Polish Journal of Microbiology
2006, Vol. 55, No 3, 203–210

The Growth, Ferrous Iron Oxidation and Ultrastructure of *Acidithiobacillus ferrooxidans* in the Presence of Dibutyl Phthalate

RENATA MATLAKOWSKA, EMILIA SKUDLARSKA and ALEKSANDRA SKŁODOWSKA*

Laboratory of Environmental Pollution Analysis, Faculty of Biology, Warsaw University
Miecznikowa 1, 02-096 Warsaw, Poland

Received 30 June 2006, revised 13 July 2006, accepted 17 July 2006

Abstract

The iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* is an example of strictly chemolitotrophic extremophile occurring in acidic environments. The prime niche of these microorganisms is an environment with low pH and high concentrations of iron, sulfide minerals or sulfur. Besides these environments, *A. ferrooxidans* is also isolated from heavy metal contaminated environments such as soil and sewage sludge and is known to be useful in bioremediation processes of these environments. In the current study, the influence of dibutyl phthalate on the growth, activity and ultrastructure of *A. ferrooxidans* ATCC19859 was shown. The presence of dibutyl phthalate in 9K medium did not influence *A. ferrooxidans* growth or ability to oxidize ferrous iron although changes in growth medium were accompanied by changes in the protein expression profiles of periplasmic fractions and remarkable changes in ultrastructure of the cell.

Key words: *A. ferrooxidans*, dibutyl phthalate (DBP), ferrous iron oxidation, ultrastructure of the cell

Introduction

The iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* is an example of obligate chemolithotroph and an extremophile occurring in acidic environments. Extreme acid environments of pH 2–5 are commonly associated with coal mines, draining waters and mining effluents. In addition to a low pH, these environments are characterized by high concentrations of reduced sulfur and iron compounds (Kelly and Harrison, 1989). As an obligate extreme acidophile and chemolithotroph, *A. ferrooxidans* needs to be cultivated under special conditions. It requires inorganic energy sources and a comprehensive range of micronutrients. Additionally, it is highly sensitive to soluble organic materials, particularly organic acids, as well as anions. The 9K liquid mineral medium of Silvermann and Lungren (1959) is the best known medium for cultivation of *A. ferrooxidans* under laboratory conditions. For many years it was, however, impossible to obtain growth of different strains of *A. ferrooxidans* on solid media. One of the main reasons was the toxic effect of the hydrolysis products of gelling agents on these bacteria. The application of overlaid solid media made it possible to obtain colonies of all tested strains of this species (Johnson, 1996).

Besides strictly chemolithotrophic and acidic environments, *A. ferrooxidans* is also isolated from other heavy metal contaminated environments such as soil and sewage sludge. The occurrence of these bacteria in sewage sludge is still regarded as doubtful, although it has been reported by several research groups (Tyagi *et al.*, 1993; Zagury *et al.*, 1994, Gomez and Bosecker, 1999; Matlakowska and Skłodowska, 2003). Its presence there seems improbable, especially because of the high pH of sewage sludge, reaching 6–7, as well as the high concentration of organic matter. It should, however, be taken into account that acidic micro-niches can be created in this environment by microbial activity. Such microniches were also observed in the bioleaching of heavy metals from alkaline or slightly neutral ores (Ostrowski and Skłodowska, 1992). The existence of acidic niches in dehydrated digested sewage sludge seems possible because of the high

Corresponding author: A. Skłodowska: e-mail: asklodowska@biol.uw.edu.pl, phone: 48 22 5541005, fax: 48 22 5541006

concentration of metal sulfides as well as the presence of chemolithototrophic, neutrophilic, and moderate acidophilic sulfur-oxidizing bacteria in this environment (Tyagi *et al.*, 1993). Initial acidification of the sludge can occur by the growth of indigenous, less acidophilic thiobacilli, followed by acidophilic thiobacilli, resulting in reduction of the pH to approximately 2.0. The existence of *A. ferrooxidans* in the presence of organic matter occurring in sewage sludge may also be explained by the fact that most organic compounds are insoluble in water what results that those organic particles are not in direct contact with inner structure of cell and may have influence on outer cell structures only.

The aim of the present study was to investigate the influence of dibutyl phthalate as a model compound of benzenedicarboxylic acid esters, one of the most abundant chemical components of sewage sludge and contaminated soils, on the growth of *A. ferrooxidans*, and on oxidation of ferrous iron by this bacteria. The morphology and ultrastructure of *A. ferrooxidans* cells as well as changes in the protein profile during growth in the presence of dibutyl phthalate were also investigated.

Experimental

Materials and Methods

Bacterial strain. *A. ferrooxidans* ATCC 19859 strain obtained from the American Type Culture Collection was used.

Chemicals. Di-n-butyl phthalate (DBP) [$C_{16}H_{22}O_4/C_6H_4(COOC_4H_9)_2$] of commercial grade was obtained from Sigma.

Culture conditions. Bacteria were cultivated in either 9K mineral medium or in mineral medium supplemented with DBP at a concentration of 25% (v/v). The cultures were maintained in 500 ml flasks on a rotary shaker (100 rpm) at room temperature (22°C) for 14 days. Two controls: sterile 9K and sterile 9K + DBP media, were incubated at the same condition.

Microbial and chemical analyses. The number of bacteria cells, pH, and total iron and ferrous iron concentrations in the cultures were analyzed. The cell count of a sample was determined using a microscope counting chamber and standard deviation was calculated with Microsoft Excell software. Total iron was determined by atomic absorption spectrometry (SOOLAR M6, TJA Solution, UK) following the acidification of samples with 6 M HCl. Standard deviation (SD) and relative standard deviation were calculated using Solar MAA software (TJA Solution, UK). Ferrous iron was assayed using the *ortho*-phenantroline colorimetric method (Hermanowicz *et al.*, 1999). DU-65 Beckman UV-VIS Spectrophotometer with Soft-pac Module Quant II Linear for standard deviation calculation for colorimetric measurements was used.

Preparation of samples for transmission electron microscopy (TEM). Bacterial cells were fixed with 3% glutaraldehyde in sodium cacodylate buffer and then treated with osmium tetroxide for 4 hours. Increasing concentrations of ethanol was used for dehydration. Ultrathin sections of epon-embedded cells were cut and treated with uranium acetate and lead citrate.

Protein isolation and analysis. *A. ferrooxidans* proteins were separated into cytoplasmic, periplasmic, inner membrane (cytoplasmic) and outer membrane fractions using the procedures of Guiliani and Jerez (1999) and Neu and Heppel (1965). Protease inhibitor (50 µg PMSF ml^{-1}) was used. SDS-PAGE (10%) gel electrophoresis was carried out by the standard procedure of Schägger and von Jagov (1987). Proteins were visualized by silver staining and analyzed with Image Master 1D Elite (Nonlinear Dynamics) using molecular weight calibration kits (Amersham Pharmacia Biotech). Proteins containing hem groups were detected by *o*-dianisidine staining according to the method of Francis and Becker (1984).

MS/MS analysis. MS/MS identification of selected proteins that had been separated using SDS-PAGE was performed after trypsin digestion using a Micromas ESI-Q-TOF mass spectrometer. The results were analyzed with Mascot software (www.matrixscience.com). The MS analysis was performed at the Environmental Laboratory of Mass Spectrometry, Institute of Biophysics and Biochemistry (Polish Academy of Sciences, Poland).

Results

Comparison of growth and Fe (II) oxidation of *A. ferrooxidans* in different media. In 9K + DBP medium two fractions were visible: water fraction being mineral salt solution and organic fraction of DBP. As is shown on Figure 1, the growth of *A. ferrooxidans* in 9K + DBP occurred only in water fraction. The presence of brown sediment (jarosite) in this fraction indicated the growth of iron-oxidizing bacteria. Bacterial growth was not observed in DBP fraction.

Comparison of the growth curves for *A. ferrooxidans* cultivated in either 9K medium or in 9K + DBP medium showed only slight differences (Fig. 2). The lag phase of both cultures of *A. ferrooxidans* lasted 1 day and the stationary phase was reached after 7 days. The number of bacteria after 14 days of cultivation was 1.23×10^9 and 1.15×10^9 respectively, starting from 4×10^6cells/ml.

No difference in the ability of iron oxidation was detected. Both cultures of *A. ferrooxidans* showed complete oxidation of ferrous iron after 5 days (Fig. 3). The Fe(II) oxidation observed in both sterile control (9K and 9K + DBP media) was exclusively a chemical process. pH elevation as a result of ferrous iron oxidation was observed in both cultures of *A. ferrooxidans* between days 2 and 4 (Fig. 4). Slow acidification

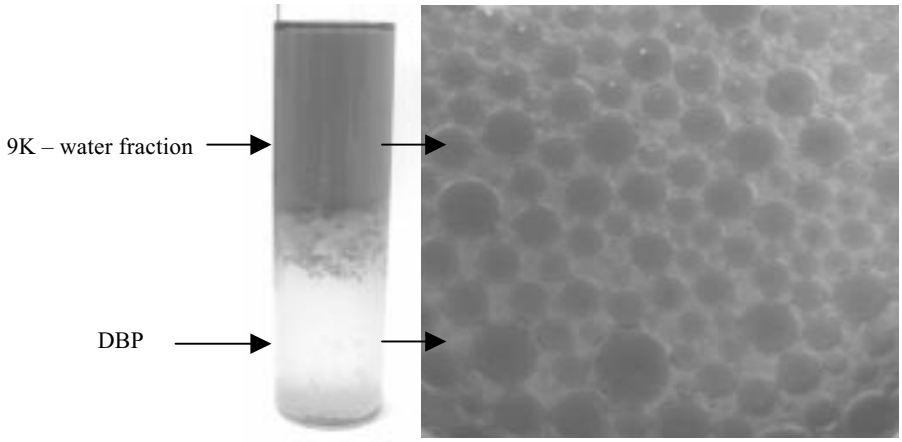

Fig. 1. Cultures of *A. ferrooxidans* in 9K + DBP medium

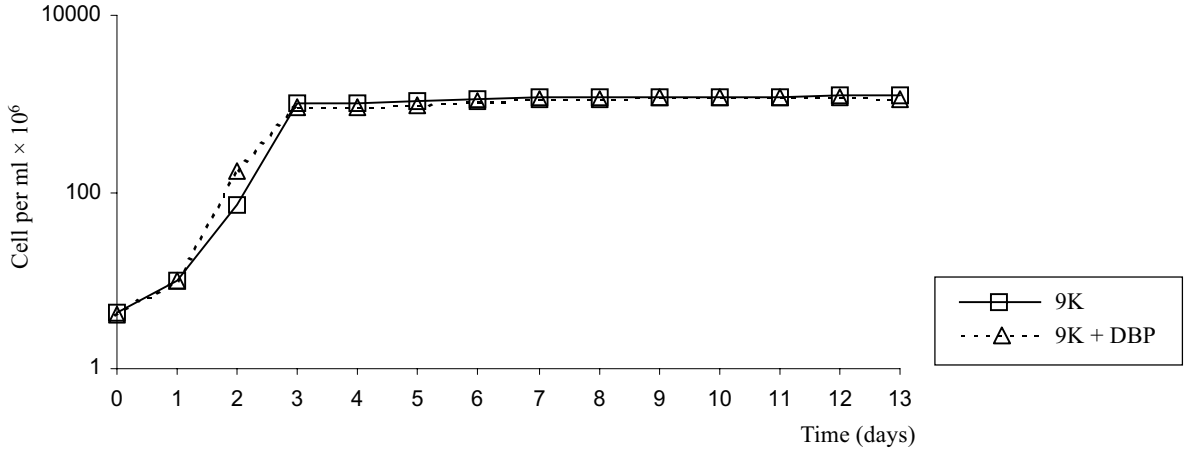

Fig. 2. Growth curves of *A. ferrooxidans* in 9K and 9K + DBP media

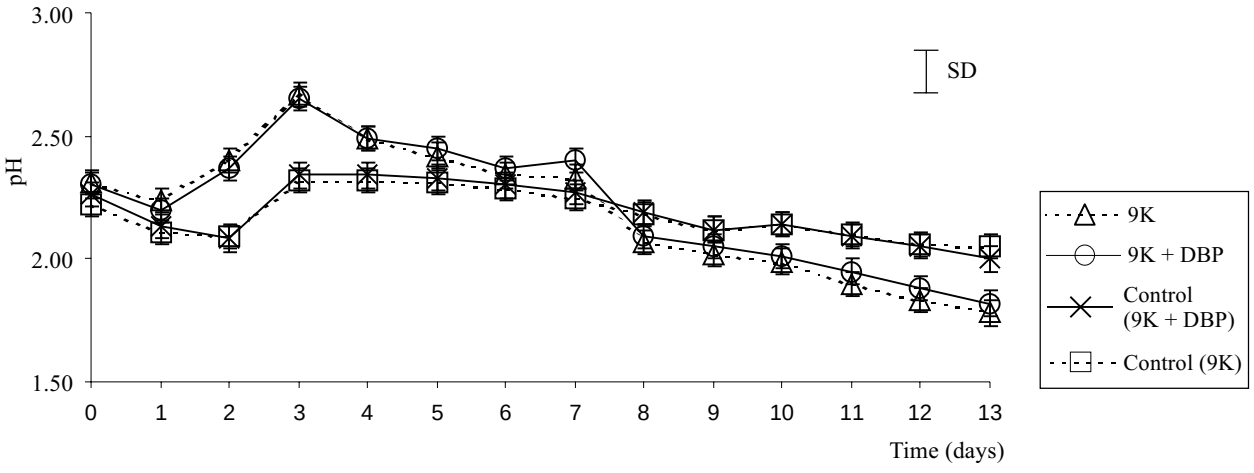

Fig. 3. pH changes during the cultivation of *A. ferrooxidans* in 9K and 9K + DBP media

subsequently happend. Figure 4 shows the concentration of total iron. Identical decreases in total iron concentrations were observed in both cultures due to precipitation of jarosite (Fig. 5).

Morphology and ultrastructure of *A. ferrooxidans.* Cross- and longitudinal ultrathin sections of bacterial cells were examined using TEM. Morphological and ultrastructural differences between cells grown

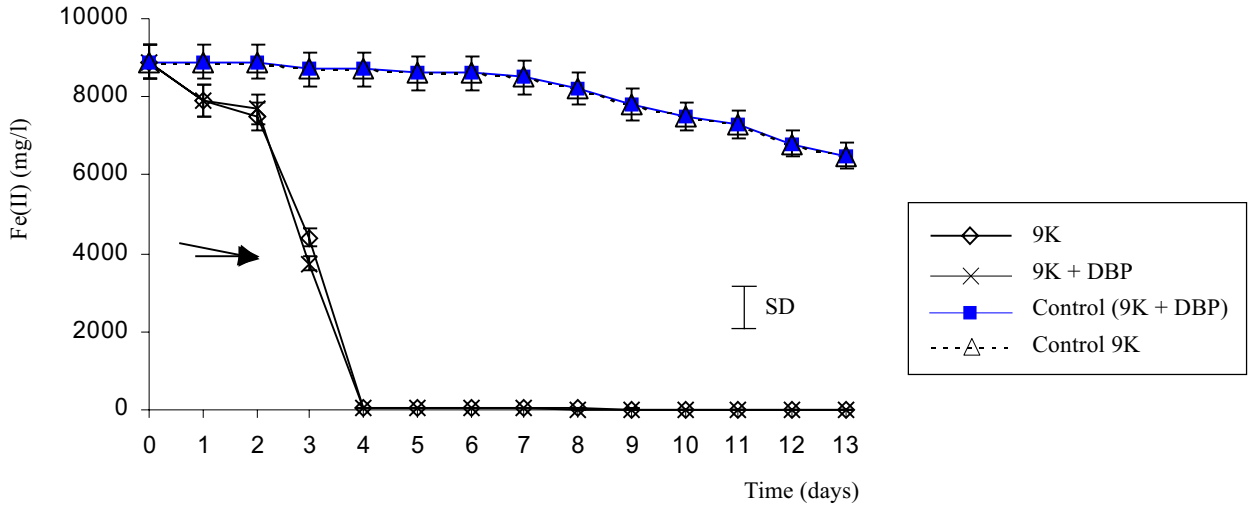

Fig. 4. Ferrous iron oxidation during growth of *A. ferrooxidans* in 9K and 9K + DBP media

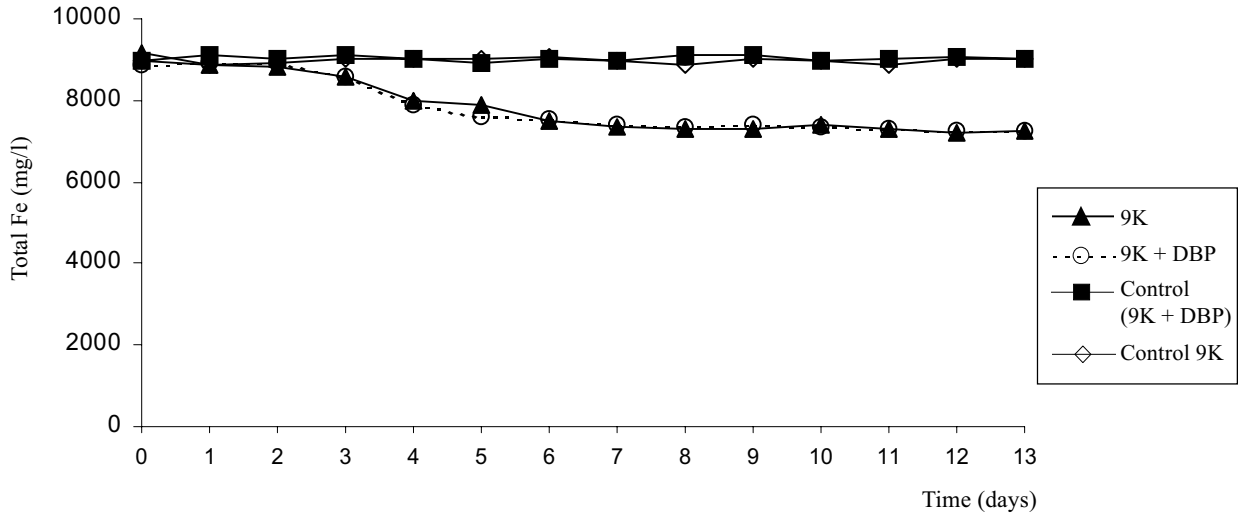

Fig. 5. Total iron concentration during growth of *A. ferrooxidans* in 9K and 9K + DBP media. Standard deviation: 5–50 mg/l and relative standard deviation: (RSD) 0.2–0.5%

in 9K and those grown in 9K + DBP media were clearly visible (Fig. 6). Cells that were grown in 9K medium showed the typical shape and ultrastructure characteristic for autotrophically grown bacteria. In contrast, cells grown in the presence of DBP were deformed and closely attached to each other. Additionally, the nucleoplasm of cells cultivated in 9K+DBP medium presented a characteristic consistency, which seemed to be very dense and tightly packed. Empty spaces in *A. ferrooxidans* cells were also observed.

Protein profiles of bacteria grown in the absence and in the presence of dibutyl phthalate. SDS-PAGE gel electrophoresis was performed to determine the patterns of protein produced by the studied *A. ferrooxidans* strain when grown in either 9K medium with ferrous iron or in the presence of dibutyl phthalate. Cells were fractionated and protein expression profiles of four cellular fractions were campared. The cytoplasmic as well as inner and outer membrane profiles did not show any significant quantitative differences. Proteins identified as: CsoS1C (10 kDa), CbbS (12 kDa) and CbbL (42 Da) were found in the inner membrane of *A. ferrooxidans* cells cultivated under both studied conditions (Fig. 7 B). Two cytochromes of molecular weights 22 and 51 kDa (detected using *o*-dianisidine staining, results not shown), as well as a subunit of cytochrome oxidase (25 kDa), were present in the inner membrane fraction of both cultures. The blue copper protein, rusticyanin required for the oxidation of iron, was identified in the periplasmic fraction of *A. ferrooxidans* cells grown in 9K medium as well as in 9K + DBP medium.

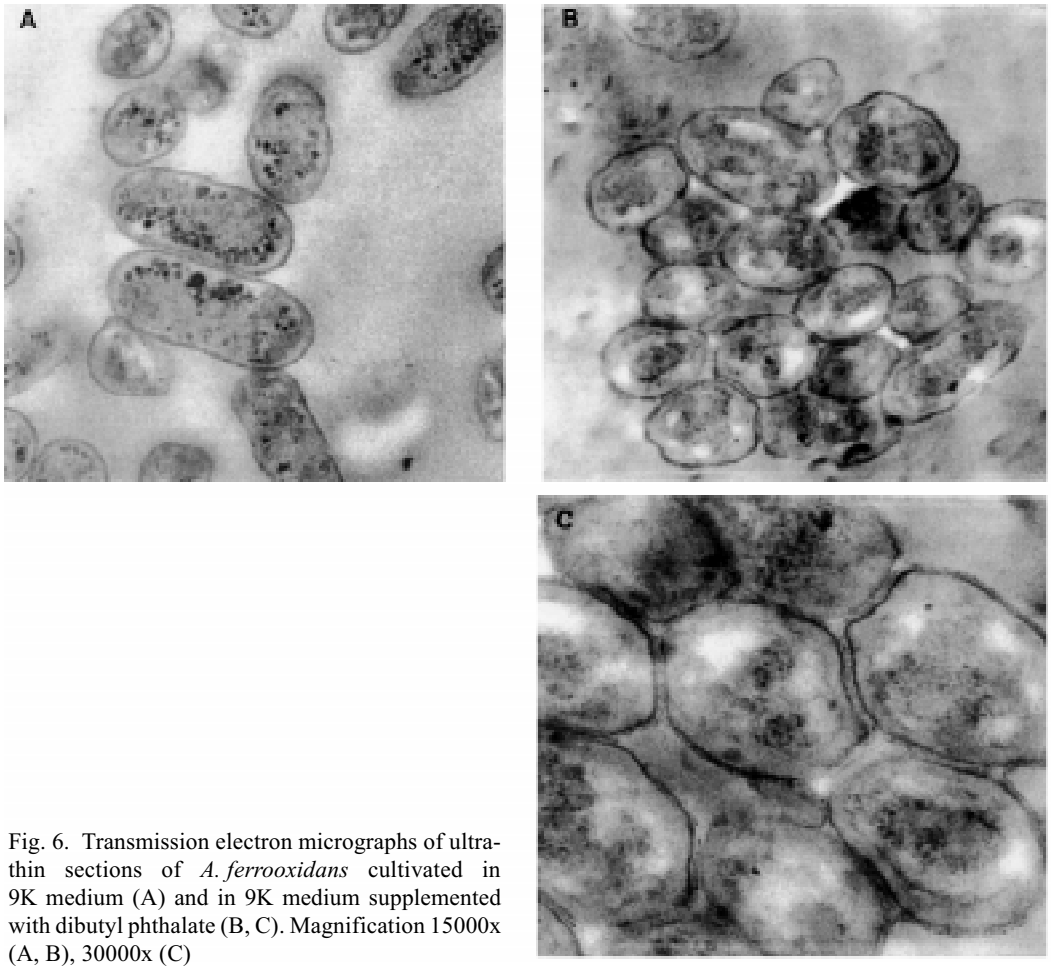

Fig. 6. Transmission electron micrographs of ultra-thin sections of *A. ferrooxidans* cultivated in 9K medium (A) and in 9K medium supplemented with dibutyl phthalate (B, C). Magnification 15000x (A, B), 30000x (C)

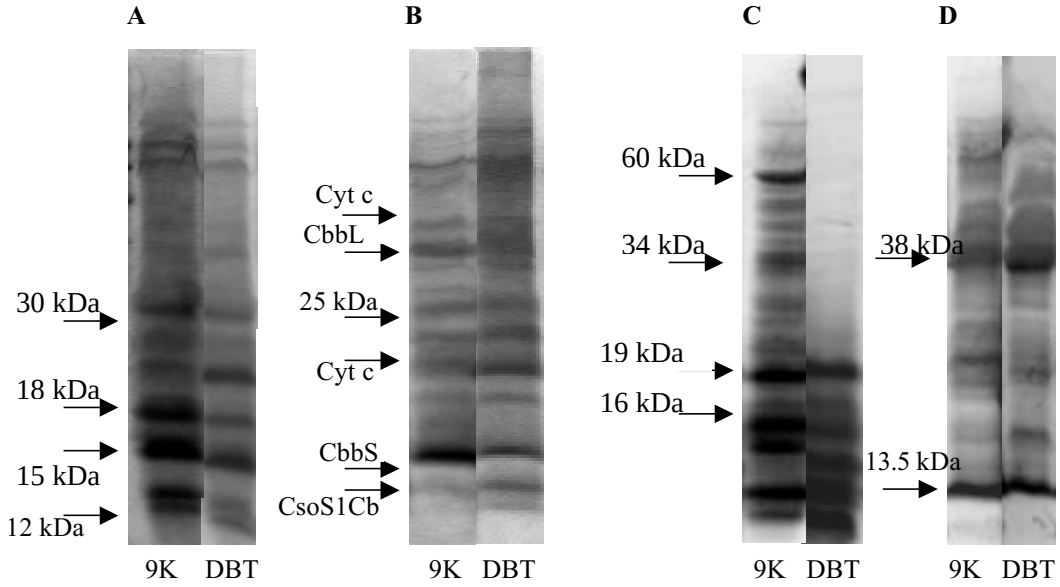

Fig. 7. Electrophoretic patterns of cytoplasmic (A), inner membrane (B), periplasmic (C) and outer membrane proteins of *A. ferrooxidans* grown in 9K and 9K + DBT media

Significant differences were observed, however, in protein profiles of the periplasmic fraction. The presence of DBP in the medium resulted in a total lack of proteins with a molecular weight more than 20 kDa (Fig. 7 C).

Discussion

In the presented study, the influence of dibutyl phthalate on the growth, ferrous iron oxidation and ultrastructure of *A. ferrooxidans* was studied. Phthalic acid esters (PAEs) are a large group of chemicals used as plasticizers in the production of plastics. They are widely distributed in sediments, natural water, wastewater and soil. PAEs are also detected at high concentrations in landfill leachate as well as in sewage sludge. As a result of low water solubility and a high octanol/water partition coefficient, PAEs tend to accumulate in soils and sediments. Phthalic acid esters have been found in sewage sludges at levels of 12–1250 mg kg^{-1} dry weight and were not degraded during sewage sludge digestion as shown by Rogers, (1996).

The adaptation of *A. ferrooxidans* to growth in sewage sludge was the aim of our previous study (Matlakowska and Skłodowska, 2003; Matlakowska and Skłodowska, 2005). We investigated the effect of sewage sludge, which combines different chemical and physical factors acting simultaneously on the bacterial cell, *e.g.* the availability of energy substrates and oxygen, presence of organic compounds and heavy metals, pH, pulp density, sludge particle size, metal speciation. The adaptation of *A. ferrooxidans* to sewage sludge and its role in metal bioleaching was shown (Matlakowska and Skłodowska, 2003).

The results presented in this paper concentrate on the action of just one factor – dibutyl phthalate on *A. ferrooxidans* under stable conditions. Bacteria were cultured in the media containing optimal concentrations of ferrous iron and micronutrients, as well as optimal pH and temperature. The concentration of dibutyl phthalate used was 25% (v/v). This concentration was selected as a representative for the sum of all esters in dehydrated digested sewage sludge. It was shown that the presence of dibutyl phthalate in 9K medium did not affect *A. ferrooxidans* growth or ability to oxidize ferrous iron. In contrast, a prolonged lag phase lasting 4–5 days was observed when bacteria were grown in dehydrated digested sewage sludge containing sulphur and small amount of ferrous iron (Matlakowska and Skłodowska, 2003). Previously, a wide variety of chemicals have been shown to affect the growth and ferrous iron oxidation of *A. ferrooxidans*. Inhibition of growth and physiological activity was observed in the presence of carbohydrates, organic acids (Tuovinen *et al.*, 1971; Fratinni *et al.*, 2000) as well as chemical compounds used for copper extraction (ACORGA, LIX 64, nonylphenol, izodecanol, Alamine) (Torma and Itzkovitch, 1976; Bosecker, 1997; Mazuelos *et al.*, 1999). Pronk *et al.*, (1991) indicated that *A. ferrooxidans* ATCC 21834 could, however, be grown on formate when the substrate supply was growth limiting, for example in formate-limited chemostat cultures. *A. ferrooxidans* grown under such conditions retained the ability to oxidize ferrous iron at high rates and assimilated carbon using the Calvin cycle. Moreover, under anoxic conditions, *A. ferrooxidans* was able to reduce ferric iron with sulfur or hydrogen as electron donors (Pronk *et al.*, 1992; Ohmura *et al.*, 2002). Drobner *et al.*, (1990) showed that *A. ferrooxidans* (ATCC 2327) and two other strains were able to grow using the oxidation of gaseous hydrogen as an energy source.

As described before, sewage sludge is known to have a destructive influence on the growth and cell structure of *A. ferrooxidans* (Matlakowska and Skłodowska, 2003). Cells grown in medium containing sewage sludge were slightly deformed and contained reserve material. Additionally, carboxysomes were not observed and these cells were embedded in slime and covered by mucosal biofilm. As mentioned above, cells grown in the presence of DBP showed structural changes in peripheral parts of the cell while the outer and inner membranes were unchanged. The mechanism by which DBP influences bacterial cells is unknown. It maybe related to some physical changes in the medium and thus is of an indirect nature. DBP is a well known solvent for a number of organic compounds and is a surfactant as well. Synthetic surfactants affect microbial surface structure, composition, properties and functions (Angelova and Schmauder, 1999). They cause changes in fatty acid compositions and the level of their saturation (Chevalier *et al.*, 1988). Anionic surfactants enhance cell surface hydrophobicity (Marchesi *et al.*, 1994) and increase membrane permeability (Laouar *et al.*, 1996; Chan and Kuo, 1997). Decreased surface tension of 9K medium containing DBP was confirmed (unpublished results) and it may cause changes in the structure of the outer membrane, allowing periplasmic leakage and contact between the surfactant and periplasmic proteins. The result was visible on ultrathin sections (Fig. 6) and as the degradation of large proteins observed in the SDS-PAGE profile of the periplasmic fraction (Fig. 7C). Moreover, increased hydrophobicity as well as decreased relative surface charge of *A. ferrooxidans* cells were observed during growth in 9K medium with dibutyl phthalate (unpublished results).

The results of SDS-PAGE clearly show that the expression of proteins, which are important to the *A. ferrooxidans* electron pathway from ferrous iron to oxygen, is not repressed when the organism is grown in the presence of DBP. The changes in expression of proteins of electron pathway were detected in *A. ferrooxidans* cells cultivated in sewage sludge, and clearly indicate that under these conditions,

the energy demand of cells is lower and partially covered by assimilation of simple organic molecules, such as anaplerotic compounds.

The results of the present study clearly demonstrate that *A. ferrooxidans* can grow and oxidize ferrous iron in the presence of dibutyl phthalate. This compound is an example of chemicals, which does not inhibit its growth or iron oxidation. The ultrastructural changes observed in *A. ferrooxidans* cells are due to physical changes of the medium such as surface tension rather than to direct influence of DBP on bacterial cells. The most important conclusion from presented studies is that the presence of benzenedicarboxylic acid diesters is not major obstacle for industrial application of *A. ferrooxidans* in bioremediation of soils and sewage sludge.

Acknowledgement. We wish to thank Dr. E. Lewandowska from Laboratory of Electron Microscopy, Institute of Psychiatry and Neurology, Warsaw for performing TEM observations.

Literature

Angelova B. and H.P. Schmauder. 1999. Lipophilic compounds in biotechnology – interactions with cells and technological problems. *J. Biotechnol.* **67**: 13–22.

Bosecker K. 1997. Bioleaching: metal solubilization by microorganisms. *FEMS Microbiol. Rev.* **20**: 591–600.

Chan E.C. and J. Kuo. 1997. Biotransformation of dicarboxylic acid by immobilized *Cryptococcus* cells. *Enzyme Microb. Technol.* **20**: 585–589.

Chevalier J., M.T. Pommier, A. Cremieux and G. Michel. 1988. Influence of Tween 80 on the mycolic acid composition of three cutaneous corynebacteria. *J. Gen. Microbiol.* **134**: 2457–2461.

Drobner E., H. Huber and K.O. Stetter. 1990. *Thiobacillus ferrooxidans*, a facultative hydrogen oxidizer. *Appl. Environ. Microbiol.* **56**: 2922–2923.

Francis R.T. and R.R. Becker. 1984. Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. *Anal. Biochem.* **136**: 509–514.

Frattini C.J., L.G. Leduc and G.D. Ferroni. 2000. Strain variability and the effect of organic compounds on the growth of the chemolithotrophic bacterium *Thiobacillus ferrooxidans. Antonie van Leewenhoek* **77**: 57–64.

Gomez C. and K. Bosecker. 1999. Leaching of heavy metals from contaminated soil by using *T. ferrooxidans* or *T. thiooxidans*. *Geomicrobiol. J.* **16**: 233–244.

Guiliani N. and C.A. Jerez. 1999. Protein genes from *Thiobacillus ferrooxidans* that change their expression by growth under different energy sources.. In Amils R and Ballester (eds), Biohydrometallurgy and the Environment Toward the Mining of the 21st Century, Process Metallurgy. **9 A**: 79–88.

Hermanowicz W., J. Dojlido, W. Dożańska, B. Kosiorowski and J. Zerbe. 1999. Fizyczno-chemiczne badanie wody i ścieków. Arkady, Warszawa.

Johnson D.B. 1995. Selective solid media for isolating and enumerating acidophilic bacteria. *J. Microbiol. Methods.* **23**: 205–218.

Kelly D.P. and A.P. Harrison. 1989. *Thiobacillus*. In: Staley JT. Bryant MP. Pfennig N. Holt JG eds *Bergey's manual of systematic bacteriology*. vol. 3. Baltimore: The Williams and Wilkinson Co. p. 1842.

Laouar L., K.C. Lowe and B.J. Milligan. 1966. Yeast responses to non-ionic surfactants. *Enzyme Microb. Technol.* **18**: 433–438.

Marchesi J.R., G.F. White, W.A. House and N.J. Russel. 1994. Bacterial cell hydrophobicity is modified during the biodegradation of anionic surfactants. *FEMS Microbiol. Lett.* **124**: 387–392.

Matlakowska R. and A. Skłodowska. 2003. Isolation and evaluation of indigenous iron- and sulphur-oxidizing bacteria for heavy metal removal from sewage sludge. In: Proceeding Materials of 15th International Biohydrometallurgy Symposium. Nereus Group Athens. 2003. 265–276.

Matlakowska R., I. Swiecicka and A. Skłodowska. 2005. Phenotypic and genotypic adaptive responses of indigenous iron chemolithoautotrophic bacteria isolated from sewage sludge. In: Harrison STL. DE. Rawlings and J. Petersen (eds), Proceedings of the 16th International Biohydrometallurgy Symposium. Cape Town. 2005. 697–704.

Mazuelos A., N. Iglesias and F. Carranza. 1999. Inhibition of bioleaching processes by organics from solvent extraction. *Process Biochem.* **35**: 425–431.

Neu H.C. and L.A. Heppel. 1965. The release of enzymes from *E. coli* by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* **240**: 3685–3692.

Ohmura N., K. Sasaki and N. Matsumoto. 2002. Anaerobic respiration using Fe^{3+}, S^0 and H_2 in the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans*. *J. Bacteriol.* **184**: 2081–2087.

Ostrowski M. and A. Sklodowska. 1992. Bacterial and chemical leaching pattern on copper ores of sandstone and limestone type. *World. J. Microbiol. Biotechnol.* **9**: 328–333.

Pronk J.T., W.M. Meijer, W. Hazeu, J.P. van Dijken, P. Bos and J.G. Kuenen. 1991. Growth of *Thiobacillus ferrooxidans* on formic acid. *Appl. Environ. Microbiol.* **57**: 2057–2062.

Pronk J.T., J.C. De Bruyn, P. Bos and J.G. Kuenen. 1992. Anearobic growth of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **58**: 2227–2230.

Rogers H.R. 1996. Sources behavior and fate of organic contaminants during sewage treatment and in sewage sludges. *Sci. Total Environ.* **85**: 3–26.

Schägger H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**: 368–379.

Silverman M.P. and D.G. Lungren. 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yield. *J. Bacteriol.* **77**: 642–648.

Torma A.E. and I.J. Itzkovich. 1976. Influence of organic solvents on chalcopyrite oxidation ability of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **32**: 102–107.

Tuovinen O.H., S.I. Niemela and H.G. Gyllenberg. 1971. Effect of mineral nutrients and organic substrates on the development of *Thiobacillus ferrooxidans*. *Biotechnol. Bioeng.* **13**: 517–527.

Tyagi R.D., J.F. Blais and J.C. Auclair. 1993. Bacterial leaching of metals from sewage sludge by indigenous iron-oxidizing bacteria. *Environ. Pollut.* **82**: 9–12.

Zagury G.J., K.S. Narasiah and R.D. Tyagi. 1994. Adaptation of indigenous iron-oxidising bacteria for bioleaching of heavy metals in contaminated soils. *Environ. Technol.* **15**: 517–530.