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Leaching of zinc from an industrial filter dust with *Penicillium*, *Pseudomonas* and *Corynebacterium*: citric acid is the leaching agent rather than amino acids

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SUMMARY

Heterotrophic microorganisms are able to solubilize metals via excreted metabolites—most often di- or tricarboxylic acids but also amino acids. With amino acids Cu, Zn, Au, Ni, U, Hg and Sb have been solubilized from metal oxides, metal sulfides or elementary metals. In this work it was investigated if excreted amino acids play a role in the leaching of zinc from a zinc oxide containing industrial filter dust. Two bacteria—*Pseudomonas putida* and *Corynebacterium glutamicum*—and a fungus—*Penicillium simplicissimum* were used. *P. putida* and *P. simplicissimum* have already been used to solubilize zinc oxide, whereas *C. glutamicum* was used because of its known ability to excrete amino acids. Amino acids in culture fluids were analyzed via derivatization with phenyl isothiocyanate, separation on a RP-18 column and UV-detection. All three microorganisms solubilized zinc from the filter dust and excreted much more citric acid than amino acids. Thus citric acid rather than amino acids was regarded to be the leaching agent. Of the two bacteria *P. putida* was more resistant towards the heavy metal-containing filter dust.

INTRODUCTION

Heterotropic microorganisms can solubilize considerable amounts of metals via excreted metabolites [4,15]. The main classes of excreted metabolites are: (i) acids derived from the tricarboxylic acid cycle and (ii) amino acids. Both groups of compounds provide protons and complexing capacity for metal solubilization [4,15]. In most cases of metal solubilization by heterotrophic microorganisms, carboxylic acids are the lixiviant. However, excreted amino acids are also able to solubilize metals [1,2,6,7,9,12–14,16,18–23]. This work provides data which support the conclusion that amino acids are of minor importance in the solubilization of zinc from an industrial filter dust by a fungus and two bacteria.

With amino acids the following metals have been solubilized: copper [6,14,16,18,20,22,23], zinc [6,19,23], gold [7,9,12,13,21], nickel [2], uranium [1], mercury [14] and antimony [20]. These metals were solubilized from elementary metals [14,18], metal sulfides [22] and metal oxides [6,19,23]. Solubilization of metal compounds via amino acids generally occurred at a pH around neutral. One representative example is the solubilization of copper and zinc oxide by aqueous solutions of histidine, glycine, serine, aspartic acid and methionine at pH 8.5 [6]. Culture fluids of *Bacillus licheniformis*, *Pseudomonas putida* and other bacteria containing most of the amino acids mentioned above were also used to solubilize copper and zinc [6].

It has never been determined if sufficient amounts of amino acids are excreted by microorganisms to be of relevance to a large scale metal leaching process. In most of the cited publications amino acids actually excreted by metal-solubilizing microorganisms were only estimated or not quantified. One purpose of the present work was to establish a reliable method for quantifying the 20 common amino acids in culture fluids of metal-solubilizing microorganisms and in the presence of a leaching substrate. Therefore the method of amino acid analysis is discussed in some detail.

A second purpose was to test if amino acids were of importance for leaching of zinc from a converter filter dust of a copper works (approximately 50% Zn). This filter dust was already leached successfully with citrate excreted by the fungus *Penicillium simplicissimum* [5]. The excretion of amino acids as additional complexing agents besides citric acid was assumed, because the amount of zinc and citric acid found in culture fluids during the leaching process exceeded the solubility product of zinc citrate (Zn_3Cit_2) [5]. In addition to *P. simplicissimum* two bacteria known to excrete amino acids were tested: *P. putida* [6] and *Corynebacterium glutamicum* [10].

MATERIALS AND METHODS

Chemicals

Phenyl isothiocyanate (PITC) was purchased from Pierce, Rockford, IL, USA, triethylamine (TEA) and acetonitrile

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(HPLC-grade) from Merck, Darmstadt, FRG. The amino acid standard mixture contained 2.5 mM of each of the following amino acids (in 0.1 N HCl): L-Asp, L-Glu, L-Ser, L-Asn, L-Gly, L-Gln, L-His, L-Thr, DL-Ala, L-Arg, L-Pro, L-Tyr, DL-Val, L-Met, L-Cys, L-Ile, L-Leu, L-Phe, L-Trp and L-Lys. This mixture was also used at half-strength.

Derivatization of amino acids with PITC

The derivatization procedure employed was essentially that of Siebert et al. [17] and comprised three drying steps under vacuum. One hundred microliters of a sample or a standard mixture of amino acids were dried at 50 °C for 30 min. After resuspension in 10 μl of ethanol-TEA-water (2:2:1, v:v:v) the solution was redried at 50 °C for 10 min. The sample was then resuspended in 20 μl of the coupling solution (ethanol-water-TEA-PITC; 7:1:1:1, v:v:v:v) and incubated at room temperature for 20 min. The final drying step lasted 45 min (at 50 °C) in order to remove all PITC. At this stage the samples were either stored dry at −20 °C or resuspended in 800 μl 12.5 mM phosphate buffer/pH 6.4, mixed, centrifuged (2 min; 10000 r.p.m.) and stored at 4 °C or −20 °C.

Analysis of amino acids

Analyses were performed with a Varian (Palo Alto, CA, USA) Model 5000 liquid chromatograph and a Pye (Philipps, Eindhoven, Netherlands) Unicam 4020 UV-detector. The column was a hypersiloctyldecylsilane C 18 (particle size 5 μm, length 250 mm). Optimal column temperature was 36 °C. The binary gradient was composed of 12.5 mM phosphate buffer/pH 6.4 (= solvent A) and acetonitrile/25 mM phosphate buffer (1:1; = solvent B). The course of the gradient was: 0–15% B for 15 min, 15–45% B for 20 min, 45–80% B for 1 min, 80% B for 2 min, 80–100% B for 2 min and 100% B for 7 min (washing step). Equilibration was achieved by changing from 100% B to 100% A in 3 min and then flushing the column with 100% A for 5 min. Further gradients with a shorter time until reaching 100% B were tested in order to reduce the run time. The flow rate was 1 ml min^{-1} and the injection volume was 20 μl. Amino acid derivatives were detected at 245 nm and 0.02 absorption units full scale (AUFS).

Identification and quantification of amino acids

The position of each amino acid in the standard-mixture was determined by adding low amounts of each amino acid to a half-strength standard mixture. The mean retention time of each amino acid was calculated from ten analyses (Table 1). The linear range for each amino acid was determined by analyzing amino acid-standard mixtures of different concentrations (0.3, 0.6, 1.25, 2.5, 5 mM). γ-Aminobutyric acid and hydroxy proline were tried as internal standards.

Microorganisms

The microorganisms used were *Pseudomonas putida* (NCIMB 10015), *Corynebacterium glutamicum* (DSM 20300) and *Penicillium simplicissimum* [5].

TABLE 1

Retention times of amino acids[a]

Amino acid	Minutes	Amino acid	Minutes
Aspartic acid	6.40	Proline	18.55
Glutamic acid	7.59	Tyrosine	25.14
Serine	12.39	Valine	26.67
Asparagine	12.82	Methionine	27.97
Glycine	13.24	Cysteine	30.59
Glutamine	13.98	Isoleucine	31.44
Histidine	16.10	Leucine	31.86
Threonine	17.00	Phenylalanine	34.18
Alanine	17.48	Tryptophan	35.16
Arginine	17.94	Lysine	35.83

[a] Applying the gradient described in Materials and Methods.

Submerged leaching

One hundred milliliter flasks containing 20 ml of medium were incubated at 30 °C and 250 r.p.m. (200 r.p.m. for *C. glutamicum*). Media were inoculated with 0.1% (v/v) of a preculture (10^9 cells per ml; synthetic medium, see below), the medium for cultivating the fungus with 5×10^6 spores per ml of medium. The substrate to be leached was an industrial filter dust containing 50% zinc in the form of zinc oxide [5]. Filter dust was autoclaved separately and was used at concentrations of 0.2% (w/v) for *P. putida*, 0.2%–1%–2.5% for *C. glutamicum* and 2.5% for *P. simplicissimum*. The following media were used: a synthetic medium for *P. putida* [6]; a complex medium for *P. putida* [3]; a synthetic medium with and without biotin for *C. glutamicum* [8]; the same complex medium for *C. glutamicum* as for *P. putida*; a synthetic medium for *P. simplicissimum* [5]. Starting pH for the two bacteria was 7.0. For the fungus the pH was not adjusted.

Other methods

For determination of biomass the culture fluids were filtered (0.2-μm pore size) and the biomass was dried at 80 °C. For cultures which contained filter dust, the difference beween the weight after 24 h at 80 °C and after 4 h at 500 °C was considered to be the biomass [5]. Zinc was measured by atomic absorption spectrophotometry using standard procedures [5]. Organic acids were analyzed by HPLC [5]. All data are the mean of three parallel flasks (in case of *P. simplicissimum* the mean of four flasks).

RESULTS AND DISCUSSION

Determination of amino acids

The conditions of derivatization and separation of amino acids were varied in order to find the optimal procedure. Selected results of these variations are given below.

Duration and temperature of the three drying steps, which are necessary during derivatization (see Materials and Methods), were optimized using a standard mixture of 20 amino acids. Drying was carried out with a rotary evaporator. Therefore only 16 samples could be treated within 2 h. More

samples can be treated simultaneously if drying is carried out by vacuum centrifugation, freeze-drying or with liquid nitrogen. Automation of the drying procedure has also been described. If the phenylthiocarbamoyl-amino acids (PTC-amino acids) were re-suspended after the third drying step, the derivatives were stable for a maximum of one week at 4 °C, but unstable at −20 °C. If the PTC-amino acids were not dissolved after the third drying step, they could be stored dry at −20 °C at least for one month without a significant loss.

The position of each of the 20 amino acids in the chromatogram was verified by addition of single amino acids to the standard mixture. The standard deviation for the retention times was in all cases lower than 2% and for the peak areas lower than 5% (except for threonine, arginine, cysteine, tryptophane and lysine which were between 5 and 8%). A linear correlation between different amounts of amino acids and the corresponding peak areas was demonstrated ($r > 0.993$) within a range of 0.3 mM to 5 mM (6 nmol and 100 nmol absolute amount injected) for all amino acids except phenylalanine and lysine. The respective range for phenylalanine and lysine was between 0.3 mM and 3 mM.

An increase in column temperature did not give better resolution. If steeper gradients were used the total analysis time (including washing steps) could be reduced from 55 min to 25 min. Despite the decreased resolution observed with steeper gradients such gradients can be used for a preliminary estimating of concentrations.

Hydroxyproline and γ-aminobutyric acid were tried as internal standards. Both substances co-eluted with certain amino acids. Therefore external standards were used and standard mixtures of amino acids were derivatized and separated with each change of samples. In preliminary experiments the standard mixture of amino acids was dissolved in the different media in order to prove that there was no negative interaction between medium components and amino acids.

Leaching of filter dust

With *P. simplicissimum* solubilization of zinc occurred only if the fungus excreted citric acid, and citric acid was excreted only if the fungus grew in the presence of filter dust [5]. In order to compare *P. simplicissimum* with the two bacteria the leaching experiments with the bacteria were also carried out as a one-step process (the bacteria were grown in the presence of the filter dust).

In a medium with 2.5% filter dust *P. simplicissimum* solubilized 60% of the zinc. The culture filtrate contained amino acid concentrations around one millimole per liter compared to more than 50 mM citric acid (after 96 h). Because zinc is solubilized from zinc oxide by protons ($ZnO + 2\ H^+ \rightarrow Zn^{2+} + H_2O$) [5] the low amounts of amino acids were surely not able to solubilize the measured amount of zinc.

C. glutamicum was used in order to test an amino acid-excreting bacterium with regard to zinc solubilization from the filter dust. In the absence of filter dust, glutamate excretion was considerably higher in the synthetic medium with biotin limitation (8 mM after 96 h) than in the synthetic medium with added bitoin or in the complex medium (in both media less than 1.5 mM glutamate after 96 h). This is consistent with the literature [10]. Additionally, citric acid (30 mM after 96 h) and lactate (30 mM after 96 h) were excreted into the biotin-limited synthetic medium without filter dust. When 0.2% filter dust was added simultaneously with inoculation the excretion of glutamate, citric acid and lactate was completely inhibited. Similar inhibition could be seen if the filter dust was added 20 h after inoculation. In contrast, addition of filter dust 40 h after inoculation did not reduce excretion of glutamate. The highest zinc yield achieved with this procedure was 50% (0.2% filter dust). With 2.5% filter dust the zinc yield decreased to 6%. According to the pK_a values of the excreted metabolites (citrate: 3.1, 4.8, 6.4; glutamate: 2.2, 4.3; lactate: 3.9), zinc solubilization in *C. glutamicum* cultures could be due to all three acids. The addition of filter dust also slightly changed the spectrum of excreted amino acids: threonine and proline were excreted in higher amounts in cultures with filter dust added.

P. putida is a bacterium with several valuable properties concerning environmental biotechnology: there are strains which degrade xenobiotics and cyano-compounds, accumulate heavy metals, which are resistant to heavy metals and which can solubilize metal compounds. Unlike the strain used by Golab and Orlowska [6], the strain of *P. putida* used in this work did grow in the synthetic medium in the presence of filter dust. Less than 5% of the zinc was solubilized if *P. putida* grew in the synthetic medium. In contrast, 100% of the zinc contained in the filter dust was solubilized (Fig. 1) during growth in the complex medium (0.2% w/v filter dust; this corresponds to 0.12% w/v zinc oxide—Golab and Orlowska [6] used 0.1% w/v zinc oxide). Golab and Orlowska achieved a zinc yield of 34% after 96 h compared to 100% in this work. The complex medium used in this study contained glucose whereas that used by Golab and Orlowska did not. The glucose presumably enabled the excretion of citric acid (Fig. 1) causing the higher zinc yield.

Amino acids were excreted only in amounts lower than

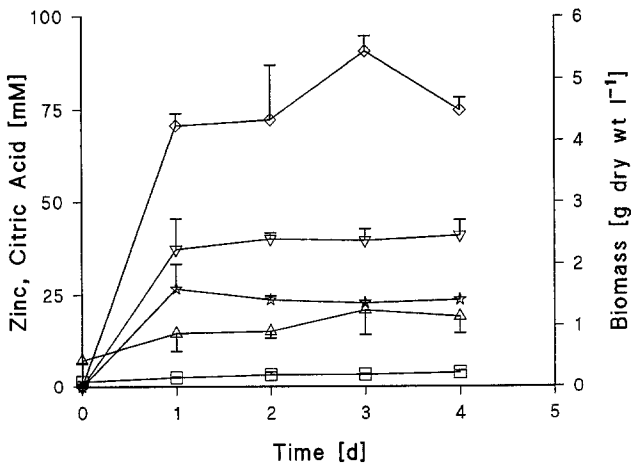

Fig 1. *Pseudomonas putida*: formation of biomass, solubilization of zinc and excretion of citric acid (0.2% filter dust, complex medium). ◇, biomass formed in cultures without filter dust; ☆, biomass formed in the presence of filter dust; □, zinc solubilized by the medium without bacteria; △, zinc solubilized by the action of the bacteria; ▽, citric acid formed in the presence of filter dust.

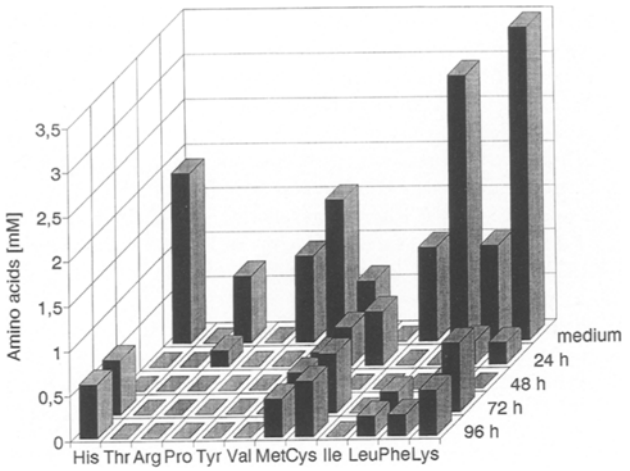

Fig 2. Amino acids found in the culture fluid of *Pseudomonas putida* cultivated in the complex medium in the absence of filter dust.

5 mM. Zinc is solubilized from the filter dust by protons. From this and the observation that *P. putida* excreted considerably more citric acid than amino acids it was concluded that citric acid was the main leaching agent. That amino acids per se are not very effective in solubilizing zinc oxide is further supported by the results of the leaching of the zinc oxide with aqueous solutions of amino acids [6] and the observation that only histidine out of 20 amino acid solutions (25 mM) reached a zinc yield of 11% with 0.5% filter dust [19].

The presence of filter dust changed the amino acid pattern found in the culture fluid of *P. putida* (Fig. 2, Fig. 3): in the absence of filter dust *P. putida* took up almost all amino acids contained in the complex medium within 48 h. Between 48 and 96 h low amounts of some amino acids were excreted again or freed via autolysis. The only amino acid which was not contained in the medium but excreted by *P. putida* was cysteine. In cultures with filter dust a few additional amino acids could be detected compared to cultures without filter dust. Furthermore, amino acids already present in the complex

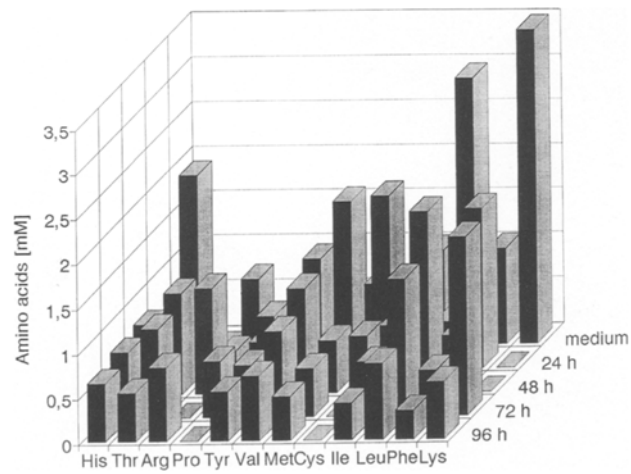

Fig 3. Amino acids found in the culture fluid of *Pseudomonas putida* cultivated in the complex medium in the presence of 0.2% (w/v) filter dust.

medium were taken up more slowly in the presence of filter dust.

CONCLUSIONS

Three major conclusions were drawn from these results: (i) amino acids are not of great importance for solubilization of zinc from filter dust by *P. simplicissimum* and this is probably true for fungi in general, because amino acid efflux is rare among yeasts and filamentous fungi [11]; (ii) although *C. glutamicum* solubilized zinc from filter dust, this bacterium is not very suitable for biohydrometallurgical processes because it needs a complex medium and the substrate to be leached could not be added at the time of inoculation; and (iii) the most promising result is that *P. putida* was able to solubilize considerable amounts of zinc from an industrial filter dust containing several heavy metals: *P. putida* produces citric acid, a compound which is particularly useful if metals are to be leached from metal oxides, silicates or carbonates [4]; additionally, the fact that *P. putida* is a bacterium may be an advantage concerning oxygen supply in bioreactors; it must be determined if *P. putida* can tolerate filter dust concentrations as high as those used with *P. simplicissimum* (up to 10% w/v).

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