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Extraction of Zinc from Industrial Waste by a *Penicillium* sp.

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Zinc was extracted from a filter residue of a copper works (58.6% zinc) by a *Penicillium* sp. isolated from a metal-containing location. By isotachophoresis citric acid was identified as the leaching agent. Citrate was only formed when the leaching substrate was present. This production of citrate was different in several ways from that achieved by *Aspergillus niger*: glucose was utilized before fructose; the initial concentration of zinc was 50 to 500 times higher than usual in citrate fermentations with *A. niger*; citrate production stopped when 80 to 90% of the zinc was leached, although sufficient sugar for further synthesis was still present; and in synthetic media citrate production by *A. niger* needs an acidic environment (pH 2), while the formation of citric acid by *Penicillium* sp. occurred in a pH range of 7 to 4. Tests with different concentrations of waste material (0.5, 2.5, and 5%) showed that the highest yield of solubilized zinc occurred with a 2.5% substrate (93% zinc extracted after 13 days).

The selective extraction of metals from industrial wastes both diminishes disposal problems and opens up new possibilities of recycling this refuse by use of pyrometallurgical methods. A promising technique in this field is leaching with microorganisms. With this technique both energy requirements and environmental damage are kept low. Three groups of microorganisms are used for the leaching process: autotrophic bacteria (*Thiobacillus* spp.; 17), heterotrophic bacteria (*Serratia* spp., *Pseudomonas* spp., *Bacillus* spp., and other species; 8, 11–13), and fungi (*Penicillium* spp., *Aspergillus* spp., and other species; 4–6, 9, 15, 16, 19). On the one hand the use of autotrophic thiobacilli is advantageous because no organic carbon source is needed for their growth. On the other hand heterotrophic bacteria and fungi can be used with higher pHs (alkaline, acid-consuming materials). A further advantage of leaching with heterotrophic microorganisms is that because of the formation of complexes between metals and metabolites, precipitations can be avoided when high concentrations of metals and alkaline pHs are present. At the same time complexation often reduces the toxicity of metal ions (1, 2). Because of their capacity for oxidizing the substrate only partially and secreting it again in partly oxidized form fungi are of particular interest for leaching. This "incomplete oxidation" is strongly influenced by the composition of the medium and causes the accumulation of organic acids, which are able to extract metals from solid substrates. The most important primary metabolite for leaching is citric acid (4).

Products of secondary metabolism might also add to the process of leaching. The production of secondary metabolites especially by actinomycetes and fungi imperfecti leads to a series of unusual compounds, the investigation of which, from the leaching point of view, has been grossly ignored up to now.

To elucidate and optimize leaching processes an exact analysis of the leaching fluid is necessary. We have therefore studied in this work the process of leaching with *Penicillium* sp. by means of atomic absorption spectrophotometry (zinc), isotachophoresis (citric acid), and high-pressure liquid chromatography (HPLC) (sucrose, glucose, fructose, and citric acid) for 13 days.

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MATERIALS AND METHODS

Conidium suspension. The *Penicillium* sp. was incubated on agar slants at 30°C (medium: peptone-glucose-yeast agar, recipe 197, with 0.5 ml of recipe 72; 18). The pH was adjusted to 6.0. The conidia were washed off the slants with a solution of sterile 0.5% Tween 80 (2 ml per slant) by shaking them briefly. All conidium suspensions obtained in this way were pooled in a sterile flask.

Leaching. Leaching was carried out in 500-ml Erlenmeyer flasks with 0.5, 2.5, or 5.0 g of substrate and 100 ml of medium (medium for citrate production; 14). The substrate used for the study was a filter residue from the converter of a copper works (58.6% Zn, 11.3% Pb, 6.1% Sn, 1.5% S, 0.47% Cu, 0.11% As, 0.07% Fe, 0.03% Sb, and 0.02% Ni). The pH was adjusted with HCl to 4.0 before addition of the leaching substrate, and the flasks (medium and substrate) were sterilized at 121°C for 30 min. Both substrate addition and autoclaving caused the pH to rise to 7.0. A 2-ml suspension of conidia was used for inoculation. The flasks were incubated at 30°C and 150 rpm. Two control flasks (medium and substrate; medium and fungus without substrate) were incubated at the same time. For sampling, 5-ml samples were removed and replaced with 5 ml of fresh medium. The samples were mixed with 50 μl of Tween 80 (diluted 1:100) and centrifuged at 12,000 × *g* for 15 min. The supernatant solution was collected with a pipette, and the pH was measured. The samples were stored at −20°C and raised to +20°C before analysis.

Atomic absorption spectrophotometry. The conditions for the determination of zinc were as follows: wavelength, 307.6 nm; slit, 0.7; hold mode; measuring time, 3 by 3 s; standards, 15.3 and 45.9 mM Zn ($ZnCl_2$ in 1% HCl); and air-acetylene flame (35:15). A Perkin-Elmer instrument (model 2380) was used for measurements. Samples from days 1, 2, and 3 were measured undiluted, and all other samples were diluted 1:10 with double-distilled water.

Isotachophoresis. A Tachophor 2127 (LKB Instruments, Inc.) with a Teflon capillary (23 cm; inner diameter, 0.55 mm) was used. Table 1 shows the composition of the electrolyte systems used for the determination of negatively charged organic acids (Merck & Co., Inc.: HCl 317, β-alanine 1008, hexanoic acid 197; Fluka Chemie AG: Triton X-100 93420). The separations were started at 150 μA. The

TABLE 1. Electrolyte systems used for the determination of organic acids by isotachophoresis

Electrolyte system	Anion (M)	Counter ion	pH	Additive	Solvent
Leading	Chloride (HCl) (0.010)	β-Alanine	3.5	0.3% Triton X-100	H_2O
Terminating	Hexanoic acid (0.005)	β-Alanine	3.7	None	H_2O

current was reduced to 50 μA when the voltage reached 8 to 10 kV (depending on the citrate content of the sample). The linear signal and the differential signal of the conductivity detector were recorded (chart speed, 1 cm/min). For the quantitative determination of citric acid the step length was measured according to the differential signal. Calibration was carried out with a 5 mM solution of citric acid (1, 2, 4, 8, 16, and 32 μl). The injection volume of the samples was 1 μl. All separations were carried out at 20°C.

HPLC. The separation of sucrose, glucose, fructose, and citric acid was carried out with an Aminex HPX 87H column (Bio-Rad Laboratories; precolumn, Aminex Micro Guard Cation H) with 0.005 N H_2SO_4 as the eluent and a flow rate of 0.5 ml/min. For the separations a temperature of 20°C was chosen. By injecting three 50-μl samples a 20-μl loop was rinsed and filled. The substances were quantified by using a refractive index detector (model 156; Beckman Instruments, Inc.). Citric acid was additionally measured with a variable-wavelength monitor (model 2151; LKB; 220 nm). Areas were integrated by a computer program. Calibration was carried out with a 25 mM solution of each sugar and citric acid. A common base line was set for all peaks. One run took about 15 min. The samples were prepared by dilution (1:100; double-distilled water) and ultrafiltration (cutoff, 10 kilodaltons).

RESULTS

The highest concentration of soluble zinc was reached with a pulp density of 2.5%. After sterilizion of the medium mixed with the 2.5% substrate the content of soluble zinc in the medium was 0.8 mM. It then increased continuously from day 2 on. On day 13, 209.6 mM was reached (93% of the total zinc content; Fig. 1). After day 3, the increase in the soluble zinc concentration paralleled that of the citric acid concentration (149.6 mM as determined by isotachophoresis after 13 days; Fig. 1) in the menstruum, and the pH decreased from 7.0 to 4.1 (Fig. 1). The fungus formed pellets, and the substrate was totally adsorbed to the pellets after a few days. The pellets were dark brown and about 0.5 mm in diameter.

Tests with a pulp density of 0.5% resulted in a yield of only 74% (33.2 mM) after 6 days (Fig. 2). Leaching and the production of citric acid started on day 1 and stopped on day 6, although incubation went on for 9 days.

With a pulp density of 5% the phase between the start of the culture and the beginning of leaching and citrate production was prolonged to 5 days, and leaching stopped on day 9 (23.9% extracted; Fig. 2).

In the control flask containing medium and fungus no citrate could be detected by isotachophoresis. The phosphate present in the medium could be detected during the entire incubation by isotachophoresis, whereas the phosphate present in the leaching culture was not detectable even

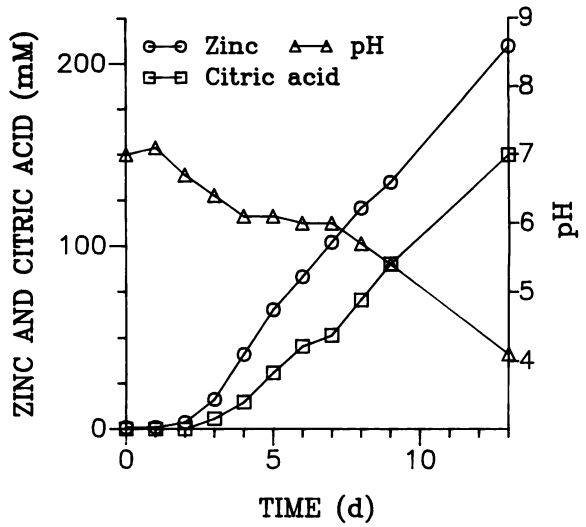

FIG. 1. Zinc extraction from industrial waste material by *Penicillium* sp. A 500-ml flask containing 100 ml of medium, 2.5 g of substrate, and 2 ml of conidium suspension was shaken at 30°C for 13 days. Zinc (atomic absorption spectrophotometry), citric acid (isotachophoresis), and pHs were determined with each sampling. d, Days. The data are from a single experiment chosen from three which gave very similar results.

in the day-zero sample (after sterilization and before inoculation).

Figure 3 shows the results of the HPLC analysis of the leaching culture with the 2.5% substrate. Only 84% of the weighed-out 138.9 g of sucrose per liter (405.8 mM) could be detected after autoclaving of the substrate-containing medium. Without autoclaving about 100% of the sucrose could be recovered. Part of the difference was split into glucose and fructose.

In the leaching culture sucrose started to decrease markedly on day 3. From day 6 on sucrose could not be detected. The concentrations of glucose and fructose increased markedly from day 3 on and decreased after day 6 (glucose) and day 9 (fructose). In this process glucose was consumed immediately, whereas fructose only started to decrease

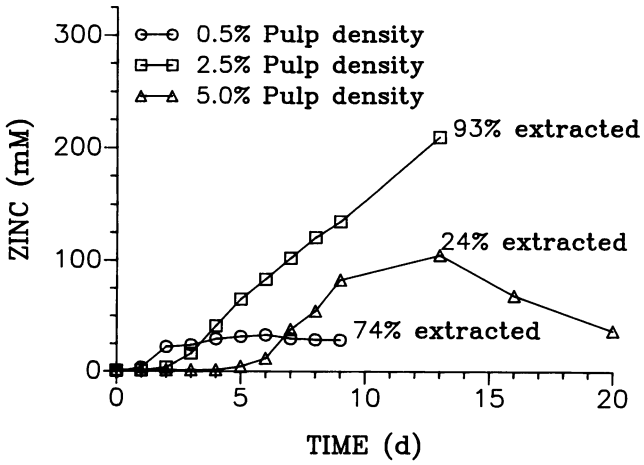

FIG. 2. Comparison of zinc extraction from industrial waste material by *Penicillium* sp. at various pulp densities (0.5, 2.5, and 5.0%). d, Days. The data are from a single experiment chosen from three which gave very similar results.

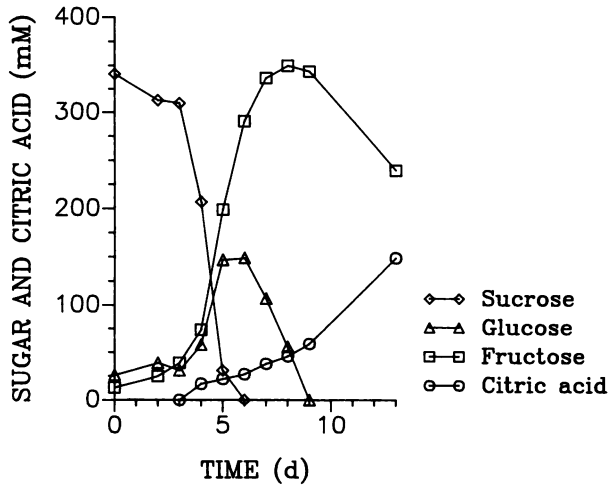

FIG. 3. HPLC analysis of the production of citric acid and the utilization of sucrose, glucose, and fructose during leaching at a 2.5% pulp density. d, Days. The data are from a single experiment chosen from three which gave very similar results.

when all the glucose was spent (day 8). The citric acid concentrations determined by HPLC were considerably lower than those determined by isotachophoresis, except for the results on day 13.

DISCUSSION

Zinc extraction and citric acid production. As preliminary tests with isotachophoresis showed that citric acid was the only organic acid produced by the fungus under test conditions, a medium that produced a high yield of citrate was chosen. When the substrate to be leached was added to the medium, the initial zinc concentration (0.8 mM) was much higher than that described as inhibitory for citrate production by *Aspergillus niger* (1.5 μM; 20). This high zinc content did not inhibit the formation of citric acid by *Penicillium* sp. at all; in contrast, it was the presence of the substrate that induced citrate production, as can be deduced from the control experiments in which no substrate was added. When *Penicillium* sp. and *A. niger* (CBS 120.49) were incubated without substrate, citric acid could only be found in the *A. niger* culture. In contrast, only *Penicillium* sp. could produce citric acid in the presence of the substrate.

Incubation of *Penicillium* sp. with different pulp densities showed that the amount of citrate produced and, consequently, the amount of leaching were higher when high concentrations of zinc in the form of the leaching substrate were present in the medium. With a 0.5% pulp density (initial phase, 1 day), the leaching stopped after 6 days (Fig. 2), although sufficient glucose and fructose were present to continue the production of citric acid (data not shown). With a 2.5% pulp density (initial phase, 2 days), the leaching stopped on day 13, although again sufficient fructose was available (Fig. 3). With a 5% plup density, the initial phase was prolonged to 5 days and the extraction stopped on day 9. These results indicate that citrate production is correlated with the quantity of zinc (substrate) added to the medium (Table 2). The low extraction rate with the 5% substrate can not be due to a zinc concentration too high for the fungus, as the zinc concentration in the mixture with the 2.5% substrate was much higher. Unidentified substances contained in the substrate and only having a strong inhibitory effect with a pulp density of 5% must be held responsible.

TABLE 2. Relative amounts of citrate produced with respect to the substrate (zinc) content of the medium

Day	Citrate (mM) produced with:	
	0.5% Substrate	2.5% Substrate
3	10.5	5.8
5	27.0	30.9
7	32.0	51.5
9	31.0	90.2
13		150.0

Sugar consumption. When sucrose is autoclaved hydrolytic cleavage as well as transformation of the sugar into toxic substances may occur (hydroxymethylfurfural; 3). The lower the pH of the medium, the more sucrose is split (about 30% at pH 4). The longer the autoclaving takes, the more hydroxymethylfurfural is produced. When investigating the utilization of sucrose it is best to keep the time of autoclaving short, the pH of the medium not too low, and the temperature of the HPLC column low.

The conditions we used for the separations by HPLC were not suitable for the determination of sugars and of citric acid at the same time. In comparison with isotachophoresis, too-low citrate values were obtained with HPLC because of an overlap of the peaks of glucose and citrate. Determination of citrate by use of peak heights and quantification of citrate with a UV detector could not improve the results. Only in the day-13 sample, in which glucose was not present any longer, were the HPLC and isotachophoresis results identical (Fig. 1 and 3).

With sugar a distinct difference could be seen between *Penicillium* sp. and *A. niger*. With *A. niger* all the sucrose is hydrolyzed within 30 h, and then glucose and fructose are utilized at the same time (7). With *Penicillium* sp. it took 6 days for all the sucrose to be hydrolyzed. From the products of the hydrolysis *Penicillium* sp. attacked first glucose and then fructose.

We observed that immediately after autoclaving (and before inoculation) the phosphate zone on the isotachopherogram could no longer be seen if substrate was present in the medium, indicating that phosphate may play a part in the production of citrate by *Penicillium* sp. With *A. niger* limitation of phosphate may lead to an increase in citrate production. This effect only occurs when the supply of trace elements is not well balanced (10).

From the experiments carried out so far we can deduce that citrate production in *Penicillium* sp. is regulated differently from that in *A. niger*. It may be supposed that *Penicillium* sp. produces citric acid to protect itself against too-high zinc concentrations. The following model can be proposed: the zinc released through chemical extraction induces the production of citric acid, which reduces the toxicity of the zinc ions by complexation. As the produced citric acid increases the acidity of the medium, more zinc is extracted, in turn increasing the production of citric acid.

The reason why 100% of the zinc substrate was not extracted may reside in the observation that some substrate was adsorbed to the reactor wall at the gas-liquid interface, making it unavailable for leaching.

The objects of further research for obtaining optimal leaching of industrial wastes with *Penicillium* sp. are to overcome the inhibition occurring with substrate concentrations above 2.5%, to minimize the length of the process, and to elucidate what might be responsible for inducing the formation of citric acid.

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