the maximum for *P. aeruginosa* and *P. fluorescens*, respectively) except for Fe and Fe-TM (mainly in case of *P. fluorescens*), as already mentioned above (see Section 3.1.) where this substrate had an adverse effect on the siderophore activity irrespective of the culture technique (free *versus* immobilized cells).

With Fe-free media, the siderophore activity by free cells was greatly higher than that of their immobilized counterparts (13 and 3 times at the maximum for *P. aeruginosa* and *P. fluorescens*, respectively). In Fe-containing media (with or without TM), the contrary was shown. The siderophore activity of immobilized *P. aeruginosa* and *P. fluorescens* cells with skim milk was higher than that of free cells – up to a factor of 9 and 3 for *P. aeruginosa* and *P. fluorescens*, respectively – which confirmed our previous results [11]. Indeed we postulated that the combination of metal

Table 2
Siderophore activity ((a) and (b)) and specific siderophore activity ((c) and (d)) of free (FC) and immobilized (IC) cells of *P. aeruginosa* and *P. fluorescens* cultivated in the presence of Fe and/or TM and supplied with skim milk (concentration C2)

	Control-FC	Control-IC	Skim milk-FC	Skim milk-IC	Skim milk-IC/ skim milk-FC	Control-IC/ control-FC	Skim milk-IC/ control-IC	Skim milk-FC/ control-FC
(a) *P. aeruginosa*								
LIS	0.872 (i)	0.068 (n)	8.005 (a)	1.345 (g)	0.2	0.1	19.8	9.2
HIS	0.796 (ij)	0.094 (mn)	7.546 (b)	1.605 (f)	0.2	0.1	17.1	9.5
LIS-TM	0.390 (l)	0.191 (ln)	5.583 (d)	1.109 (h)	0.2	0.5	5.8	14.3
HIS-TM	0.635 (jk)	0.169 (ln)	6.205 (c)	2.138 (e)	0.3	0.3	12.7	9.8
LIS-Fe	0.236 (ln)	0.230 (ln)	0.026 (n)	0.239 (ln)	9.2	1.0	1.0	0.1
HIS-Fe	0.211 (ln)	0.223 (ln)	0.033 (n)	0.246 (ln)	7.5	1.1	1.1	0.2
(b) *P. fluorescens*								
LIS-Fe-TM	0.322 (lm)	0.308 (lm)	0.568 (k)	0.700 (jk)	1.2	1.0	2.3	1.8
HIS-Fe-TM	0.354 (l)	0.372 (l)	0.604 (k)	1.352 (g)	2.2	1.1	3.6	1.7
LIS	0.305 (bc)	0.131 (hi)	0.325 (b)	0.107 (hj)	0.3	0.4	0.8	1.1
HIS	0.082 (im)	0.128 (hi)	0.079 (im)	0.103 (hk)	1.3	1.6	0.8	1.0
LIS-TM	0.057 (jn)	0.102 (hk)	0.470 (a)	0.232 (df)	0.5	1.8	2.3	8.2
HIS-TM	0.094 (hl)	0.211 (df)	0.470 (a)	0.252 (ce)	0.5	2.2	1.2	5.0
LIS-Fe	0.046 (jn)	0.081 (im)	0.012 (n)	0.031 (ln)	2.6	1.8	0.4	0.3
HIS-Fe	0.036 (kn)	0.083 (im)	0.016 (mn)	0.032 (ln)	2.0	2.3	0.4	0.4
LIS-Fe-TM	0.190 (fg)	0.225 (df)	0.093 (hl)	0.201 (eg)	2.2	1.2	0.9	0.5
HIS-Fe-TM	0.267 (cd)	0.306 (bc)	0.153 (g)	0.156 (gh)	1.0	1.1	0.5	0.6

	Control-FC	Control-IC	Skim milk-FC	Skim milk-IC
(c) *P. aeruginosa* ($\times 10^6$ cells ml^{-1})				
LIS	10830 (a)	986 (hi)	9303 (b)	1117 (hi)
HIS	4444 (f)	1192 (hi)	8404 (c)	1348 (hi)
LIS-TM	4623 (f)	1129 (hi)	7581 (d)	1195 (hi)
HIS-TM	2996 (g)	1400 (hi)	7439 (d)	1968 (h)
LIS-Fe	886 (hj)	1329 (hj)	12 (i)	213 (i)
HIS-Fe	681 (hi)	683 (hi)	15 (i)	167 (i)
LIS-Fe-TM	5966 (e)	4646 (f)	290 (i)	703 (hi)
HIS-Fe-TM	1148 (hi)	1734 (hi)	275 (i)	1177 (hi)
(d) *P. fluorescens*				
LIS	2729 (de)	3309 (cd)	512 (gj)	1358 (fj)
HIS	352 (hj)	2878 (de)	56 (j)	4035 (c)
LIS-TM	2562 (df)	1865 (eg)	765 (gj)	158 (j)
HIS-TM	887 (gj)	1741 (ei)	287 (hj)	146 (j)
LIS-Fe	221 (ij)	536 (gj)	18 (j)	381 (j)
HIS-Fe	158 (j)	353 (hj)	22 (j)	434 (gj)
LIS-Fe-TM	9835 (a)	6445 (b)	131 (j)	226 (ij)
HIS-Fe-TM	1739 (ei)	1771 (eh)	86 (j)	182 (j)

LIS, low inoculum size; HIS, high inoculum size. Results show means. In parentheses, letters correspond to homogenous groups from ANOVA irrespective of the time of incubation: (a) and (c) *P. aeruginosa*; (b) and (d) *P. fluorescens*.

adsorption onto Ca-alginate matrix of beads along with the limited diffusion of metals inside beads most probably modified the environment in the vicinity of immobilized cells. A gradient of metals appeared inside beads with a concentration which was minimal at the center of the bead. This impoverishment avoided the inhibition of siderophore by Fe and the toxicity of TM. Conversely, all metals were available in free cell cultures which explained the high magnitude of the values, depending on the treatment.

The level of the specific siderophore activity (Table 2c and d) depended on the skim milk supply, the microorganism and the culture technique (free *versus* immobilized cells). Skim milk had an adverse effect on the siderophore synthesized by *P. fluorescens*, irrespective of the medium and the culture technique except for HIS in beads. With *P. aeruginosa*, skim milk showed contrasting effects: a decrease of the siderophore synthesis with Fe-containing media, an increase with free cells in iron-free media, except for LIS, and no effect with immobilized cells both cultivated in Fe-free media.

4. Conclusions

Our results suggested that co-location of Pseudomonads (especially *P. aeruginosa*) with skim milk allowed the level of the siderophore activity to be maintained in a narrow margin with a culture medium containing or not metals (TM, Fe), by comparison with free cells and showed even better results with Fe-containing media. Concentration in metals (Fe and TM) in the culture medium referred to that of the bioavailable part of a contaminated agricultural soil [12]. Thus, this culture technique

could be relevant to the stabilization of the bioremediation performances in soil where environmental conditions change, one of the main reasons explaining the failure of these biological processes.

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