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Discipline : Sciences du vivant

Aspects moléculaires et cellulaires de la biologie

par

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Arsenic oxidation of *Cenibacterium arsenoxidans* : Potential application in bioremediation of arsenic contaminated water

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Jury

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Summary

Arsenic is a naturally occurring metalloid present in many organic and inorganic compounds. The most abundant arsenic species are the inorganic As[III] and As[V]. The prolonged exposure (occupational or natural) of humans to nonlethal arsenic doses causes chronic health effects, but in long time period usually causes death. Therefore, different chemical technologies were developed for arsenic decontamination of water. Most of them have two stages – the oxidation of As[III] into As[V] and the subsequent immobilization of As[V]. The main disadvantage of these technologies is the use of strong chemical oxidants, which causes a secondary pollution of the environment. The replacement of the chemical oxidation step by a biological one has potential for development, mainly due to the lack of secondary pollution and the low impact on the environment.

We focused our interest on the studies of an arsenic-oxidizing β-Proteobacterium, recently named *Cenibacterium arsenoxidans*, which possess high arsenic –oxidation capacity. These studies are the preliminary step in order to develop a microbial oxidation step for an arsenic contaminated water cleanup technology. We investigated the optimal growth conditions of the strain, and new nutrient media were tested and developed. In addition to the studies of the As[III] oxidation from free cells, the As[III] oxidation from immobilized *C. arsenoxidans* cells were studied. Thereafter, a tracking of the growth of *C. arsenoxidans gfp*-tagged cells in an "open" system was performed, which aimed to clarify the colonization and survival ability of the strain in such system, where randomly introduced microorganisms were presented. Also a method for rapid screening of arsenic-transforming bacteria was developed.

Titre en Français :

Oxydation de l'arsenic chez *Cenibacterium arsenoxidans* : Applications potentielles dans la bioremédiation des eaux contaminées par de l'arsenic

Résumé

L'arsenic est un métalloïde naturellement présent dans différents environnements. Les formes inorganiques, l'arsénite (As[III]) et l'arséniate (As[V]) sont les plus abondantes. Ce sont aussi les formes les plus toxiques. L'ingestion d'arsenic, en particulier via l'absorption d'eau contaminée, est à l'origine de graves problèmes de santé publique dans des nombreuses parties du monde. C'est pourquoi, différentes méthodes de bio-réhabilitation ont été mises au point. La plupart de ces méthodes utilisent deux étapes : une oxydation chimique de As[III] en As[V], suivie de l'immobilisation de l'As[V]. L'utilisation d'oxydants puissants est à l'origine de pollutions secondaires.

L'oxydation par voie microbiologique de l'As[III] permet de proposer une méthode alternative intéressante puisque non polluante. Notre travail s'est focalisé sur l'analyse d'une β-protéobactérie, *Cenibacterium arsenoxidans*, capable d'oxyder efficacement l'As[III] en As[V]. Nos études constituent des étapes préliminaires pour le développement de méthodologies destinées au traitement d'eaux contaminées par l'arsenic. Nous avons établi les conditions d'obtention de la biomasse d'intérêt en testant de nouveaux supports de culture, basé sur la valorisation de déchets d'industries agroalimentaires. L'oxydation d'As[III] par *C. arsenoxidans* a été testée avec des cellules en suspension ainsi qu'avec des cellules immobilisées dans des billes d'alginate. En utilisant des cellules marquées avec la protéine GFP, nous avons étudié la survie et l'implantation de *C. arsenoxidans* en milieu non stérile. Enfin, dans le but d'isoler d'autres bactéries utilisables dans les processus de traitements de milieux contaminés par l'arsenic, nous avons développé une méthode simple et rapide pour le criblage de bactéries capables de réaliser l'oxydation d'As[III].

DISCIPLINE : Aspects moléculaires et cellulaire de la Biologie

MOTS-CLES : arsenite oxidation , arsenate reduction, arsenic bioremediation,

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CONTENTS

CHAPTER I

INTRODUCTION

I. BIOGEOCHEMICAL CYCLE OF ARSENIC

Arsenic is a naturally occurring metalloid, which is classified as the 20th most abundant element in the earth's crust. In the seawater it is the 14th most abundant element and the 12th in the human body. Arsenic is relatively wide spread, with average concentration of 5ppm and it is found in the crystal structures of more than 245 minerals, most of which are ores containing sulfide. The biogeochemical cycle of arsenic is represented in Fig. 1.

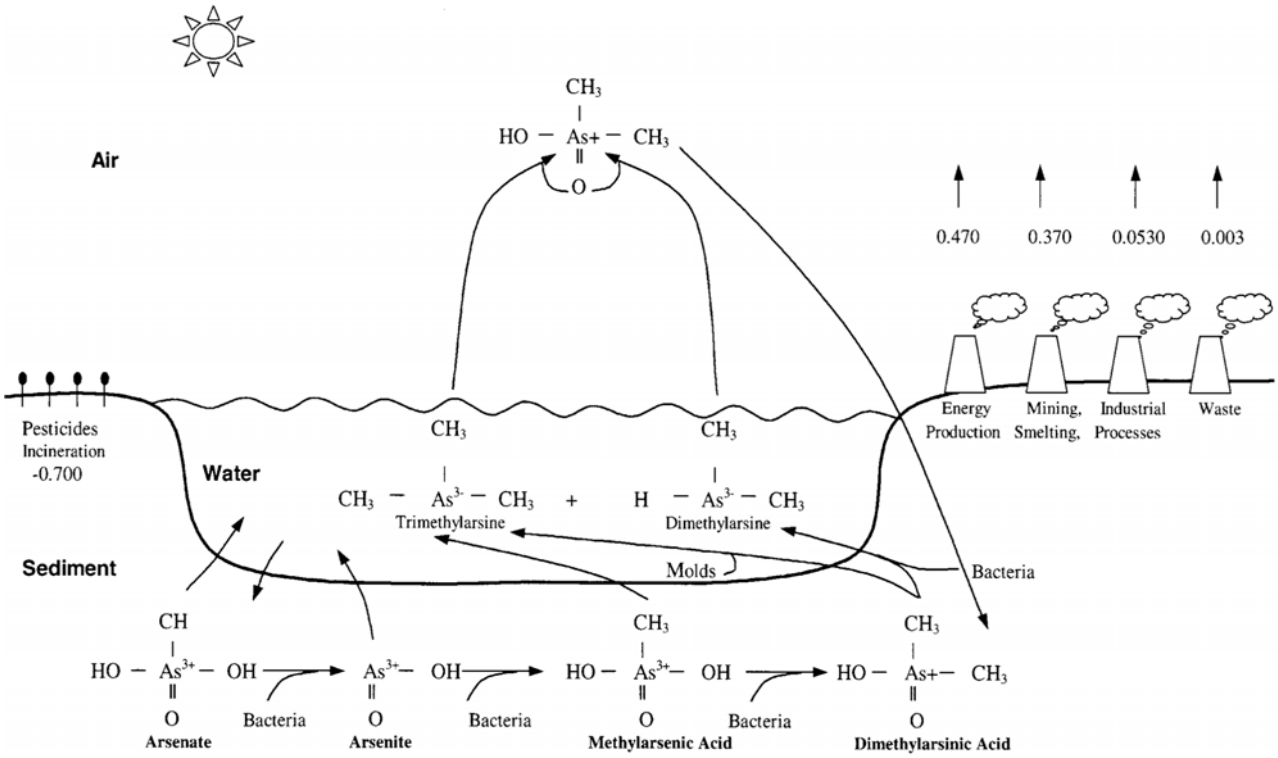

Fig.1. Biogeochemical cycle of arsenic and effects from anthropogenic sources (Ayers and Ayers, 1999).

Arsenic is present in many inorganic and organic compounds in which it has different oxidation states: As [-III] (arsine), As [0] (zero valent arsenic), As [+III] (arsenite) and As [+V] (arsenate). The most abundant arsenic species are the inorganic As[III] and As[V]. The relevant proportions of all states in a particular environment are a function of a diverse array of natural processes and /or anthropogenic activities. The most important natural process involving arsenic is the watering of rocks. This process leads to conversion of arsenic sulfides

to arsenic trioxide, which enters the arsenic cycle as dust or by dissolution in rain, river and groundwater. Another natural mobilization of arsenic is due to bacterial biotransformation of arsenic compounds, which includes reduction (including dissimilatory reduction), oxidation and methylation. Other natural ways for distribution of arsenic are the volcanoes and forest fires. From the natural sources arsenic is released into air, water and soil.

The main sources for anthropogenic release are: i) ores - mining and smelting industries; ii) metallurgic industry (additive for special lead and copper alloys); iii) agriculture-pesticides (mainly sodium arsenite) and insecticides such as lead arsenate, Ca_3AsO_4, copper acetoarsenite, monosodium methanearsonate (MSMA), disodium methanearsonate (DSMA); iv) wood preserving industry, mainly sodium arsenite and Fluor-Chrome-Arsenic-Phenol (FCAP), Chromated Copper Arsenate (CCA), Ammonical Copper Arsenate (ACA), etc. (Mandal and Suzuki, 2002); v) feed additivities, pharmaceutical industry, glass production, semiconductors production, electrical industry (combustion of wood and low quality coils) etc. The anthropogenic sources of arsenic exceed the natural sources in the environment.

1. Main forms and transformations of Arsenic in the nature

1.1. Inorganic arsenic species

The inorganic arsenic forms are the widest spread. In the environment arsenic is generally in the form of arsenite or arsenate. The former is the dominant form in the deep (anaerobic) water or in the acid water effluents, while the latter dominates under aerobic conditions. Under the form of arsine, arsenic is in [-III] oxidation state and is the most reduced form of the element and slightly soluble in water. This is also the only gas form of arsenic (Fig.2). In fruits, fish and vegetables, only 10% of the arsenic exist in inorganic form, although the arsenic content of many foods (i.e. milk, beef and pork, poultry and cereals) is mainly inorganic, typically 65-75%. A few recent studies report 85-95% of inorganic arsenic in rice and vegetables (Mandal and Suzuki, 2002).

In soil, arsenic occurs mainly as inorganic species. In aerobic environments arsenate As[V] is the most stable species and is strongly sorbed onto iron and manganese oxides/hydroxides, clays and organic matters. In contrast, in anaerobic environments (reducing conditions) the arsenite [III] is the dominant species. Inorganic arsenic compounds can be oxidized, reduced and methylated by microorganisms, producing under aerobic conditions monomethylarsonic acid (MMAsV), dimethylarsinic acid (DMAsV) and trimethylarsine oxide (TMAO). Under anaerobic conditions, inorganic arsenicals are subjected to reduction or reductive methylation to volatile and easily oxidized methylarsines.

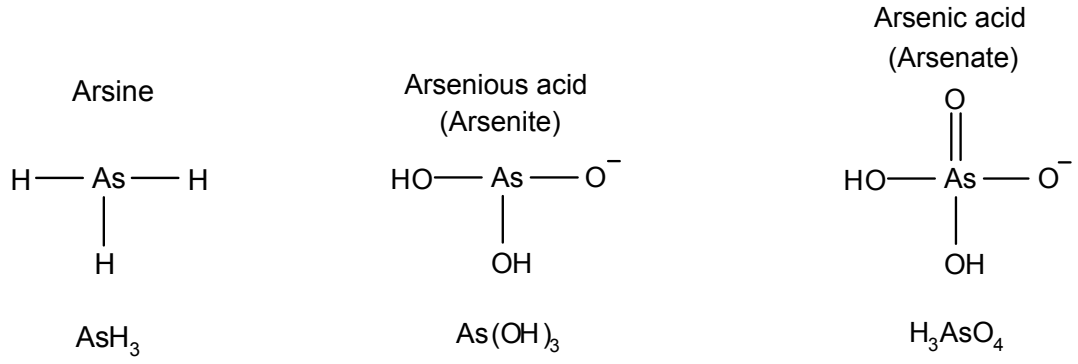

Fig.2. Inorganic arsenic species

In natural water arsenic is found in low concentrations. The major chemical form in which arsenic appears to be thermodynamically stable is the arsenate ion. The concentrations in unpolluted fresh waters range from 1–10µg l^{-1} rising to 100–5000µg l^{-1} (Smedley et al., 1996). At moderate or high redox potentials arsenic is stabilized under series of pentavalent (arsenate) oxyanions. However under acid and mildly alkaline conditions and lower redox potential, the trivalent arsenite species predominate. In aquatic environments the As[0] and As[-III] oxidation states are very rare.

In air, arsenic exists predominantly absorbed on particle matters or under the form of arsine and it has being of negligible importance except in the areas of arsenic pesticides application or biotic activity.

1.2. Organic arsenic species

In soil, soil-water and sediment water interfaces, inorganic arsenic compounds get biomethylated, through the activity of microorganisms, to form organic forms of arsenic.

Normally ground water does not contain methylated form of arsenic, but lake and pond waters contain inorganic forms of arsenic as well as methylated forms, i.e. monomethylarsonic acid (MMAsV), dimethylarsinic acid (DMAsV), monomethylasonous acid (MMAsIII) and dimetilarsinous acid (DMAsIII). The spatial distribution in the nature of organic forms of arsenic is presented in the Fig. 1 and the chemical structure of these compounds is presented in Fig.3.

In plants, animals and humans, arsenic is found accumulated in living tissue. Some of the inorganic species are converted to organic species and then excreted out of the living organism, or they can be included in macromolecules and stored in the body.

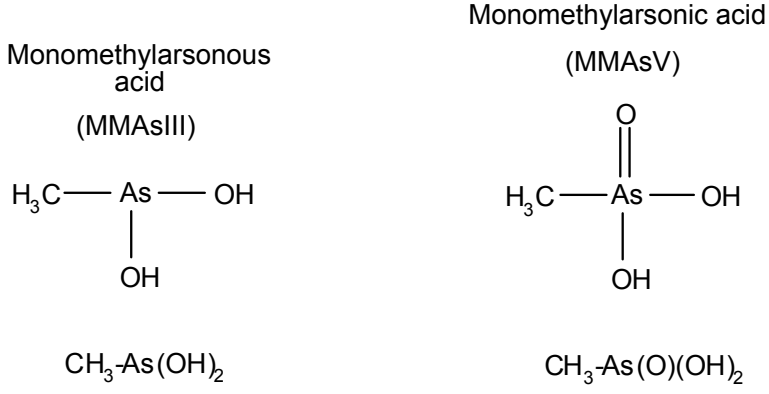

Fig.3. Main organic arsenic compounds. Part I.

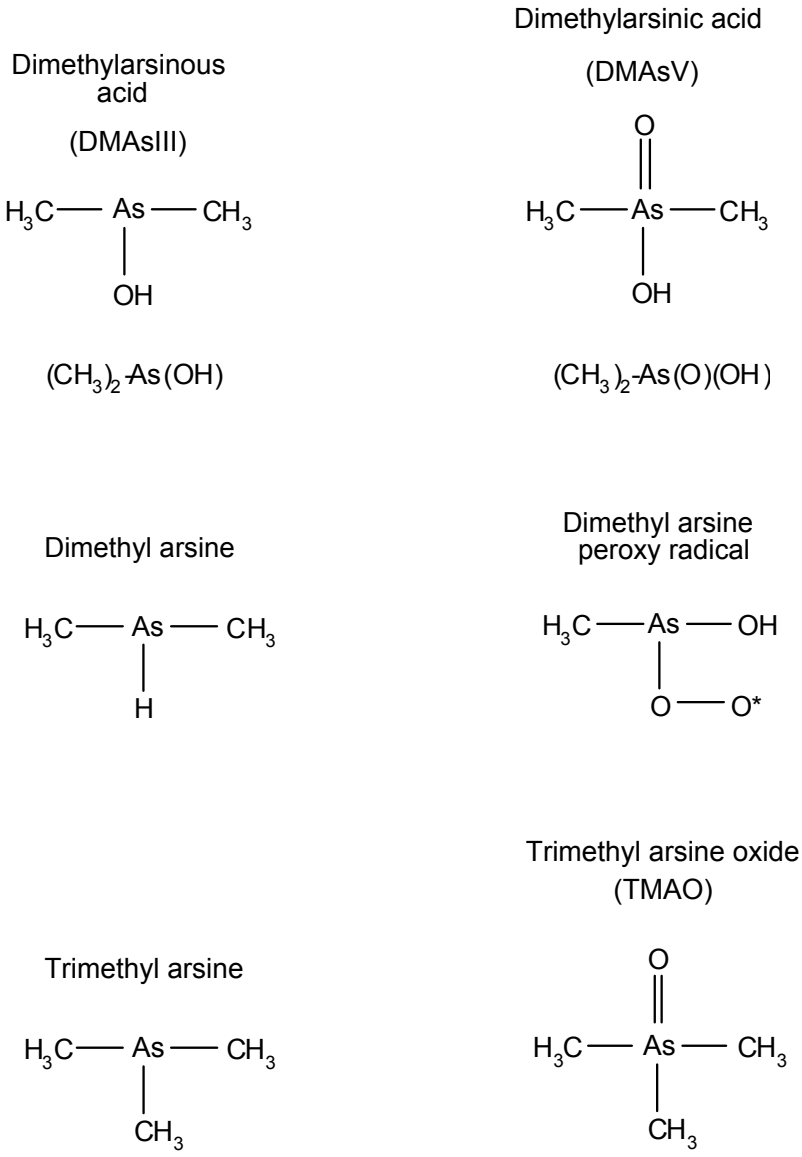

Fig.3. Main organic arsenic compounds. Part II.

2. Bioavailability and toxicity of Arsenic species

2.1. Bioavailability

The health risk to humans does not correlate directly with the external exposure dose of a given metal or metalloid. Importance for the risk has the bioavailability of the component. This represents the percentage of the external dose that reaches the systemic circulation, which is the fraction of the external dose of the examined metal or metalloid that is solubilized and absorbed from the gastrointestinal tract into the body of humans and animals (Caussy, 2003; Caussy et al., 2003). Bioavailability can be absolute or relative. The absolute bioavailability of an element is the fraction or percentage of the absorbed dose to the administrated dose (Candy et al., 1997).

$$\text{Absolute bioavailability} = \frac{\text{Absorbed dose}}{\text{Administrated dose}} \times 100$$

Relative bioavailability is the extent measurements of the absorption between two metal or metalloid compounds (Caussy, 2003). For example: if we assume that the absolute bioavailability of arsenic in the water is 100% then the relative bioavailability of arsenic in any matrix (soil, fruits, vegetables, etc.) is the quotient between absolute bioavailability of arsenic in the matrix divided by the absolute bioavailability of arsenic (Grissom *et al*., 1999):

$$\text{Relative bioavailability} = \frac{\text{Absolute bioavailability from the matrix}}{\text{Absolute bioavailability from water}} \times 100$$

2.2. Toxicity

The consequences for the human health from an arsenic exposure depend on the type of arsenic species, its dose, modality and the duration of exposure. According to the Word Health Organization (WHO), there are two types of exposure – acute, short exposure and prolonged (natural or occupational) exposure to nonlethal doses (WHO, 2001a). The First type of exposure to arsenic results in acute effects such as vomiting, abdominal colics and diarrhea. The second type of exposure to nonlethal doses causes chronic health effects which can be respiratory, pulmonary, cardiovascular, gastrointestinal, hematological, hepatic, renal, dermal, neurological, developmental, reproductive, immunologic, mutagenic and carcinogenic. The toxicity of arsenicals decrease in the order: arsines > inorganic As[III] > arsenoxides (org As[III]-containing compounds) > inorganic As[V] > arsonium compounds > As (Hindmarsh, 2000). Lately another toxicity order of arsenicals has been reported: inorganic As[III] > monomethylarsine oxide (MMAO[III]) > dimethylarsinous acid (DMAsIII) > dimethylarsinic acid (DMAsV) > monomethylarconic acid (MMAsV) > inorganic As[V] (Vega et al., 2001).

Inorganic forms

Inorganic forms of arsenic are more toxic than organic forms. The As[V] is less mobile and potentially less bioavailable than As[III] due to its stronger adsorption to mineral surfaces (Korte and Fernando, 1991). The uptake of arsenite in the cell is considered to be much faster than the uptake of arsenate, since the As[III] is uncharged at physiological pH. However, now it is believed that both forms of inorganic arsenic are actively transported into the cell. Arsenite is transported by aquaglyceroporins, which transport water and glycerol (2004) and arsenate is transported by the phosphate transporter. As[III] reacts via three mechanisms: the first mode of action is related to binding (or complexing) with electron – rich groups, -SH or -OH. In this type of action the reaction is a metal and the biological targets are the proteins. The second type of action mode is the oxidation-reduction reactions in which the chemical reaction type are metal and non-metal, and as target are used molecules that can transfer electrons. The third mode of action is the substitution of C and N in which the biological targets are choline and lipids and the chemical type of reaction is nonmetal.

The main difference in disposition between As[III] and As[V] is that As[III] is absorbed directly from the cell and As[V] competes with phosphate for absorption. The dispositions and reactions in which As[III] is involved are shown in Fig.4.

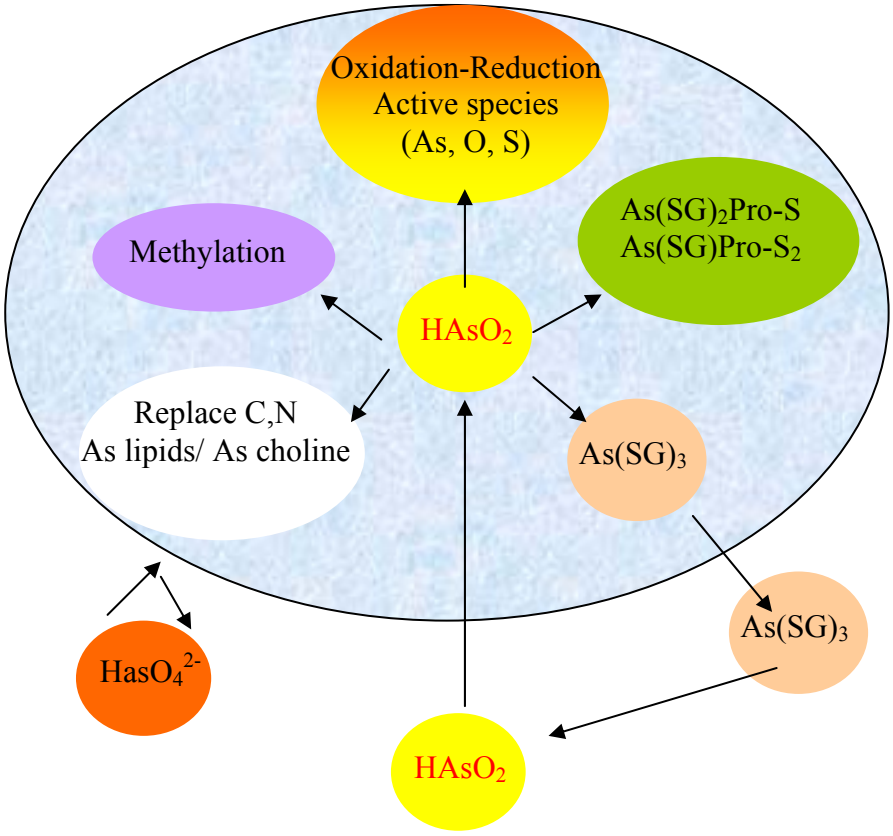

Fig.4. The disposition of arsenic [III] in the cell. (Carter et al., 2003)

Liu *et al.*, (2004) showed that As[III] enters into the cells through the aquaglyceroporins channels. These channels allow the downhill movement of uncharged solutes such as glycerol and urea. Nevertheless, the authors have proved that the human hAQP9 is the most efficient channel for As[III] uptake into the cell. The pathway for arsenite uptake into prokaryotic cells was found to be connected to a homologue of the human aquaglyceroporins- the GlpF transporter.

The general mode of action for As[V] in the cell is the substitution of phosphate, since these two ions have identical structure and acid dissociation constants. In the prokaryote *Escherichia coli* two phosphate transporters Pit and Pst, were found (Rosen, 2002). Both of

them catalyze the arsenate uptake, but the Pit system was found to be the predominant. In eukaryotes the phosphate uptake systems also are responsible for the arsenic transport into the cells. Arsenate reacts with ADP to form a mixed arsenate-phosphate bond in ATP-like molecules. That bond can be easily hydrolyzed and the mixed arsenate-phosphate molecule rapidly dissociates without generating ATP (Goyer and Clarkson, 2001). The uptake of arsenate and its subsequent transformation are shown in Fig. 5.

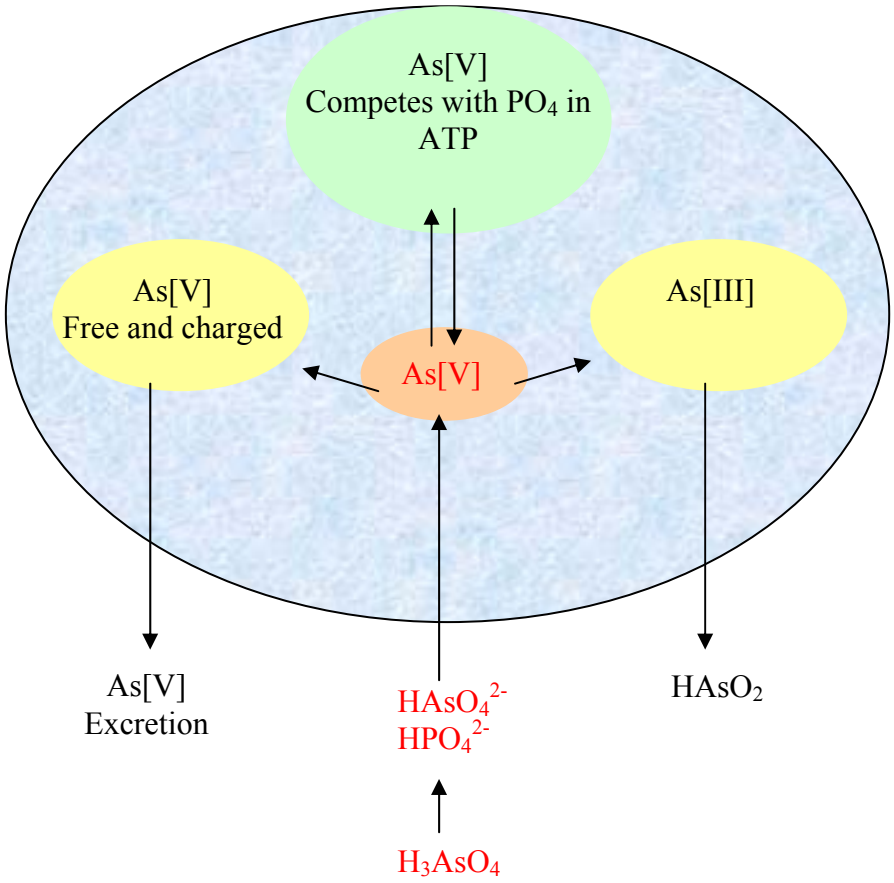

Fig.5. The disposition of As[V] in the cell (Carter et al., 2003).

Another mode for the toxic action of arsenate is the reduction process in which molecules transferring electrons are involved. The arsenate does not react directly with the active sites of enzymes. It is first reduced into As[III] *in vivo*, before exerting its toxic effect.

Organic forms

The term "Biomethylation" describes the formation of nonvolatile and volatile methylated compounds. The major volatile compounds of arsenic, formed by methylation have the structure $(CH_3)_nAsH_{3-n}$, where n = 1, 2 or 3 methyl radicals. The major nonvolatile compounds are the methylarsonate and dimethylarsinate (Bentley and Chasteen, 2002).

Organic arsenic species important in toxicity are the monomethylarsonous acid (MMAsIII) and dimethylarsinous acid (DMAsIII) for arsenic in oxidation state +III. For As[V] the organic forms are monomethylarconic acid (MMAsV) and dimethylarsinic acid (DMAsV). MMAsV is a methylated metabolite of As[III], consequently metabolized to DMAsV. The MMAsIII is a reduced metabolite of MMAsV and the DMAsIII is a reduced metabolite of DMAsV. The DMAsV is a dimethylated metabolite of As[III] and methylated metabolite of MMAsIII. Recent comparative investigations on the toxicity of methylated and dimethylated arsenicals showed that those containing As[III] are more cytotoxic, more genotoxic and more potent inhibitors of the activities of some enzymes even than inorganic As[III] (Rossman, 2003).

In humans, arsenic species are metabolized by methylation and then they are excreted much faster than inorganic ones. The first step in the As[V] transformation is the reduction to As[III], before methylation can occur. The enzyme involved in the reaction in vivo is purine nucleoside phosphorylase, with dithiol reductant, but not glutathione (GSH) (Gregus and Németi, 2002), which is able to reduce arsenate nonenzymatically also (Scott et al., 1993).

Through enzymatic transfer of the methyl group from S-adenosylmethionine (SAM) to arsenite, is formed monomethylarsonic acid (MMAsV). The same enzyme (purine nucleoside phosphorylase) or glutathione-S-transferase omega class 1-1 (GSTO1-1) then reduces MMAsV to monomethylarsonous acid (MMAsIII). A second reaction methylates MMAsIII to dimethylarsinic acid (DMAsV) (Rossman, 2003). Some DMAsV can then probably be reduced to DMAsIII (Fig.6).

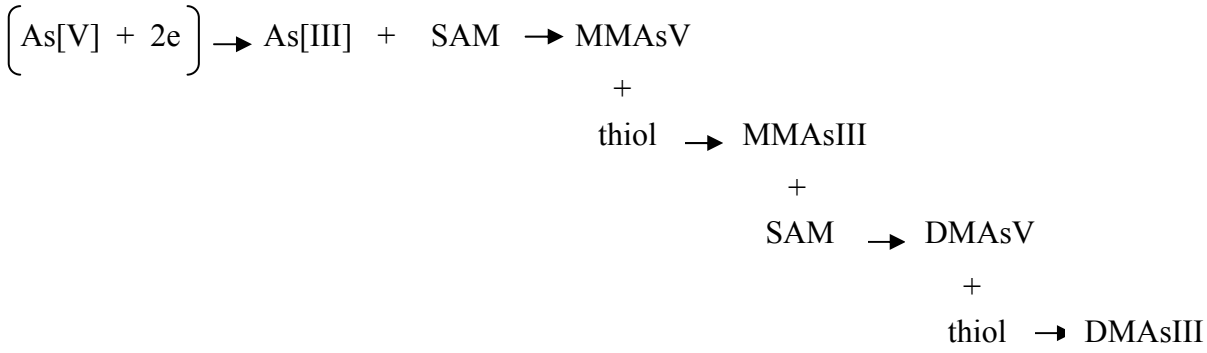

Fig.6. Metabolism of arsenic compounds in eukaryotes (mammals).

In the case of microorganisms (fungi, yeasts and bacteria) the use of arsenic as methyl group acceptor has a major emphasis on the production of volatile compounds. For example *Alcaligenes* sp. and *Pseudomonas* sp. have been reported to be able to reduce arsenate and arsenite anaerobically to arsine (Cheng and Focht, 1979). A strict anaerobe belonging to Archae *Methanobacterium* M.o.H. (now *M. bryantii*), has been shown to produce dimethylarsine from arsenate with H_2 (McBride and Wolfe, 1971). The fungi from genera *Aspergillus*, *Mucor*, *Fusarium*, *Paecilomyces* as well as *Candida humicola* and *Scopulariopsis brevicaulis* are able to convert arsenite to trimethylarsine (Alexander, 1977; Cullen and Reimer, 1989; Pickett et al., 1981). In the case of algae and phytoplankton, is proved that As[V] is the most toxic species for these organisms and they reduce it to As[III], which is consistent with conventional view that this serves as a detoxification mechanism for As[V] (Knauer et al., 1999).

To proceed from arsenate to trimethylarsine for the fungi and yeast, four two electron reductions are required. They are likely to be enzyme catalyzed reactions with the reductants providing the necessary $2e^-$. The reductants are predicted to be thiols, and a particular attention was given to glutathione (GSH) and lipoic acid (6,8-dithiooctanoic acid). Nevertheless, there is still little experimental evidence from microbial systems. Thus, it is assumed the reaction resembles those for formation of methylarsonate and dimethylarsinate of mammalians (Thompson, 1993).

Kuroda *et al.* (2001)**,** showed that in *E. coli* cysteine, but not GSH was required for dimethylarsinate metabolism. Thus, there may be different arsenic metabolic pathways in

bacteria. Moreover there is no general consensus as to a mechanism for anaerobic dimethylarsine formation.

The three oxidation states of this element and the small amounts of arsenic compounds present in biological specimens complicate the enzymology of arsenic biomethylation. The chemical intermediates and reactions in the arsenate metabolism are generally assumed to be the same in microorganisms and animals. However, there is a difference: in microorganisms the reactions tend to proceed to volatile species (methylarsines), whereas in mammalians turn to nonvolatile species, which are excreted via urine. The major urinary metabolite is the dimethylarsinate (Bentley and Chasteen, 2002).

3. Microorganisms involved in the arsenic cycle

3.1. Oxidation of inorganic As[III]

The most abundant arsenic species in nature are the inorganic As[III] and As[V]. The inorganic As[III] is widely subjected to oxidation in nature, mostly by bacteria and some archea. The first report of an As-oxidizing bacterium named *Bacillus arsenoxidans* was done from Green in 1918. Nevertheless, to date there are only a few bacterial strains, which have been described as arsenite-oxidizers with heterotrophic or chemoautotrophic growth (Table.1).

Species	Phylogeny	Reference
a. Bacteria		
Achromobacter (Alcaligenes) faecalis ■	β-Proteobacteria	Osborne and Enrlich, 1976
Agrobacterium albertimagni AOL15 ■	α-Proteobacteria	Salmassy *et al*., 2002
MLHE-1 ●	γ-Proteobacteria	Oremland *et al.*, 2002
NT-26 ●	α-Proteobacteria	Santini *et al.*, 2000 a
Pseudomonas putida ■	γ-Proteobacteria	Abdrashitova *et al.*, 1985
Pseudomonas arsenitoxidans ●	γ-Proteobacteria	Ilialetdinov *et al.,* 1981
ULPAs1(*Cenibacterium arsenoxidans*)■	β-Proteobacteria	Weeger *et al*., 1999
b. Archea		
Thermus thermophilus HB8 ■	Thermales	Gihring *et al*., 2001b
Thermus aquaticus YTI ■	Thermales	Gihring *et al*., 2001b
Thermus sp. HR13 ■/●	Thermales	Gihring and Banfield, 2001a
Sulfolobus acidocaldarius BC ■/●	Sulfolobales	Sehlin *et al*., 1992

Table 1. a) Arsenic-oxidizing bacteria; b) arsenite-oxidizing Archea; ■-heterotrophic arsenite oxidizers; ●- chemoautotrophic arsenite oxidizers. In some cases the microorganism is found to be able both-respire As[V] and oxidize As[III].

The arsenic oxidation is a potential detoxification mechanism amongst bacteria. The genetic basis of the arsenite oxidase was elucidated first by Muller *et al.* (2003) by knockout mutants deficient in arsenite oxidation of heterotrophic strain ULPAs1, recently named *Cenibacterium arsenoxidans*.

The enzyme responsible for the oxidation was purified and characterized by Anderson *et al.* (2001). It is located on the outer surface of the inner membrane of Gram negative chemoheterotrophic bacterium *Alcaligenes faecalis*, and exhibits activity when azurin and/or cytochrome c serves as electron acceptor. The overall structure of the enzyme is presented in Fig.7. The arsenite-oxidase contains two subunits, a larger one 88 kDa polypeptide containing the Mo-pterin and a HiPIP (high potential iron protein) 3Fe-4S centers and a smaller 14kDa subunit with the Rieske active center 2Fe-2S (Anderson et al., 2001).

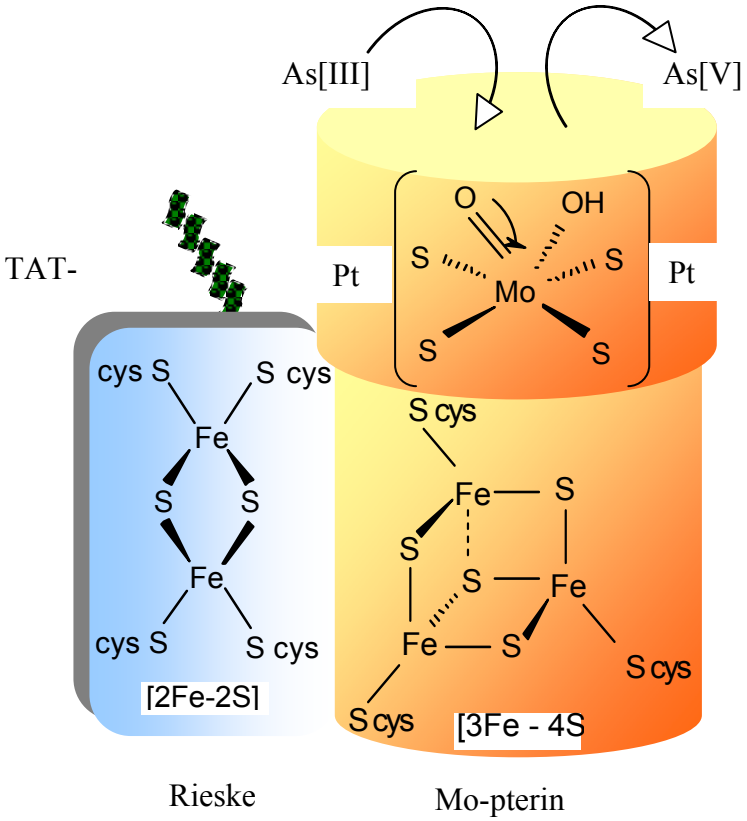

Fig.7. Arsenite oxidase model, with active sites, co-factors and linking amino acids (Silver and Phung, 2003).

At the beginning of gene for each Rieske subunit a Twin Arginine translocation (TAT) leader sequence was found. The function of TAT is the final re-assemblage of the enzyme and its

translocation from the cytoplasm to the periplasm of the cell. The arsenite oxidase enzyme is in the large super-family of dimethyl sulfoxide (DMSO) reductase proteins. This is a diverse family, in which enzymes vary in substrates, midpoint electric potential and direction. That is why, some enzymes of this family function physiologically as oxidases as is the case with arsenite oxidase and others function as reductases (Mukhopadhyay et al., 2002; Silver and Phung, 2003).

Santini and vanden Hoven (2003) recently described the purification and preliminary characterization of the arsenic oxidase of chemolitoautotroph strain NT-26 a member of α-Proteobacteria together with the cloning, sequencing and molecular analysis of the gene structure. The enzyme consist of two heterologous subunits, named AroA (98 kDa) and AroB (14 kDa), and has two molybdenium and 9 or 10 iron atoms per unit. The suggested configuration of the protein is $\alpha_2\beta_2$.

3.2. Reduction of arsenic species

To date two different mechanisms of arsenate reduction have been found in bacteria, fungi and algae. One of them is common for all microorganisms and is considered as a means of detoxification. The second one is specific only for Bacteria and some Archea. It represent the capability of microorganisms to use the arsenic as final electron acceptor in their respiratory chains, in autotrophic or heterotrophic growth.

3.2.1. Mechanism of arsenic detoxification (periplasmic As reduction)

There are several microorganisms described in the literature, which possesses the reduction capacity of inorganic and organic As[V] species.

The intracellular mechanism of arsenate-reduction in prokaryotic cells differs in the enzymes involved in the transformation. Nevertheless, the common points in the different detoxification systems as regards to the different type of operons includes a cytoplasm arsenate reductase (ArsC) and an excretion system, which can consist simply of one pump ArsB or of ArsAB ATPase (consisting of two subunits-the ArsB pump and the ArsA

ATPase). The differences in the type of the enzymes involved in the intracellular arsenic reduction and the way of their action are presented in Fig 8A.

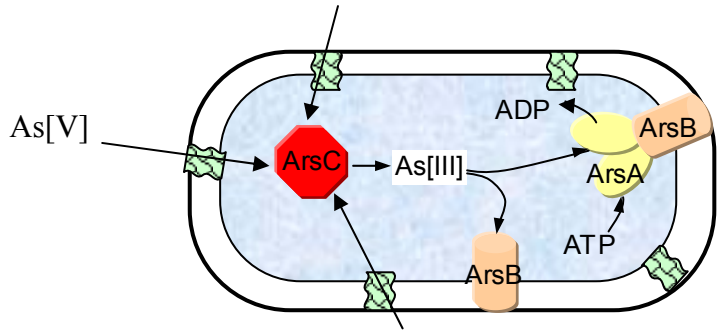

Fig. 8A. General detoxification mechanism in prokaryotes.

The arsenate reductase ArsC is coded by gene, which is widely found in Bacteria and in some Archea, in *ars* operons. This is the enzyme responsible for the reduction of As[V] to As[III]. Currently, three unrelated clades of ArsC sequences are recognized, with a common basic function, but without evolutionary relationship (Mukhopadhyay et al., 2002). All of them are glutaredoxin/glutathione (Grx/GSH) coupled enzymes and it is now believed that they are associated with the arsenite oxidase of *Alcaligenes* sp. and the respiratory arsenate reductase of *Shewanella*. The *ars* operon and ArsC reductase genes in Gram negative bacteria are found on plasmids and in the chromosomes. The ArsC from glutaredoxin/glutathione clade is about 135 amino acid residues protein, with 3 essential cysteine residues.

The thioredoxin-coupled ArsC (Trx ArsC) was firstly found in a Gram positive *Staphylococcus* (Ji and Silver, 1992). This enzyme also utilizes 3 cysteines all of them in the primary sequence of the polypeptide. The clade of thioredoxin-coupled ArsC enzymes is widespread on the plasmids and genomes of Gram positive bacteria, but it is also found in some Gram negative bacteria. For example the genome of *P. aeruginosa* possesses genes for both ArsC reductase families. (Mukhopadhyay et al., 2002).

The mechanism of arsenic detoxification in an eukaryotic type of cell is connected to the third clade of cytoplasmic arsenate reductases, represented by the Acr2p *Saccharomyces cerevisiae* arsenate reductase. Its overall function in the cell is illustrated in Fig. 8B. This Acr2p *S. cerevisiae* arsenate reductase is the only currently identified enzyme in the eucaryotic organisms, known to convert arsenate to arsenite. This is a homodimer of 130 monomers and it has common substrates of GSH and Grx with those for Grx/GSH ArsC reductase in

bacteria. The whole schema of arsenic detoxification mechanism in eukaryotes is presented on figure 8B (Mukhopadhyay et al., 2002).

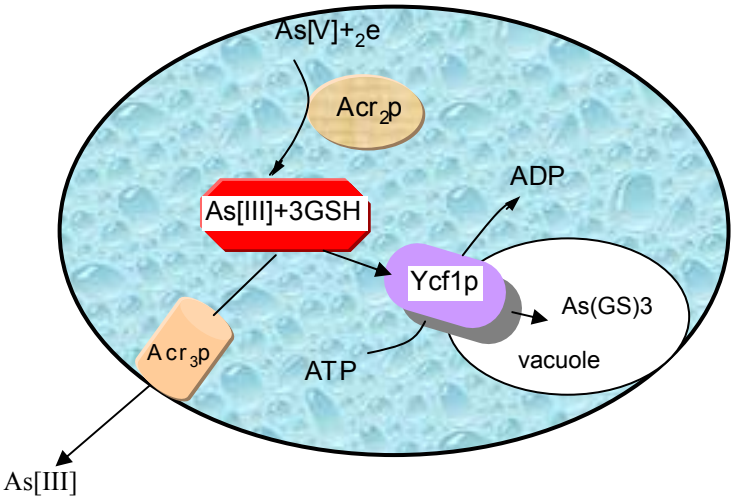

Fig.8B. General detoxification mechanism in eukaryotes.

The specific part of the intracellular detoxification mechanism in the eukaryotic type of cell is connected with the possibility to stock the As[III] in the cytoplasm vacuoles.

3.2.2. Arsenate respiration (a second type of arsenic reduction)

The arsenic respiration is the second mechanism for reduction of As[V], which is a specific one for the Bacteria and Archea. Using this mechanism bacteria couple the reduction of oxidized arsenic compounds with the final electron acceptors from their respiratory chain. Most of them reduce arsenate only as far as arsenite (Table 2).

Species	Phylogeny	Reference
a. Bacteria		
Bacillus arsenicoselenatis	Gram (+)	Switzer Blum *et al*., 1998
Bacillus selenitireducens	Gram (+)	Switzer Blum *et al*., 1998
Bacillus sp. SF-1	Gram (+)	Yamamura *et al.*, 2003
Chrysiogenes arsenatis	Gram (+)	Macy *et al.*, 1996
Desulfitobacterium GBFH	Gram (+)	Niggemyer *et al*., 2001
Desulfomicrobium sp. Ben-RB	δ- Proteobacteria	Macy *et al*., 2000
Desulfosporosinus (Desulfotomaculum) auripigmentum	δ- Proteobacteria	Newman *et al.*, 1997
Desulfovibrio sp. Ben -RA	δ- Proteobacteria	Macy *et al*., 2000
Sulfurospirillum arsenophilum	ε- Proteobacteria	Stolz *et al*., 1999
Sulfurospirillum barnesii	ε- Proteobacteria	Laverman *et al*., 1995
Sulfurospirillum deleyianum	ε- Proteobacteria	Stolz *et al*., 1999
Shewanella trabarsenatis	γ- Proteobacteria	Saltikov *et al*., 2003a
SES-3	ε- Proteobacteria	Laverman *et al*., 1995
Strain Y5	δ- Proteobacteria	Liu *et al*., 2004 (in press)
b. Archea		
Pyrobaculum aerophilum	Crenoarchaea	Huber *et al*., 2000
Pyrobaculum arsenaticum	Crenoarchaea	Huber *et al*., 2000
Thermus sp. HR13	Thermales	Gihring *et al*., 2001a

Table.2 Arsenate-respiring bacteria and Archea isolated in pure cultures up to date.

To the present time the respiratory-linked reductases are poorly characterised at both- protein and gene levels. The anaerobic arsenate reductase (Arr) is a heterodimer periplasmic protein, which consist of a larger molybdo-pterin subunit (ArrA) of 854 amino acids and a smaller subunit (ArrB) of about 234 amino acids sown in Fig.9. The ArrA subunit has an iron-sulfur center and the ArrB subunit contains a Fe-S center protein, which is not homologous to the Rieske polypeptyde from the arsenite oxidase and it can contain up to four 4Fe-4S clusters (Krafft and Macy, 1998; Macy and Santini, 2002; Saltikov *et al*., 2003). Thus, some authors believe there is a relationship between arsenate oxidase and the respiratory-linked reductases, which only difference is that they work in a opposite direction (Mukhopadhyay *et al*., 2002; Silver and Phung, 2003).

Recently, Saltikov and Newman, (2003a) and Afkar, (2003) have found the genetic determinants of two respiratory arsenate reductases- the first one from γ-Proteobacteria, the Gram (-) *S. trabarsenatis* and the second one from the Gram (+) *B. selenitireducens*, respectively. The genetic determinants from *S. trabarsenatis* include the genes for *arrAB* and the contiguous *arsBCD* operon (known as arsenic resistance operon). For both bacteria the sequence of ArrA, the large Mo-pterin subunit have 47% identity in amino acid sequences (Silver and Phung, 2003). According to the phylogenetic trees of ArrA and related sequences, the protein appears to be a distantly related member of DMSO family of oxireductases of Mo-pterin and Fe-S center - containing enzymes. The second subunit of enzyme - ArrB appears to be an iron-sulfur protein related to DmsB of DMSO reductase and NrfC of nitrite reductase (Silver and Phung, 2003).

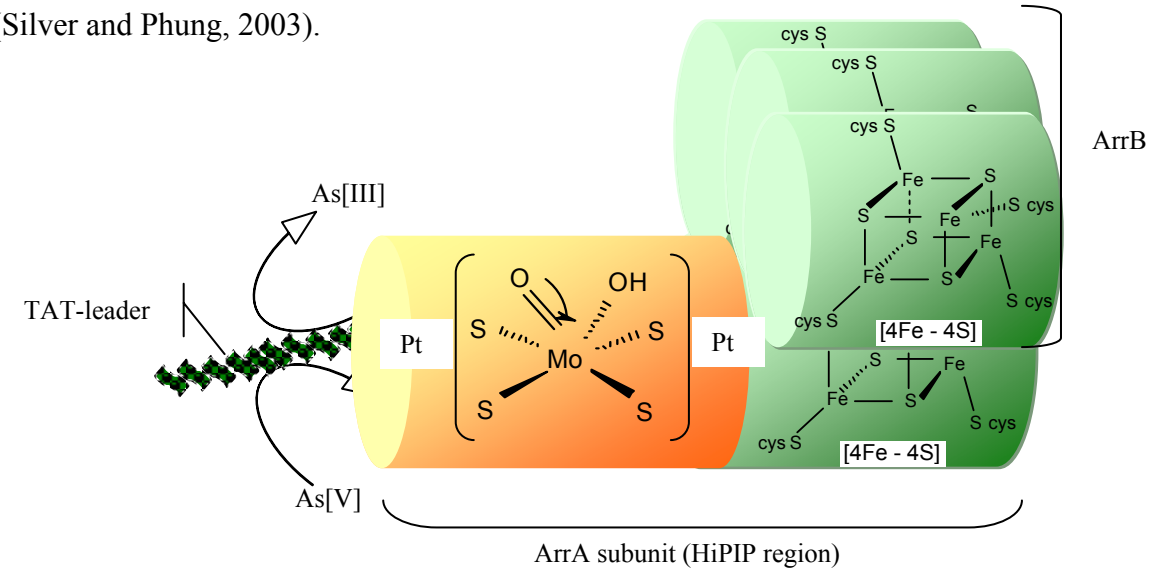

Fig.9. Respiratory arsenate reductase (ArrAB), model, with active sites, co-factors and linking amino acids reductase (Silver and Phung, 2003).

II. METHODS FOR ARSENIC DECONTAMINATION

1. Chemical methods and technologies for remediation of arsenic-containing water

To date most of the technologies for arsenic decontamination of polluted water are based on chemical methods and treatment techniques. Predominantly, they consist of two steps. The first step is generally the oxidation of As[III] into As[V]. The As[V] is easily removed from water by precipitation and sorption due to its high reaction activity. This form of arsenic has low mobility and less toxicity (Arai *et al.*, 2001; Meng *et al*., 2001; Goldberg and Jonhston, 2000). Ionic strength effects more the As[III]sorption. It is generally held that As[V] is more strongly bound and forms predominantly inner-sphere complexes regardless of pH and ion strength (*I*). Therefore, most of the chemical treatment technologies in a given effluent are based on the preliminary oxidation of total arsenic to As[V].

1.1. Chemical arsenite-oxidation

Generally chemical methods for arsenite oxidation are based on the use of strong oxidizing agents such as hypochlorite, permanganate and hydrogen peroxide or Fenton's reagent (FR). Fenton's reagent is a widely applied oxidative technology, which utilizes the reaction of hydrogen peroxide with ferrous iron (generally added as $FeCl_2$ or $FeSO_4$) to produce free radicals capable to oxidize aqueous phase contaminants. The addition of ferrous salts containing chloride or sulfur or the use of strong oxidants is undesirable, because of the generation of organochloride byproducts, which cause the additional destruction and impact on the environment. Recently Arienzo *et al*. (2002), proposed an electrochemical peroxidation (ECP) technique for the oxidation of As[III], which is based on the application of direct electric current to steel electrode, complemented with a small addition of H_2O_2.

Zaw and Emett (2002), proposed an advanced oxidation method for arsenic removal, since the oxidation rate of dissolved As[III] by oxygen is extremely low. The advance oxidation method is based on the use of ultraviolet light and a photo absorber. The maintenance of dissolved oxygen was achieved by air sparging. This process needed a pH adjustment of treated water, which was made through the addition of HCl. The targets of the photolytic reaction were the Fe[III] ions, involved in the transfer of one electron from the complexed ligand, such as organic, hydroxide or chloride species, to a Fe[III] -centered orbital, forming

Fe[II] and a free radical (Zafiriou *et al*., 1984). The generated free (hydroxyl) radicals was then used to oxidize As[III].

1.2. Coagulation and co-precipitation techniques

Coagulation encompasses all reactions, mechanisms and results in the overall process of particle growth and particle aggregation within water. Coagulation involves the removal of colloidal and settleable particles, but the term commonly refers to the removal of dissolved ions, which is actually precipitation. The precipitation is the process in which dissolved ions in solution form an insoluble solid as a result of a chemical reaction. A co-precipitation indicates the cases in which an inorganic contaminant forms an insoluble complex with a coagulant. The valence of the inorganic contaminant and the pH of the solution are important for the removal by co-precipitation. There are four types of co-precipitation:

- inclusion – mechanical entrapment of a portion of the solution surrounding the growing particle (significant for the large particles);
- adsorption – the attachment of an impurity onto the surface of a particle or precipitate (significant for the small particles);
- occlusion – entrapment of a contaminant in the interior of a particle of precipitate, which continues to grow;
- solid-solution formation – a type of occlusion in which a particle of precipitate becomes contaminated with a different type of particle that precipitates under similar conditions and is formed from ions whose sizes are nearly equal to those of the original precipitate.

Throughout coagulation dissolved arsenic is converted into insoluble reaction products, which can be further eliminated by sedimentation and/or filtration. This arsenic removal involves three mechanisms:

- Precipitation: the formation of insoluble or less soluble compounds of $Al(AsO_4)$ or $Fe(AsO_4)$
- Co-precipitation: the incorporation of soluble arsenic species into the metal hydroxide flock

- Adsorption: based on the electrostatic binding of soluble arsenic to the external surfaces of the insoluble metal hydroxides.

The direct precipitation plays the least important role in arsenic removal, therefore is the least abundant technique.

The co-precipitation is much more active arsenic removal mechanism, and different techniques still are under amelioration and development. The advantages of these techniques are the high adsorption capacity for arsenate (As[V]) of iron hydroxides formed from ferric salts and the low leachability of arsenic in the sludge, when compared to the As-precipitates formed with soil minerals such as clays (Sadiq, 1997). The disadvantages are the necessity of As[III] oxidation (usually in this cases are used oxidizing agents as hypochlorite, permanganate and hydrogen peroxide), low level of phosphates and silicates in treated water (they decrease the effectiveness of the process and phosphates enhances the mobility of As[V]), requirement of high Fe/As ratio (in average 40 to reach about 85% of arsenic removal). This leads to the addition of Fe-salts in treated water (Meng *et al*., 2000).

Nishimura and Umetsu, (2001) have developed a co-precipitation technique, based on the use of ozone for As[III] and Mn[II]oxidation and a consequent co-precipitation of As[V] with Mn [III]. This method is useful for removal of arsenic from dilute acidic sulfate solutions in the pH range of 1.0-3.0, in which the co-precipitation of arsenic with ferric hydroxide does not take a place. Nevertheless, it also requires a mole ratio of Mn/As = 10-100.

Lackovic and co-workers (2000) have proposed an arsenic removal system by zero-valent iron. Recently Nikolaidis and co-workers (2003) improved this technique. They state that both As[III] and As[V] are removed efficiently and that the efficiency is related to the surface area or the type of iron used. However, ferrous iron was leached from the columns, with average concentration of 50mg/L and finally 72% of the iron was exported from the system. The major advantage of this method is the direct removal of arsenite over the two-step systems.

In general the adsorption of arsenic is based on its chemical specificity and that at natural pH values the As[III] exists only as H_3AsO_3 and $H_2AsO_3^-$ with high pK_a values of 9.2 and 12.7, respectively. The As[V] can exist in solution as H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$ and AsO_4^{3-}, with pK^1_a 2.3, pK^2_a 6.8 and pK^3_a 11.6. The mechanisms of arsenic adsorption were investigated and it was found that the As[V] forms inner- sphere surface complexes on Al and Fe oxide surfaces while As[III] forms inner- and outer-sphere surface complexes on amorphous Fe oxides and outer- sphere complexes on Al oxides. It was also found that the position of the As-O stretching bands, for both As[III] and As[V], are strongly pH and ionic strength (*I*)

dependent (Goldberg and Johnston, (2001). The inner-sphere complexes from a ligand exchange reaction with a surface functional group, results in lost of water molecules between surface functional groups and the adsorbate ions. Outer-sphere complexes mainly due to electrostatic interactions contain more than one water molecule between the adsorbate and the adsorbent functional group (Arai, 2001). In general, As[V] sorption on Al and Fe-oxides is characterized with a maximum at a pH value of 4 and As[III] has a maximum sorption in the pH range of 7 to 8.5.

1.3. Filtration

The conventional filtration process represents the separation of solid particles from water (liquid effluents) by passing the solution through a medium. The solid particles are removed during this process as a result of any one or combination of mechanisms: mechanical straining, flocculation, sedimentation, adsorption and/or biological metabolism. The filter medium can consist of different materials such as: sand, activated carbon or paper, that retains the solid on its surface and allows the water to pass through. Common particles removed by filtration are silt, clay, colloidal and precipitated natural organic matter, naturally-occurring iron and manganese precipitates, precipitates from different metal salts or polymer coagulation, microorganisms and/or their metabolic products. Filters can be classified in various ways, according to the hydraulic system (gravity, up-flow, etc.), rate of filtration, the type of granular medium used and/or by the location of particle accumulation (e.g. cake filtration, depth filtration, etc).

The technology for treatment of arsenic contaminated water, proposed by Meng et al. (2000) is based on the conjugation of two chemical methods – the co-precipitation of arsenic with iron hydroxides, combined with filtration of the treated water through a bucket sand filter. Nikolaidis and co-workers (2003), developed an iron filing filter system for arsenic remediation, in which a part of the As[III] is oxidized into As[V] and both are simultaneously removed due to precipitation on the Fe^0 surface during the filtration of the treated water.

Wang and Reardon (2001), developed a two-column reactor, to remove the dissolved As. In this system the treated water is filtered through two columns, fulfilled with crushed siderite and limestone, respectively. Although the arsenic is removed by the sorption mechanism, not by the filtration, because the Fe[III] hydroxide was formed from the siderite and it is a sorbent with high affinity towards As[V] ions.

1.4. Adsorption

The process of adsorption is the accumulation of material of an interface or the liquid/solid boundary layer. It is a mass transfer process where a substance is transferred from the liquid phase to the surface of a solid and is bound through chemical or physical forces. The adsorption takes place also within suspended particles, as a part of the process coagulation/co-precipitation or on fixed medium. Therefore, adsorption is a surface phenomenon and the greater the surface, the greater is its capacity to accumulate material. Each adsorbent medium possesses different associated properties, performances and costs. Arsenic is adsorbed onto the surface of various granular, usually activated clay, metal oxides or hydroxides of Fe, Al or Mn, organic adsorbents based on cellulose, such as:

1. Oxides – hydrated ferric oxide, aluminum oxide, titanium oxide, silicium oxide, etc.;
2. Sand – Fe-oxide or MnO_2-coated;
3. Minerals containing Fe, Mn and Al – bauxite, hematite, feldshpar, trolite, etc.;
4. Clay minerals – kaolinite, bentonite, Bijoypur clay, zeolite, kutnahorite, khabazite etc.;
5. Synthetic anion exchange resins;
6. Chitin and chitosan;
7. Cellulose materials – sawdust, newspaper pulp.

The most abundant technologies and applications of adsorption of arsenic are connected with the use of oxides, Fe- or MnO_2-coated sands, minerrals containing Fe, Mn and Al and clay minerals. Lièvremont and co-workers (2003) proposed the use of kutnahorite (Mn, Ca-carbonate ($CaMn(CO_3)_2$)) and chabazite (microporous aluminosilicate zeolite ($CaAl_2Si_4O_{12}.6H_2O$)) as a support and at the same time as the sorption materials for As[V]. Wang and Reardon (2001) developed a reactor for dissolved arsenic decontamination of water. The system functions by equilibrating the targeted water with CO_2 and directing it via saturated flow through crushed siderite. The result was a siderite dissolution and an increase of dissolved Fe[II]. The second step of the system includes aeration in the input of the treated water, before its passage through limestone. The aeration in the second step of the system, is supposed to increase the oxidation of the Fe[II] into Fe[III] and to help to remove the CO_2 from the feed water, resulting in precipitates of Fe[III]-oxyhydroxides, which are an effective sorbents of AsO_4^{3-}.

In anoxic environments, mobility and availability of arsenic are largely controlled by sorption on sulfide minerals. Bostick and Fendorf, (2003) have investigated the reactions of As[III], since this is the dominant form under these conditions, and more precisely its sorption on troilite (FeS) and pyrite (FeS_2). They found that As[III] sorbed most strongly at pH (>5 to 6), and it was coordinated to both sulfur and iron, which is a characteristic of As coordination in arsenopyrite. In parallel the arsenic reduction was accompanied by oxidation of surface S and Fe[II]. Although the formation of arsenic sulfides, such as orpiment (As_2S_3), realgar (AsS), or arsenopyrite (FeAsS) is the way for explaining the arsenic uptake in anoxic environments, arsenic solubility seldom conforms to that of pure mineral sulfides (Sadiq, 1990; Sadiq, 1997). Therefore, the specific mechanism of As retention in anoxic systems remains to be elucidated, despite the relationship between the sulfide minerals and arsenic solubility.

1.5. Ion exchange

Ion exchange is the reversible interchange of ions between the solid and the liquid phase and there is no permanent change in the structure of the solid. All applications based on this method are developed for large-scale usage. Synthetic ion exchange resins are based on a cross-linked polymer matrix usually composed of polystyrene cross-linked with vinylbenzene. Charged functional groups are attached to the matrix through covalent bonding and fall into four groups: strongly acidic, weakly acidic, strongly basic and weakly basic. Various strong base anion exchange resins are available. They can effectively remove arsenic from water, producing effluents with less than 1µl/L. Arsenic, being uncharged, can not be removed from the water, unless an oxidation step to convert As[III] into As[V] is included in the process. The sulfate-selective resins, which are conventional, are suited also for arsenic removal. The nitrate-selective resins also remove arsenic, but arsenic breakthrough occurs earlier. Ion exchangers are typically down-flow, packed bed columns with ion resins beads pre-saturated with an exchangeable ion. The water is passed through the packed bed until the appearance of unwanted contaminant in the effluent. At this stage a reactivation of the ion exchange media with a regenerating solution is necessary and it should be rinsed with water in preparation for another treatment cycle. The redox potential and the pH are important factors with regard to arsenic removal by this technique.

1.6. Membrane / Reverse osmosis

Membrane separation is based on the use of semi-permeable membranes. Their permeability is selective for water and certain solutes to separate impurities from water. Membranes are able to remove dissolved solids, including arsenic. However they are usually expensive and therefore are typically considered in applications such as brackish water conversion and for removal of specific ions, such as arsenic, that are difficult to remove by other means. There are many different membrane alternatives including microfiltration, reverse osmosis, electrodialysis, ultrafiltration and nanofiltration. Membrane process treatment performance depends on the quality of the treated water and the desired quality of the product water. Generally the main problems are the scaling, fouling caused by particle matter and the biofouling of the membranes.

1.7. Sedimentation of arsenic containing particles

The sedimentation is the gravity separation of solid particles from liquid by settling. Generally it is used in conjunction with coagulation or precipitation.

2. Biological methods and technologies for remediation of arsenic-containing water and soils

The biological remediation of arsenic from water and soils has enormous advantages, most of them are still to be elucidated. The biological remediation techniques are based on the use of natural utilities such as the plants and bacteria. Moreover these techniques can prevent and preserve the treated environment from secondary pollution. The mechanisms of biological remediation can be classified by the type of organisms involved in the processes:

- plants
- microorganisms

Or by the type of the precise reaction of the organism:

- biological oxidation
- biological reduction
- biosorption
- bioprecipitation / biomineralization
- rhizofiltration
- phytoextraction

The main part of bioremediation methods is attributed to microorganisms, and especially prokaryotic microorganisms (Bacteria and Archea), due to the high resistance and tolerance of microorganisms toward the inorganic forms of the element. Lately the interests of plant application for remediation of arsenic increase. Several studies for the arsenic uptake from wild and some genetically modified plants have been done.

2.1. Properties and application of plants in arsenic remediation

Plants can be used in biosorption, bioaccumulation, rhizofiltration and phytoextraction. These techniques are named after the processes which represents the assimilation of an element from the environment and its retention and accumulation in the plant tissues.

The phytoremediation is a promising method, based on the use of green plants to assimilate or detoxify metals and organic chemicals. The term dates from 1991 and describes the accumulation of metals from the plants in soil and ground water (Liht *et al*., 1995; Visoottiviseth *et al*., 2002).

The plants, which accumulate high concentrations of metals are referred as ”hyperaccumulators”, a term first used by Brooks *et al*. (1977) to describe those plants that take up more than 1000 μg metal g^{-1} dry mass.

To date the main area of studies in the domain of phytoremediation are represented by the research of wild types hyperaccumulators (soil and water plants) and a few recent studies of genetically modified plants *Brassica napa* and *Arabidopsis thaliana*. The biochemistry of arsenic uptake and the subsequent transformations in the plants are yet to be elucidated.

2.1.1. Studies of plants for phytoremediation of arsenic contaminated water and wetlands

The research of water hyperaccumulators plant of arsenic, still do not encompass the wild type vegetation and all the studies are based predominantly on the investigation of the more potent arsenic accumulator between the plants in a given water ecosystem. Lee *et al*. (1991), have observed that aquatic plants accumulate arsenic and another metals to concentrations far excess of environmental levels. One of the hypothesis suggested that arsenic (arsenate) bioaccumulation and uptake are intimately associated with phosphate uptake, because of the strong similarity between these two elements.

Dushenko *et al*. (1995), have studied the bioaccumulation in aquatic macrophytes in regard to the relationship with environmental partitioning, specific metal uptake and nutrients. Rooted aquatic macrophytes play an important role in the cycling and toxicity of arsenic, because they are in the base of the aquatic food chain and are closely associated with sediments. The following emergent aquatic plant species were studied cattail (*Typha latifolia* L.), water horsetail (*Equisetum fluviatile* L.) and arrow-grass (*Triglochin palustre* L.) and as submergent – pondweed (*Potamogeton pectinatus* L.), water milfoil (*Myriophyllum exalbescens*) and bur-reed (*Sparganium* sp.). The authors found that macrophytes tended to bioconcentrate arsenic relative to sediment concentrations, up to a factor of ten, and that the submerged species contained much higher levels of arsenic than the emerged. Nevertheless, this study did not revealed the main reason for that phenomenon - the specific potential of plants or the

importance of environmental conditions and their influence on the plants development and arsenic uptake.

Del Rio *et al* (2002) studied 99 different wild plant species, belonging to 89 genera and 29 families from the Donana National Park in Spain in order to identify most suitable metal accumulator species for a phytoremediation. This is the largest wetland area in Europe, which was polluted with arsenic from spilling of pyrite sludge. Among the 99 different plant species studied, above all *Amaranthus blitoides* was highlited as the most promising to be used in the remediation of the affected area.

Robinson *et al*. (2002) studied the watercress (*Lepidum satvium*) an aquatic macrophyte and consumed as a vegetable in New Zealand. They found that this plant can uptake arsenic from substrates containing relatively low concentration of the element (reduction of arsenic concentration in solutions by 7.3% in average), which indicate that the plant may have potential for phytoremediation by extracting arsenic from contaminated soils and water. Thus, this plant should not be used as a diary food, especially from arsenic containing environments.

2.1.2. Studies of plants suitable for phytoremediation of arsenic contaminated soils

The phytoremediation of soils contaminated with arsenic is focused on the research of wild type plants hyperaccumulators as well as on the use of genetically modified plants. Visoottiviseth *et al*. (2002) and Francesconi *et al*. (2001) nominated the fern species *Pityrogramma calomelanos* as the most proficient plant in arsenic accumulation from soil, attaining concentrations of 8350 $\mu g\ g^{-1}$ (dry mass) in the frond. The authors estimated the potential for phytoremediation of that plant at 2% of the soil arsenic load per year. Visoottiviseth *et al.* (2001) studied 36 plant species amongst which only four species seemed suitable for phytoremediation - two fern species *Pityrogramma calomelanos* and *Pteris vittata*, a herb *Mitosa pudica* and a shrub *Melastoma malabrathricum*.

Recently, three other ferns *Pteris cretica*, *Pteris longifolia* and *Pteris umbrosa* were also identified as arsenic hyperaccumulators. Zhang *et al.* (2004) investigated the total thiols and acid-soluble thiols in the Chinese brake fern *Pteris vittata*, when grown under arsenic exposure. This study objective were to elucidate whether thiols are involved in the arsenic detoxification in the plant and what is the possible relationship of arsenic and the

phytochelatin production, which represents a new research domain in the arsenic – plants interactions.

The hyperaccumulation of *Pteris* species seems to be constitutive in nature. Moreover, all of them have shown similar capacity to accumulate arsenic.

The interest of use of genetically modified plants in the arsenic bioremediation arose lately and it could be a good possibility to get inside in the structural, signal and overall regulation in arsenic uptake process in the plants. Nie *et al*. (2002) have shown the first clear evidence of arsenic phytoremediation using transgenic plants *Brassica napa*. These plants express the *Enterobacter cloacae* UW4 1-aminocyclopropane-1-carboxylate deaminase (ACC), responsible to lower stress levels induced by ethylene in the plant. As a consequence, these plants can tolerate several environmental stresses. The most advantageous characteristic, however, was their ability to uptake up to four times more arsenate compared with non-modified canola plants.

Assays with genetically modified *Arabidopsis thaliana*, transformed with bacterial genes which led to accumulation of arsenic in the plant leaves of quantities otherwise lethal, also were done (Chaffey, 2002).

2.2. Properties and application of microorganisms in arsenic remediation

Microorganisms have been found to have the capacity to concentrate or remediate the metals into forms that are further precipitated or volatilized from solution and hence less toxic or easily disposable. Thus, the microorganisms can only alter the speciation of the metal contaminants and convert them into less toxic form (Singh and Cameotra, 2004). The main two processes of metal form alterations, in which the microorganisms are involved, are the extracellular transformation and the metal uptake (Fig.10). The oxidation of arsenite had a special place in the extracellular transformation of this element.

The microbial influence on metal mobility can be applied for bioremediation purposes. The microorganisms can mobilize metals through autotrophic and heterotrophic leaching, chelation by microbial metabolites and siderophores, methylation (which can result in volatilization), (Gadd, 2004). For arsenic remediation, most important processes are the oxidation, biomethylation, biosorption, dissimilative arsenate-reduction or sulfate-reduction. Biosorption can be defined as a microbial uptake of organic and inorganic metal species, soluble and insoluble on the cell surface or intracellular.

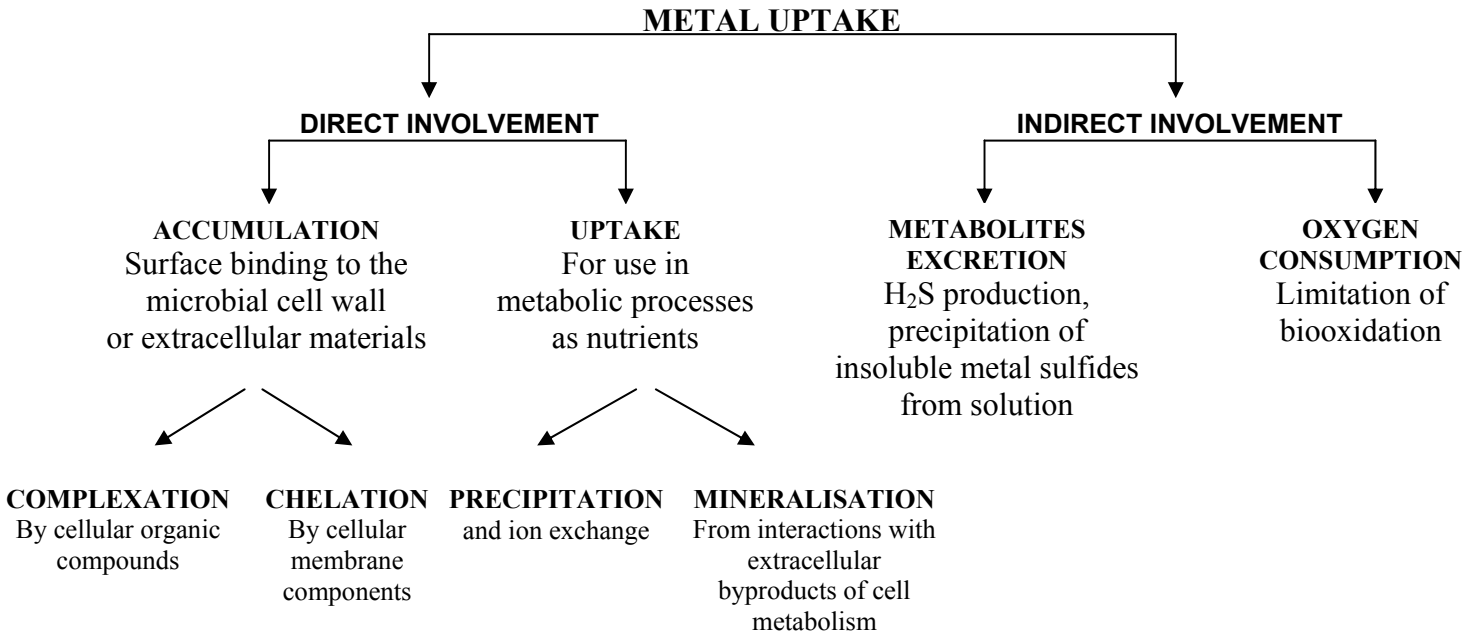

Fig. 10. Microbial processes for metal uptake and sequestration (Singh et Cameotra, 2004)

Biosorption has been demonstrated to be a useful alternative to conventional treatment systems for the removal of metals from the dilute aqueous solution. Nevertheless, there are a few authors who have worked on the application of fungi in the arsenic treatment technologies. Loukidou *et al.* (2003) proposed employment of fungal, non- living biomass of *P. chrysogenum* as As[V] biosorptive agent.

In the domain of bacteria some work being carried out and also a few patents for their possible application in arsenic remediation were published. Most of works for the biological removal of arsenic are based on the filtration of water trough sands or another support on which is possible to form a biofilm from arsenic oxidizing bacteria. Another type of treatment is based on the production of H_2S from sulfate-reducing bacteria, which consequently leads to the precipitation of arsenic under arsenic sulfides (Groudeva et al., 2001).

Katsoyanidis and Zouboulis (2004) proposed the use of biological iron oxidation for the removal of arsenic. The main incertitude in this processes comes from the usage of consortia of bacteria, which are often natural formed, but the precise function of different types of microorganisms are not investigated in depth.

Lièvremont *et al.* (2003) demonstrated a new insight to the arsenic removal process, based on the use of arsenic-oxidizing bacteria for the conversion of As[III] into As[V] followed of a chemical sorption of the arsenate in chabazite or kutnahorite.

3. Immobilization Techniques

The immobilization techniques are useful tool in biotechnology and pharmaceutical industries. They can be applied for whole cells immobilization as well as for the development of enzymatic biocatalysts.

The whole cell immobilization was defined as ”the physical confinement of localization of intact cells to a certain region of space with preservation of some desired activity” (Karel *et al*., 1985). The immobilization techniques can be divided into four major categories based on the physical mechanism employed (Fig.11), according to Pilkington and co-workers, (1998):

- attachment or adsorption on solid carrier surfaces;
- entrapment within a porous matrix;
- selfaggregation by flocculation (natural) or with cross-linking agents (artificially induced);
- cell containment behind barriers

3.1. Attachment or adsorption on solid carrier surfaces

The cell immobilization on a solid carrier is due to the physical adsorption from the electrostatic forces or by entrapment of bacteria in biofilm. The thickness of this film usually ranges from one layer of cells to 1mm or more. The strength with which the cells are bonded to the surface of the support and the depth of the biofilm often varies and is not readily determined.

The examples of solid carriers used in this type of immobilization are the:

- cellulose materials (DEAE- cellulose, wood sawdust, delignified sawdust);
- inorganic materials (polygorskite, montmorilonite, hydromica, porous porcelain, porous glass, etc.)

Solid materials like glass or cellulose can be treated with polycations, chitozan or other chemicals to enhance their adsorption ability (Norton and D'Amore, 1994; Navarro and

Durand, 1977). The examples for the cell immobilization on a solid carrier are shown in Fig.11a.

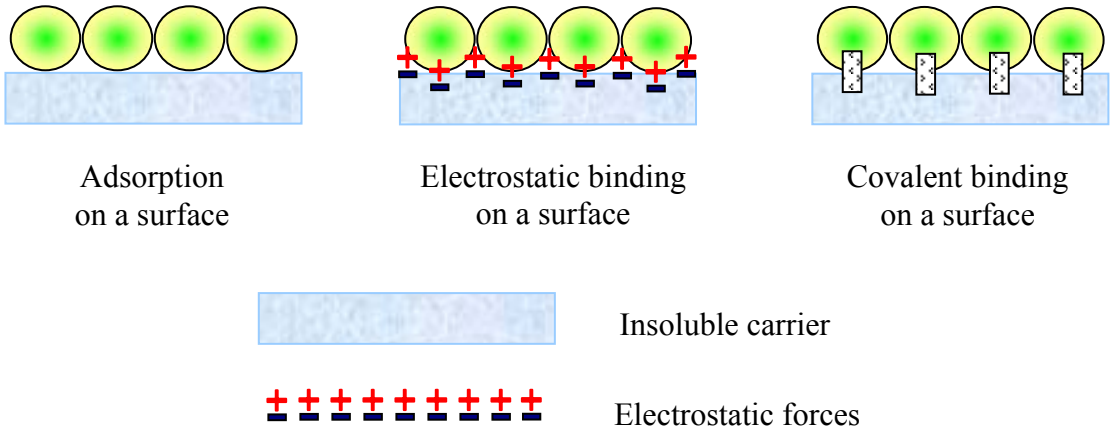

Fig. 11a. Basic methods of cell immobilization. Immobilization on the surface of a solid carrier (Kourkoutas et al., 2003).

3.2. Entrapment within a porous matrix

Here, the cells are either allowed to penetrate into the porous matrix until their mobility is obstructed by the presence of other cells, or the porous material is formed *in situ* into a culture of cells. Examples of this type of immobilization are the entrapment into:

- polysaccharide gels like alginates, *k*-carrageenan, agar, chitosan, polygalacturonic acid;
- other polymeric matrixes like gelatin, collagen, polyvinyl alcohol

The examples of entrapment are according to Norton and D'Amore (1994), and Park and Chang (2000), and the general way of realization is shown in Fig.11b.

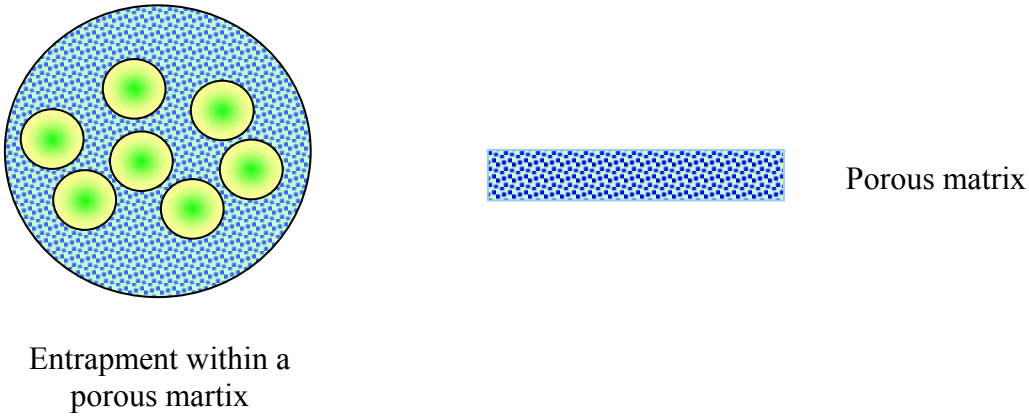

Fig. 11b. Basic methods of cell immobilization. Entrapment within a porous matrix (Kourkoutas et al., 2003).

3.3. Selfaggregation by flocculation (natural) or with cross-linking agents (artificially induced)

Cell flocculation has been defined as an aggregation of cells to form a larger unit. It is also the property of cells in suspension to adhere in clumps and sediment rapidly (Jin and Speers, 1998). Flocculation can be considered as an immobilization technique as the large size of the aggregates makes their use in reactors possible. The two types of cell flocculation, a natural one and an artificially induced one are presented in Fig.11c.

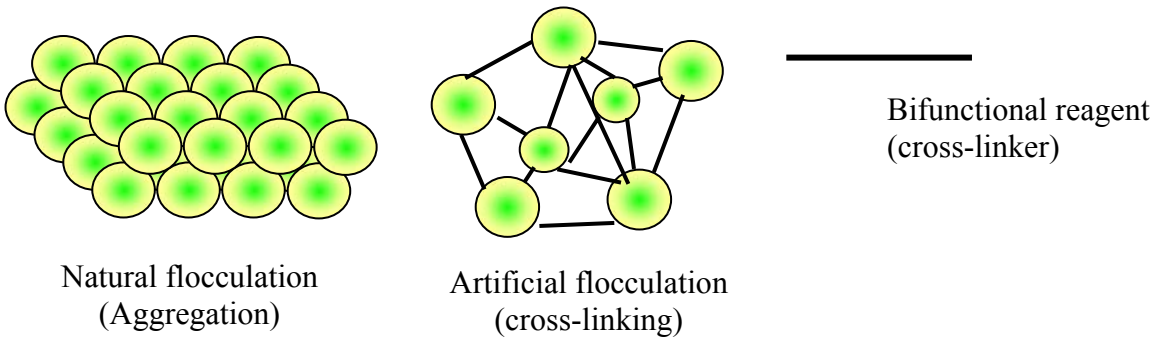

Fig. 11c. Basic methods for cell immobilization. Cell flocculation (Kourkoutas et al., 2003).

3.4. Cell containment behind barriers

The mechanical containment of cells behind a barrier shown in Fig.11d, can be achieved either by the use of microporous membrane filters or by entrapment of cells in a microcapsule. Another approach is the immobilization of the cells onto an interaction surface of two immiscible liquids. The membrane bioreactor is widely used in cell recycling and continuous process (Lebeau et al., 1998; Kargupta et al., 1998).

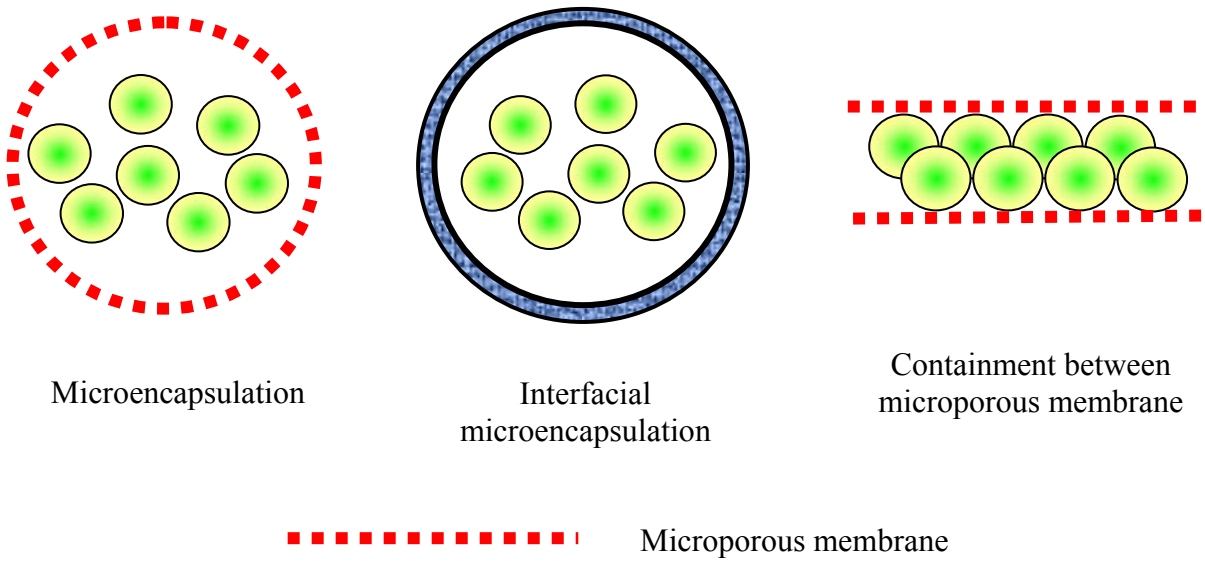

Fig. 11d. Basic method of cell immobilization. Mechanical containment behind a barrier (Kourkoutas *et al*., 2003).

For the purposes of biotechnology and pharmaceutical industries, many different supports have been developed and tested. Such are the polymers as gelatin, collagen, polyvinyl alcohol (PVA), and polysaccharides as *k*-carrageenan, chitosan, agar, etc. One of the most well known and widely used matrix for immobilization of whole living cells amongst them are the alginates.

3.5. Alginates

3.5.1. Structural units and molecular structure

Alginates are produced from brown seaweeds *Phaeophyceae*, mainly *Laminaria* and are linear polymers containing β-(1→4)-linked D-mannuronic acid (M) and α-(1→4)-linked L-guluronic acid (G) residues. The D-mannuronic acid is $^{4}C_{1}$ with diequatorial links between them and the linked L-guluronic acid are $^{1}C_{4}$ with diaxial links between them, as shown in Fig.12.

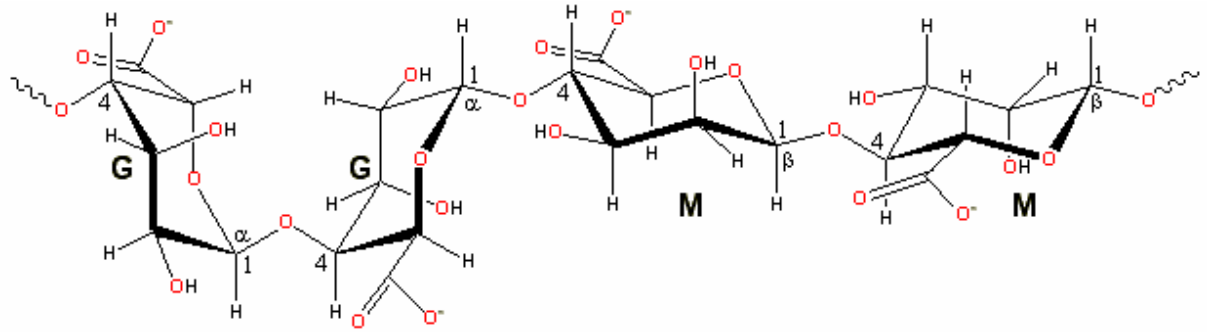

Fig.12. Chemical structures of alginate monomers.

Bacterial alginates are additionally O-acetylated on the second and/or 3 positions of the D-mannuronic acid residues (http://www.lsbu.ac.uk/water/hyalg.html). The bacterial O-acetylase may be used to O-acetylate the algal alginates, and to increase their water - binding capacity.

Alginates may be prepared with a wide range of average molecular weights (50-100 000 residues) to suit each particular application. The proportion as well as the distribution of the two monomers determines to a large extent the physiochemical properties of alginates. The importance of the quantity of each monomer and its spatial distribution in the polymer are illustrated in Fig.13 and Fig.14.

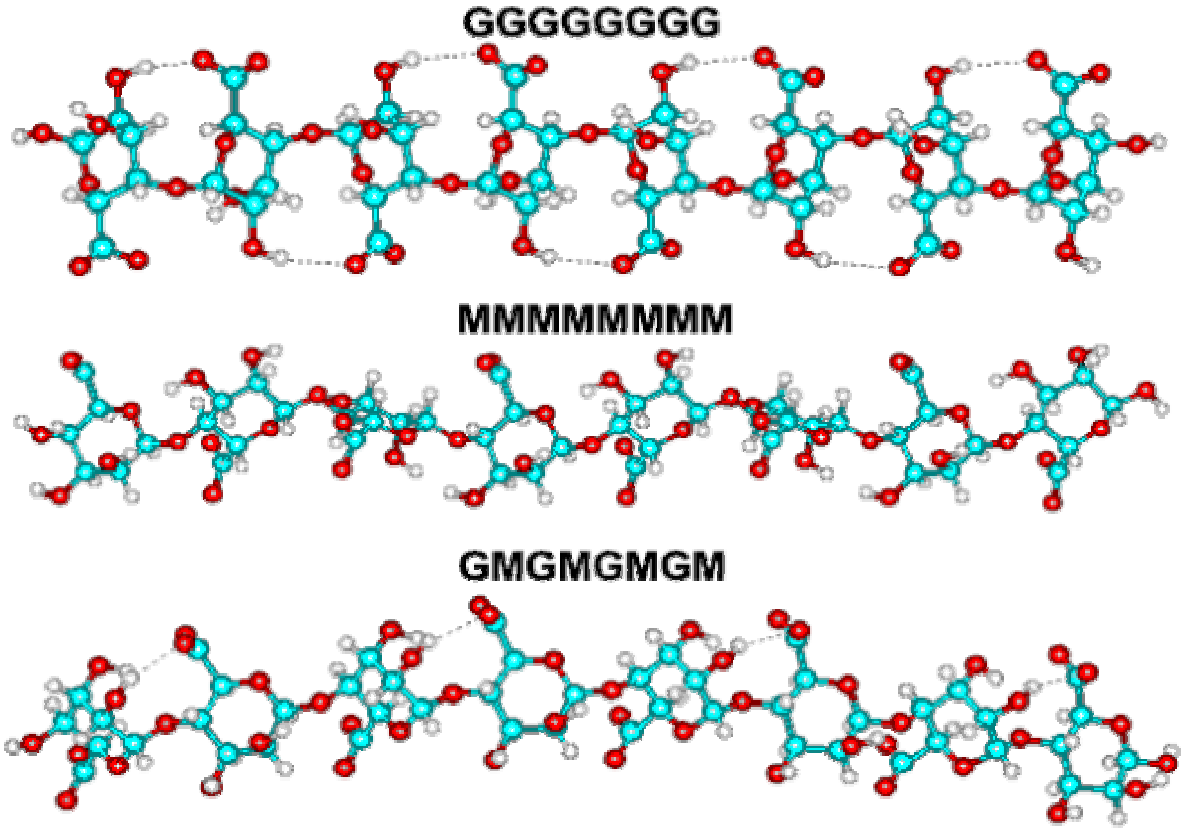

Fig.13. Spatial structures of alginates in function of the monomer units: (G) is a monomer of L-guluronic acid and (M) is a monomer of D-mannuronic acid.

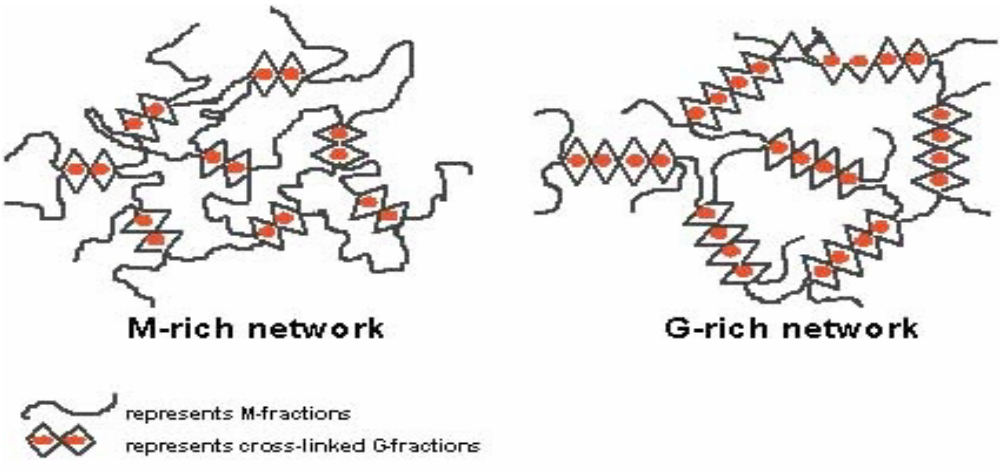

Fig.14. Simplified spatial structures of alginates in function of the percentage of each one monomer unit.

3.5.2. Mechanical properties of alginates

The alginate gel as an immobilization matrix is sensitive to:

- chelating compounds (phosphate, lactate and citrate)
- the presence of anti-gelling cations (Na^{+}, Mg^{2+}, etc.)

Therefore, for the preservation of the beads they should be kept in an calcium ions solution with Na^{+}:Ca^{2+} ratio from 25:1 to 3:1 or to replace the Ca^{2+} with other divalent cations with a higher affinity to alginate. The estimated correlation between mechanical gel strength and the affinity of divalent cations decreasing in the order:

$Pb^{2+} > Cu^{2+} = Ba^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+}$

However, the toxicity of these ions toward the living cells is the limiting factor for the use of most ions, and only Ba^{2+}, $Sr^{2+}$and Ca^{2+} are considered as nontoxic (http://www.genialab.de/inventory/alginate.htm) for bacteria, archaea and yeast.

3.5.3. Application of alginates

Entrapment within beads of Ca-alginate gel has become the most widely used technique for immobilization of living cells (algae, animal cells, bacteria, cyanobacteria, fungi, plant cells and protoplasts, and yeast). The applications of alginates, produced commercially have a wide range (Rehm and Valla, 1997):

- Textile printing;
- Paper and board treatments;
- Welding-rot production;
- Water treatment;
- Can sealing;
- Creaming of latex;
- Production of ceramics; food for human and pets;
- Pharmaceutical industry

In textile printing alginates are used for fixation, color yield and brightness, and even ensuring the printing. In paper treatment the application of alginates improve the surface uniformity. This is important prior to printing, to enable de deposition and adherence of dyes and inks (Sutherland and Elwood, 1979) In the welding-rot production, alginates are used as component of covering materials, and also as component improving bendability. The alginate application in water treatment technologies is connected mainly with increase of aggregate size in artificial flocculation.

Commercially produced alginate is used mainly in the food industry, for example: in ice-creams, cream and cake mixtures, in beer production (to enhancing the foam), fruit drinks (to assist the suspension of fruit pulp).

In pharmaceutical industry the alginates are used in dermatology, and wound healing, dental impression materials, treatment of oesophageal reflux, immunostimulants or in medical transplantation technologies (Sabra et al., 2001).

In conclusion:

Arsenic is a wide spread toxic element, which causes several problems. The main problem in regard to human health is the contamination of drinking water and agricultural soils.

The biological methods for arsenic remediation described here have several advantages, when compared to the chemical ones, mainly due to the lack of secondary pollution and the low impact on the environment.

However the biological methods for arsenic remediation are a relatively new area of study. Phytoremediation has been widely studied in arsenic remediation, but very little characterization of the plant species involved in this process has been carried out to date. In microbial remediation there is still only small amount of information known about the role of different microorganisms involved in the overall regulation of the transformation processes.
It is known already that the main processes of inorganic arsenic species transformation are the oxidation of As[III] or the reduction of As[V], but only few bacterial and yeast species are reported as arsenite-oxidizers or arsenic-reducers. There is also a lack of information concerning the microorganisms involved in the biomethylation of inorganic arsenic. Similarly, there is little information to date, regarding the use of yeast in arsenic bioremediation.

Therefore, the investigation of microorganisms (bacteria and yeast), possessing arsenic transformation abilities in regard with their further application in bioremediation processes is an open and wide area, which is worth of being explored.

CHAPTER II

AIMS OF THE PROJECT

The main aim of this project was to study the potential and suitability of an arsenite–oxidizing strain, recently named *Cenibacterium arsenoxidans*, in arsenic remediation of water in regard of further technology development. This study was done with the view of the replacement of the chemical oxidation in the treatment technologies of arsenic polluted water.

The strain, *Cenibacterium arsenoxidans* was previously isolated (Weeger *et al.*, 1999) and was chosen because of its high oxidation capacity (a maximum of As[+III] – 0.4 $mM.h^{-1}$ from free cells, with concentration of $5x10^8$ CFU/ml). This organism oxidizes the inorganic arsenic using the enzyme arsenite – oxidase. The gene structure and operon arrangement were recently identified and described (Muller *et al*., 2003).

Preliminary laboratory-scale studies with *Cenibacterium arsenoxidans* in a batch reactor system using Na-lactate as a sole carbon-source showed complete oxidation of As[III] to As[V] from the strain (Weeger *et al.*, 1999). However, the estimated cost of chemically defined medium is relatively high.

Therefore, two new nutrient media, based on the use of alternative, inexpensive industrial waste products as a carbon source, were developed and tested. In this way it is possible to join the arsenic remediation with the treatment of effluents with high organic charges, which usually causes problems when treated in industrial water purification plants. From another point of view, such media could be used only as a cheap and efficient alternative for biomass production of *C. arsenoxidans*.

The development of the strain was also studied in sterile and non-sterile conditions as a simulation of a real time process. It was achieved through a gfp-tagged clone of *C. arsenoxidans*.

As an alternative to the free cells culture application in the cleanup process, the remediation potential of entrapped *C. arsenoxidans* cells in Ca^{2+} alginate beads was studied.

Part of the research work was devoted to the development of a new screening method for arsenic-transforming bacteria. This assay was developed in order to enhance the rapid screening of bacterial strains possessing arsenite-oxidizing ability, which were of interest in this work as well as for future work.

Thus, this research work focused on the development of a microbiological method for remediation of arsenic in which the chemical oxidation is replaced by a bacterial one.

CHAPTER III

RESULTS AND DISCUSSION

In this Chapter the results obtained throughout the different stages of the study are presented and discussions included. They are arranged in four parts, according to the main aim of each set of experiments performed. The overall presentation of results in each part follows the pattern:

1. Introduction, consisting of short description of aims of the part and the experiments done.
2. Presentation of a manuscript or a paper, concerning the results.
3. Presentation of additional results, obtained during the assays, which were not included into the manuscript or the paper.
4. Conclusion and Perspectives, based on the obtained results in each set of experiments.

Part III does not follow the above pattern. In this part the results are introduced, and presented, but there is not a manuscript or a paper added.

Part I: Development of nutrient media

1. Introduction

One of the research objectives of my work was the development of a nutrient medium to increase the biomass production of arsenite-oxidizing strain. Several carbon sources were tested in order to define the growth characteristics of the strain *C. arsenoxidans*. We focused on the tests of some additional carbon sources, which were not previously tested. The growth of the strain was firstly tested on carbon sources, such as ethanol, methanol and sucrose. In parallel three different liquid organic wastes from food industry were also tested as possible organic sources for the growth of *C. arsenoxidans*. The tested by-products were molasses, sauerkraut brine and whey. An additional objective was the achievement of an optimal ratio of cost of the medium/quantity of biomass produced. Thus the importance of each component from the chemically defined medium (CDM) was assayed for its importance in the overall growth of the strain.

In this part the results obtained from the three chemically pure carbon-sources assayed and the three by-products tested as a carbon-source are presented.

- The results for the sauerkraut based media and whey media are presented in the manuscript of the paper, named: Sauerkraut brine and whey-inexpensive by-products for biomass production of an arsenite-oxidizing bacterium *C. arsenoxidans*.
- Additional results for the development of the strain in the sauerkraut broth media and whey based media are presented after the paper as additional results.
- The results obtained for the growth of the strain on molasses medium and its modifications as a carbon-source are presented as additional results after the manuscript of the paper.
- The results of the growth of the strain on ethanol, methanol and sucrose are presented as additional results.

The conclusions and perspectives concerning this part of results are presented at the end of the part.

2. Manuscript of Article I

Simeonova, D. D., Lièvremont, D., Groudeva, V. I. and Lett M-C.

Sauerkraut brine and whey – inexpensive by-products for biomass production of an arsenite-oxidizing bacterium *Cenibacterium arsenoxidans*

Manuscript submitted to Applied Microbiology and Biotechnology

SAUERKRAUT BRINE AND WHEY – INEXPENSIVE BY-PRODUCTS FOR BIOMASS PRODUCTION OF AN ARSENITE-OXIDIZING BACTERIUM *CENIBACTERIUM ARSENOXIDANS*

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Keywords: arsenic bioremediation, sauerkraut brine, whey

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Abstract

An inexpensive nutrient medim was required for the optimization of the biomass production of *Cenibacterium arsenoxidans*, an arsenite-oxidizing bacterium which can be used in arsenic remediation process. Since this strain was initially isolated on a medium containing sodium lactate, different liquid organic waste containing lactate, such as whey and sauerkraut brine, were tested. The growth of the strain, expressed by the optical density of the media containing 20% (vol./vol.) water solutions of whey or sauerkraut brine was much higher, when compared to the growth in a medium with Na-lactate (OD 0.4-0.5 ± 0.01-0.03 at 600 nm) as a unique carbon-source, which corresponded to 20 x 10^8 CFU/ml. In whey medium the OD was in the range of 0.99 ± 0.07 at 600 nm, which corresponded to 100 x 10^8 CFU/ml and in the sauerkraut brine medium the OD was in the range of 0.95 ± 0.03, which corresponded to 140 x 10^8 CFU/ml.

The determination of inorganic arsenic species and their quantification (As[III] and As[V]) were obtained by high-performance liquid chromatography coupled with inductively coupled plasma atomic emission spectrometry (HPLC-ICP-AES). Both new nutrient media had no influence on the oxidation of arsenite (As[III]) by the strain.

Introduction

Arsenic is a ubiquitous element in the earth's crust and naturally occurs in many inorganic and organic compounds in different oxidation states as a result of natural processes (weathering reactions, biological activity, volcanic emissions, etc.) or anthropogenic activities (mining, smelting, glass manufacturing, pharmaceutical industry, electronics, pesticides, chemical weapons). The inorganic arsenite (As [III]) and arsenate (As [V]) are the most stable forms of the element.

To date, there are different technologies for arsenic decontamination of waters, which are predominantly based on chemical treatments. In these technologies the first step is the oxidation of arsenite As[III] into arsenate As[V], the second step in these is then the precipitation of As[V]. The As[V] is favored for precipitation due to its high reaction activity, low mobility and less toxicity (Arai et al., 2001; Meng et al., 2001). As a consequence of the powerful oxidants (H_2O_2, $KMnO_4$, chlorine, hypochlorite, Fe^{3+}) used in the oxidation step, the chemical oxidation of arsenic causes additional destruction and pollution of the environment. Other methods used are the ion exchange or reverse osmosis remediation techniques, although these have high removal efficiency, they require high investments, running and maintenance costs.

It is well known that microbial activity strongly influences arsenic speciation and its transport in the environment. Bacterial transformation systems of both As[III] and As[V] forms are likely to be ancient (Gihring et al., 2003; Lebrun et al., 2003) and are found in numerous bacterial species encoded on chromosomes or mobile plasmids (Silver, 1996). Several bacteria are known to transform inorganic arsenic through oxidation (Coram and Rawlings, 2002; Duquesne et al., 2003; Ehrlich, 2001; Macy et al., 2000; Weeger et al.,

1999). Such biological processes can be applied to bioremediation since they generate less toxic or less mobile forms of arsenic as well as being environmental friendly.

A bacterial strain, recently named *Cenibacterium arsenoxidans* (Weeger et al., 1999) was chosen because of its high oxidation capacity (a maximum of 0.4 mM (As[III])/h from free cells, with concentration of 5 x 10^8 CFU/ml). This organism oxidizes the inorganic arsenic using the enzyme arsenite – oxidase. The gene structure and operon arrangement were recently identified and described (Muller et al., 2003).

Preliminary laboratory-scale studies with *C. arsenoxidans* in a batch reactor system using Na-lactate as a sole carbon-source showed complete oxidation of 100 mg of As[III] to As[V] from the strain. However, the cost of chemically defined medium is high. For those purpose alternatives nutrient media, based on the use of inexpensive industrial waste product as a carbon source, such as sauerkraut brine or whey, were developed. These waste products are abundantly available since sauerkraut brine is the by-product from sauerkraut production and whey is the major by-product of cheese manufacturing and casein production. Both have a high organic charge and acidic pH and usually causes problems when treated in industrial water purification plants.

Thus, this research work focused on the development of a microbiological method for remediation of arsenic in which: 1) the chemical oxidation is replaced by a bacterial one and 2) the oxidation of arsenite can be directly connected to the conversion of liquid organic waste.

Materials and Methods

Bacterial strain

A heterotrophic arsenite-oxidizing bacterial strain ULPAs1, recently renamed *Cenibacterium arsenoxidans*, was previously isolated from an aquatic environment, contaminated with arsenic (Weeger et al., 1999). The strain had a doubling time of 1.5 h and showed a minimum inhibitory concentration (MIC) of 6.65 mM (498 mg/l) for As[III].

Inoculation and growth conditions

A preculture from a single colony of the strain, was prepared in 20 ml of liquid chemically defined medium (CDM) and incubated for 72 hours at 220 rpm (Certomat, U) at 25°C in the dark. The inoculum was not induced with arsenite. The preculture was centrifuged for 15min at 5000 rpm (Jouan, GR 412), washed twice with double deionised, sterile water (Milli-Q system, Millipore) and finally resuspended in 10 fold smaller volume of sterile ddH_2O. The oxidation of As[III] in the tested media was carried out in sterile tubes, with final volume of 20 ml. The tubes were inoculated with 20 µl from the preculture of *C. arsenoxidans*, (initial concentration of 1×10^5 CFU/ml) and were incubated at 25°C, on a rotary shaker (220 rpm) in the dark. Samples for viability counts and optical density at 600 nm were taken three times a day every 4 hours during the experiments. The dilutions of the samples for CFU counting, were made in sterile ddH_2O and were plated on CDM agar in duplicate. All experiments were performed at these standard conditions and each set of experiments was carried out three separate times with samples prepared in triplicate (n=9).

109 Nutrition media

110

111 Chemically defined medium (CDM). The compounds of the CDM and its preparation, were
112 previously described in Weeger et al. (1999). The CDM had a final pH of 7.2.
113 Sauerkraut brine medium. The sauerkraut brine was kindly supplied by the producer
114 Coopchou, (Vendenheim, France). The natural pH of the sauerkraut brine was 3.5 - 3.7. The
115 sauerkraut brine was pretreated by filtration through a paper filter, centrifugation at 6000 rpm
116 for 20 min, heating twice at 90°C in a water bath - for 15 min and allowed to settle for 24 h at
117 4°C. A second filtration through a paper filter, dilution 1:5 (vol./vol.) with sterile ddH_2O and
118 final filtration through 0.20 μm pore-size membrane (Nalgene, France) were then carried out.
119 The sauerkraut brine medium was prepared in two modifications- sauerkraut brine medium
120 without salt solutions from CDM, named SBM and with addition of salts solutions named
121 SBM-A*BC. The SBM-A*BC consisted of salts solutions A*, B and C in the same quantity
122 as in the CDM, except that solution A* did not contain Na - lactate. The final pH of SBM and
123 SBM-A*BC were adjusted to 7.2, with 1 M NaOH.

124 Whey medium. For the whey based medium commercially available products from the french
125 cheese industry (fromage blanc, Auchan and Rians) were used. The initial whey pH was 4.0–
126 4.3 and was pretreated for the preparation of nutrition medium by filtration through paper
127 filter, dilution with double deionised water 1:1.5 (vol./vol.), neutralization with 1 M NaOH up
128 to pH 7.2, a second filtration through paper filter, followed by sterile filtration through
129 0.20μm pore-size membrane.
130 The whey medium was also prepared and tested in two modifications- without salt solutions
131 from CDM, named WM and with addition of salt solutions WM-A*BC. The WM-A*BC
132 consisted of solutions A*, B, and C from the CDM in the same quantities, except that solution
133 A* again did not contain Na -lactate.

D-, L- lactate assay. The measurements of initial and final quantity of D- and L-lactate as a unique carbon source in the all tested media were done as described by Gutmann and Wahlefeld (1974).

Physico-chemical parameters of the tested media

The analysis of the basic physico-chemical parameters of SBM and WM were made in the Centre d'Analyse et de Recherche (CAR, Strasbourg, France). The methods used were carried out as the Norme Française Technique (NFT). The parameters tested were: chemical oxygen demand (COD) (NFT 90 - 101), biological oxygen demand in 5 days (BOD_5) (NFT 90 - 103), ammonium concentration (NFT 90 - 015), Kjeldahl NTK (NFT 90 - 110), Nitrates (NFT 90 - 012) and Nitrites (NFT 90 - 013) concentrations.

Determination of arsenic transformations

The arsenite-oxidation from *C. arsenoxidans* during growth was investigated by taking samples of the cultures supernatants at 24 h. In parallel, as a control, uninoculated SBM and WM were assayed for arsenate-oxidation capacity. In tested media 10 mg/l of As[III] was added. The supernatants samples from the different media were filtrated through a 0.22 μm pore - size membrane (Durapore, Millipore-GV), and stored at 4°C, in the dark for no more than 1 week before measurement.

For the arsenic speciation and quantification a technique based on the coupling of HPLC and ICP-AES (Amran et al., 1997; Moll et al., 1996) was used. Arsenic was eluted with a phosphate buffer, degassed and filtered through a cellulose acetate membrane (0.65 μm pore - size, Millipore). The detection of arsenic compounds was carried out at a fixed wavelength as

159 described in Weeger et al. (1999). Detection limits were 0.12 mg/l for As[III] and 0.14 mg/l
160 for As[V], and quantification limits were 0.43 mg/l and 0.36 mg/l for As[III] and As[V],
161 respectively. All data obtained in this study were from at least two independent experiments,
162 on duplicate samples (n=4).
163

Results

Sauerkraut Brine Medium

Preliminary studies for arsenate reduction, arsenite oxidation or uptake of inorganic arsenic by the sauerkraut brine were done using the HPLC-ICP-AES method. The results showed 100% recovery of As[III] from an initial As[III] concentration of 10 mg/l. Thus, the sauerkraut brine did not oxidize As[III] or reduce As[V].

Growth experiments were performed in two concentrations of sauerkraut brine (10 and 20 % (vol./vol.)) as a sole carbon- source. The growth and the arsenate-oxidation ability of the strain were studied in SBM with its natural pH 3.6 - 4.0 and in a neutral pH 7.2 in the presence and absence of 10 mg/l of As[III]. The influence on the growth of the strain of salt solutions A*, B and C supplied to the SBM-A*BC were also tested. The control used was CDM, supplemented with 10 mg/l of As[III]. All experiments were carried out at 25°C. The growth of the strain in SBM-A*BC and SBM are presented in Fig. 1a and 1b, respectively. No growth of the strain was observed in the medium with a natural pH for the sauerkraut brine (data not shown). When sauerkraut brine was neutralized, the strain grew equally well in presence and in absence of salts solutions and in the presence and in absence of As[III].

The concentrations of lactate in 20% (vol./vol.) solution of sterile sauerkraut brine, without salts solutions, at the natural pH 3.6 - 3.9, was 1.39×10^{-2} M in the D-form and 1.11×10^{-2} M in the L-form. After neutralization of SBM up to pH 7.2 - 7.5, the concentrations of D-lactate was 1.53×10^{-2} M and no change in the L-lactate concentration, was observed.

In 20% solution of sauerkraut brine, without salts solutions (SBM) at neutral pH after 96 h of growth of *C. arsenoxidans*, the quantity of both forms of lactate decreased significantly to 3.8×10^{-5} M of each. When salts solutions A*, B and C were added, into the

189 sauerkraut brine (SBM-A*BC), the level of lactate decreased to 3.1 x 10^{-5} M (D-form) and
190 2.8 x 10^{-5} M (L-form). Therefore the strain grows better in SBM supplemented with salts
191 solutions.

192 The common physico-chemical parameters TOC, COD, BOD and N were determined
193 for SBM-A*BC. These analyses were assayed in triplicate. The results obtained for all of the
194 parameters in sterile SBM-A*BC (control) and after the growth of strain for five days
195 (sample) are presented in Fig. 2. The decrease in COD, BOD and TOC are very significant
196 after 5 days of incubation of the strain. The amount of the total carbon in SBM decreased
197 from 5415 mg/l to 2912 mg/l, which represent an assimilation of 2503 mg/l from the strain in
198 5 days. The COD decrease with 5480mg/l and the BOD with 3975mg/l, for the same time
199 period, which represent about 40% of organic and carbon discharge of the sauerkraut brine.
200 The initial concentration of nitrate was less than 1.2 mg/l and no changes in its concentration
201 were observed after 5 days in presence of the strain. The initial nitrite concentration was
202 1.3 mg/l and at the end of assay was below 1.2 mg/l.
203

204 Whey medium
205

206 The preliminary tests for oxidation, reduction and uptake of arsenic ions from the whey were
207 also performed using the HPLC - ICP - AES method. Results showed no transformation or
208 uptake of arsenic.

209 The growth of *C. arsenoxidans* in this medium was also assayed with or without
210 supplementation of salts solution A*, B and C. The growth at two levels of pH 4.0 - 4.3 and
211 7.2, and the oxidation of As[III] to As[V], were tested. All tests were assayed at two
212 concentrations (10 and 20% (vol./vol.)) of whey as a sole carbon source in the medium.
213 Results obtained are shown in Fig. 3a and 3b. In WM and WM-A*BC at the natural pH of the

214 whey, the strain did not grow (data not shown). At neutral pH of the WM, the presence of
215 salts solutions A*, B, and C improved the growth of the strain WM-A*BC. The presence or
216 the absence of arsenic in the medium had no influence on the strain growth in this medium.
217 The initial concentrations of D- and L-lactate in a 20% (vol./vol.) WM at natural
218 pH 4.0 - 4.3 were 0.075 x 10^{-2} M and 3.05 x 10^{-2} M, respectively. After neutralization of the
219 whey up to pH 7.2 - 7.5, they were 0.06 x 10^{-2} M (D-lactate) and 2.96 x 10^{-2} M (L-lactate).
220 The decrease in the concentrations of both forms of lactate after 96 h of incubation of the
221 strain, without salts and arsenic supplementation, and at neutral pH were in the range of
222 0.6 x 10^{-5} M for D-lactate and 1.7 x 10^{-5} M for L-lactate, showing a significant decrease.
223 When salts solutions were introduced into the whey, in contrast to the SBM, the use of lactate
224 was slightly lower and the levels of both forms slightly increased to 0.9 x 10^{-5} M (D-form)
225 and 2.9 x 10^{-5} M (L-form).
226 The main physico-chemical parameters of WM-A*BC (COD, BOD, TOC) were
227 determined in triplicate. The final concentration of whey in the medium used for these
228 experiments was 20%. The results obtained showed a significant level of degradation of
229 organic mater in presence of the strain after incubation for 5 days period when compared to
230 the control (the same medium, uninoculated with the strain). The results are presented in
231 Fig. 2. The decrease of total carbon was of 1205 mg/l. The necessity of oxygen for the
232 chemical oxidation decreased with 3270 mg/l and for the biological oxidation with 2975 mg/l,
233 after the 5 days period of incubation with the strain. This represented decrease of above 35%,
234 when compared to the control. The initial concentrations of NO_2^- and NO_3^- were below
235 1.2 mg/l and no changes were observed in their concentrations after 5 days growth of the
236 strain.

Discussion

The development and the application of a microbiological method for arsenic remediation is necessary to prevent secondary pollution of the environment due to the use of chemical products in the oxidation step of As[III] to As[V]. In previous studies it was shown that the strain *C. arsenoxidans* possesses a high capacity for oxidation of As[III] (Weeger et al., 1999). The high cost of the use of Na-lactate as a sole carbon-source for this strain led to the search for an alternative, cost effective medium, which would easily replace CDM in a large – scale system.

Whey and sauerkraut brine are waste products and their disposal is problematic, because of their high organic charges and acidic pH. These waste products contain a high quantity of lactate, which supports growth of the strain. Both by-products provided better nutrition for the strain when compared to the medium of reference CDM. The complete media based on the whey proved better for obtaining biomass than SBM (Fig. 1a and Fig. 3a). Nevertheless, in absence of supplemented salt solutions (Fig. 1b and Fig. 3b) the strain showed better growth in SBM (Fig. 1b). Organic charge was higher for SBM-A*BC, with COD changes decreasing in the presence of the strain from 14160 mg/l to 8680 mg/l. In contrast the organic charge for WM-A*BC showed a smaller decrease from 9750 mg/l to 6480 mg/l. Therefore biodegradation was higher in the SBM-A*BC medium as shown in Fig.2. Based on these results, the elaborated medium on the use of sauerkraut brine is more efficient when compared to the WM-A*BC and WM, although both media showed better growth of the strain then CDM, which is presently used for biomass production of the strain. Thus, successful optimization of biomass production for the arsenic-oxidizing bacterium *C.arsenoxidans*, coupled with a low-cost and highly effective media was achieved.

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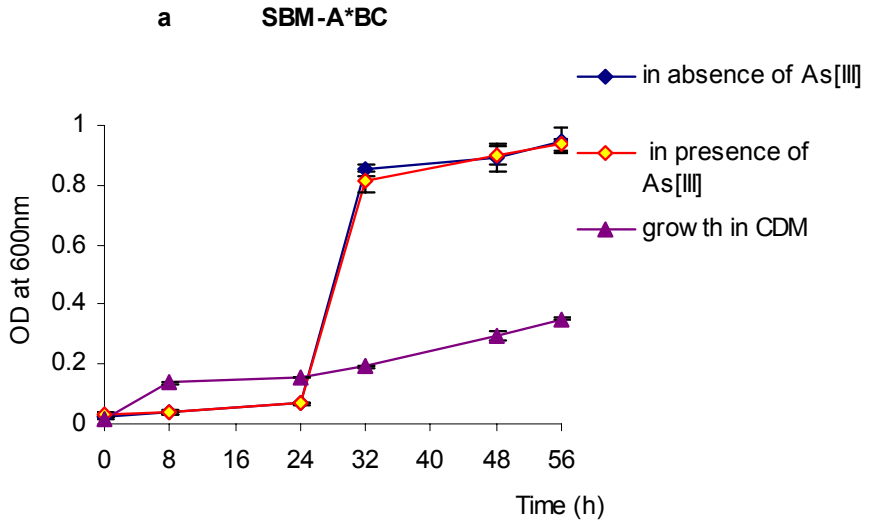
FIGURES AND LEGENDS



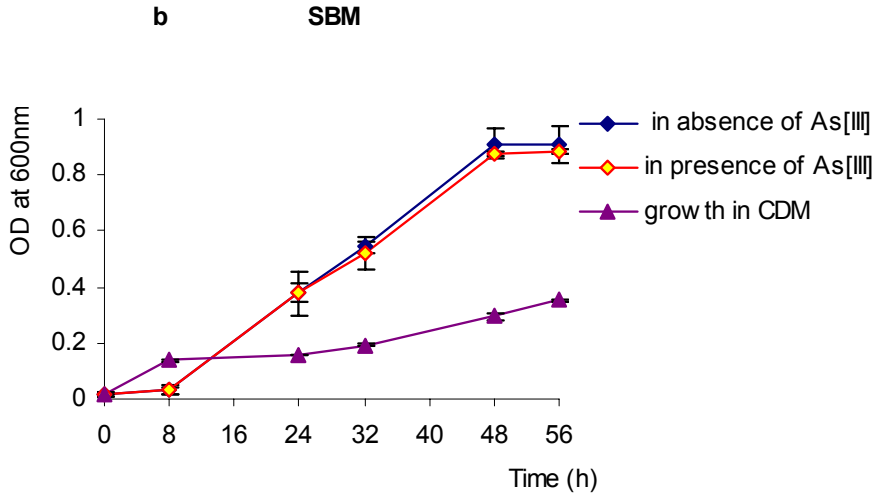
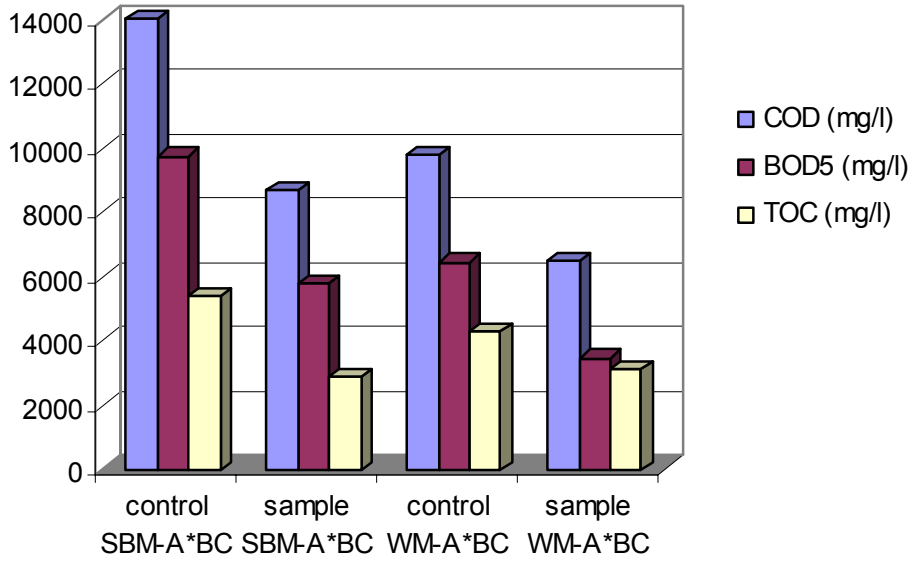


Fig. 1. Growth of *C. arsenoxidans* in 20% (vol./vol.) sauerkraut brine medium, at neutral pH 7.2. a) SBM -A*BC b) SBM.



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320 Fig. 2. Changes in main physico-chemical parameters of SBM-A*BC and WM-A*BC media
321 due to biodegradation of organic matter from *C. arsenoxidans* after 5 days of incubation.
322 Control: SBM-A*BC or WM-A*BC uninoculated with *C. arsenoxidans*; Sample: SBM-
323 A*BC or WM-A*BC inoculated with *C. arsenoxidans*. The concentration of sauerkraut brine
324 and whey - 20% (vol./vol.).

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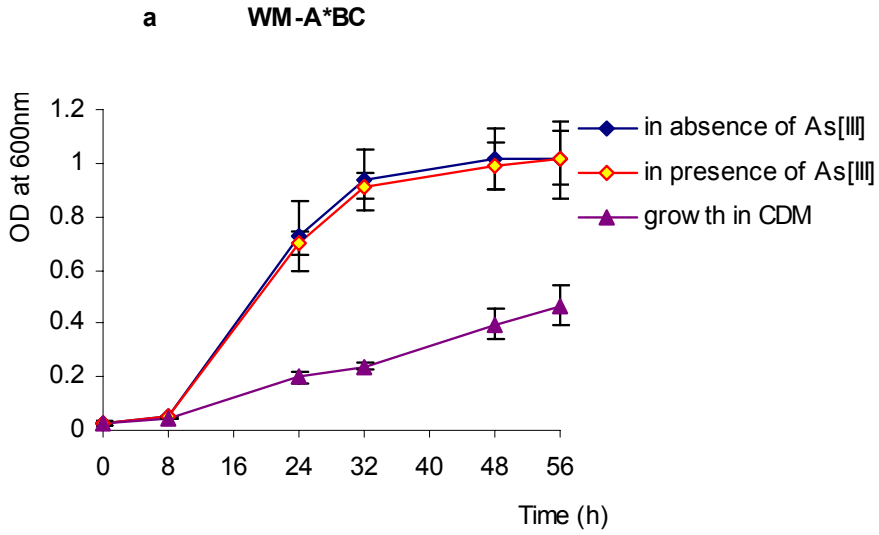

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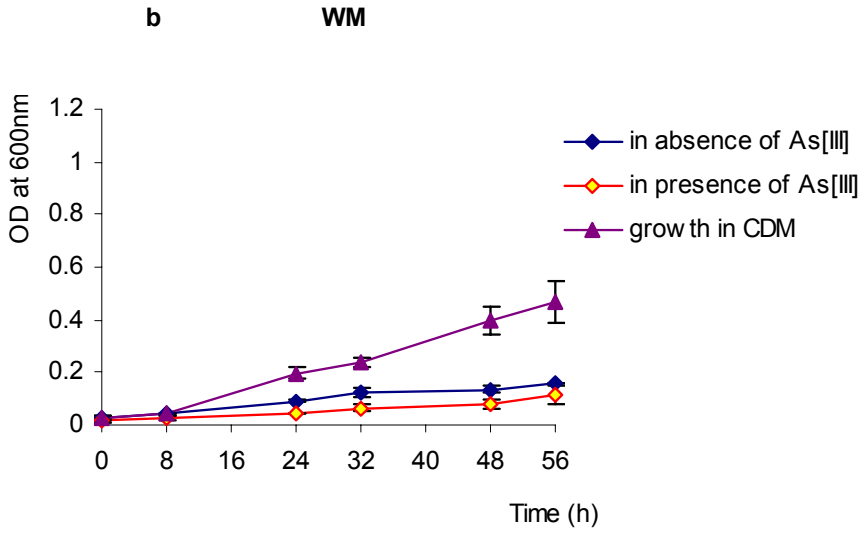

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339 Fig. 3. Growth of *C. arsenoxidans* in 20% (vol./vol.) whey medium, at neutral pH 7.2. a)

340 WM-A*BC and b) WM.

3. Additional results

3.1. Chemically defined carbon sources

The chemically grade pure and defined carbon sources ethanol, methanol and sucrose were assayed in three different concentrations as follows: 10 mM, 50 mM and 100 mM at two experiments in triplicate. The results obtained from the growth assays using ethanol, methanol and sucrose showed that these carbon-sources did not support the growth of the strain.

3.2. Liquid organic by-products

The by-product carbon-sources were assayed after the development of preliminary treatment protocols for each by-product used. The protocols concerning the sauerkraut brine based medium and the whey based medium were described in the paper. The protocol for the preliminary treatment of molasses based medium is described below.

3.2.1. Sauerkraut brine medium and Whey medium

The preliminary studies of sauerkraut brine as a carbon-source for the growth of strain *C. arsenoxidans* were assayed at two concentrations. The concentrations assayed were 10 and 20 %. The influence of the three salt solutions from the CDM on the growth of the strain were also tested in the presence as well as in absence of As[III] with concentration of 10 mg/l. The cell cultures were also plated on the agar plates of the tested medium in order to establish the correlation between the optical density of the culture and the CFU/ml. Thus the maximal specific growth of the strain (μ_{max}) and the doubling time (d_t) were determined, according to the following equations:

Determination of the specific growth (μ) of the strain, according to the growth media:

$$\mu = \frac{\ln(Nt_2) - \ln(Nt_1)}{t_2 - t_1}, \text{ where}$$

Nt_2- is the number of cell colonies from the exponential phase of growth counted at time t_2

Nt_1- is the number of cell colonies from the exponential phase of growth counted at time t1

Or

$$\mu = \frac{\ln 2}{t_d}, \text{ where}$$

d_t – is the doubling time or the time for 1 cell generation.

Therefore, the calculation of doubling time of the strain (t_d), for each media used, was done according to the equation:

$$d_t = \frac{\ln 2}{\mu}, \text{ where:}$$

$$d_t = \frac{\ln 2}{\frac{\ln Nt_2 - \ln Nt_1}{t_2 - t_1}}, \text{ therefore}$$

$$d_t = \frac{\ln 2.(t_2 - t_1)}{\ln Nt_2 - \ln Nt_1}$$

The characteristics for the growth of the strain in the different media assayed are shown in Table 1.

Table 1

Type of media	Specific growth (μ_{max})	Doubling time (d_t)
SBM-A*BC not supplemented with As[III]	$0.58.h^{-1}$	1h 11'
SBM-A*BC supplemented with As[III]	$0.55.h^{-1}$	1h 16'
SBM not supplemented with As[III]	$0.12.h^{-1}$	5h 53'
SBM supplemented with As[III]	$0.11.h^{-1}$	6h 20'
WM-A*BC not supplemented with As[III]	$0.25.h^{-1}$	2h 47'
WM-A*BC supplemented with As[III]	$0.24.h^{-1}$	2h 56'
WM not supplemented with As[III]	$0.02.h^{-1}$	37h 04'
WM supplemented with As[III]	$0.01.h^{-1}$	58h 03'
CDM in absence of arsenic	$0.27.h^{-1}$	2h 53'

The specific growth limits of *C. arsenoxidans* in CDM were obtained from 18 separate experiments at least in triplicate. These are the estimated growth parameters of the strain at 25° C on a rotary shaker, in the dark. From the table 1, it can be concluded that the best medium for the growth of the strain was the SMB-A*BC supplemented or not with arsenic, although both type of organic liquid waste used, contained lactate in the same ratio. The concentration in the SBM was of 2.63×10^{-2} M and in the WM was of 3.06×10^{-2} M. The main disproportion between two types of by-products used was in the type of lactate. In the SBM the L-form of lactate was 1.11×10^{-2} M and in the WM was 2.96×10^{-2} M. The disproportion between the D-forms of lactate was even higher: in the SBM it was of 1.53×10^{-2} M and in the WM was of 0.06×10^{-2} M. Another result obtained was that the optical density was higher, when the strain grew in WM-A*BC than in the SBM-A*BC, which corresponded to a slightly superior number of cells obtained. Thus, the quantity of lactate in the WM did not suppress the growth of the strain. Nevertheless, the highest values for the specific growth (μ_{max}), doubling time, quantity of cells per ml and per gram of substrate was achieved in the SBM-A*BC medium.

3.2.2 Molasses based medium

The preliminary treatment protocol of molasses consisted of the preparation of stock solution with concentration of 20 g/l. This solution was sterilized through filtration and used in the assays. The adjustment of this medium to a final pH of 7.2 was done with 0.1 M NaOH which was made prior to each assay. The molasses was tested in four different final concentrations as follows: 2, 4, 8 and 10 g/l. The influence of the different salt solutions from the CDM, were also assayed in order to estimate the influence of each salt on the growth of the strain. The growth of *C. arsenoxidans* was tested in a molasses medium without arsenite (As[III]) and in presence of 10 mg/l of As[III]. The cell cultures in suspension were assayed for CFU/ml using a spectrophotometer at a wavelength of 600 nm and then were transferred into Petri dishes containing the same type of medium supplemented with agar, to establish the correlation between optical density and the number of viable cells per ml.

The molasses medium was tested with two modifications. The first type of molasses medium (MM) tested, consisted only of molasses, without addition of salt solutions A*BC from the CDM. The second type of medium (MM-A*BC), consisted of molasses, but supplemented with the salt solutions from the CDM in the same quantities as in the CDM itself. The results are represented in Fig. 1.

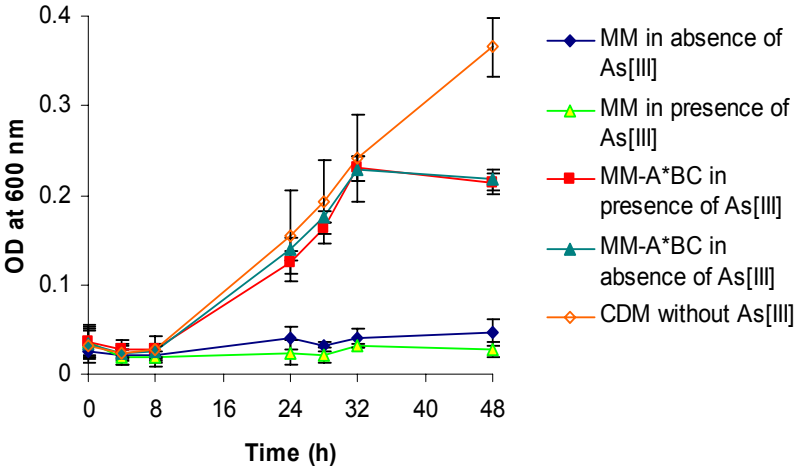

Fig. 1. Growth of the strain *C. arsenoxidans* in molasses based media in the presence and absence of As[III] with concentration of 10 mg/l and in CDM. The concentration of molasses in both types of media was 10 g/l.

The results showed that the strain did not grow in MM medium, without the salt solutions from the CDM. Another important point was that the presence of the As[III] did not influence the growth of the *C. arsenoxidans*, even in a less favorable medium such as the MM-A*BC, when compared to the SBM-A*BC or WM-A*BC or the CDM

The growth of the strain was tested in four different concentrations of molasses. The type of media was MM-A*BC and the assays were done in the absence of As[III]. The influence of the concentration of molasses on the growth of the strain is presented in the Fig. 2.

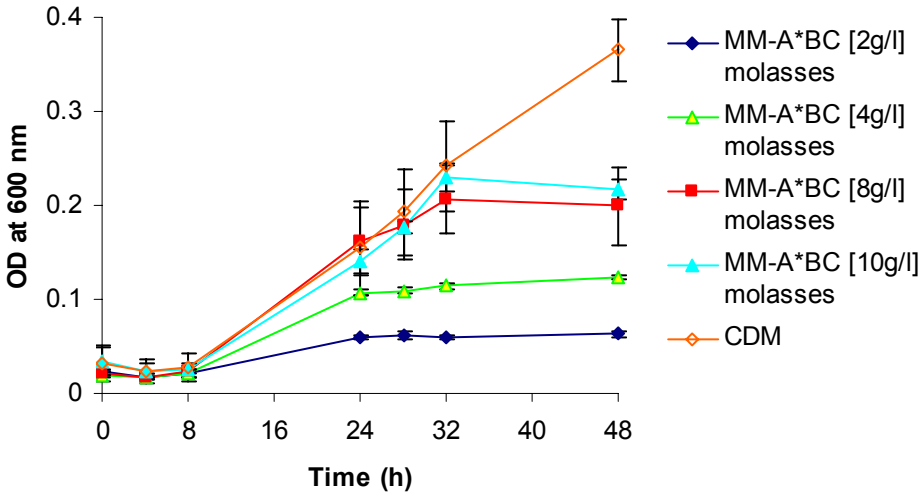

Fig. 2. Growth of strain *C. arsenoxidans* at different concentrations of molasses as a carbon-source.

These assays were performed in MM supplemented with the salt solution from the CDM. The increase of the concentration of molasses in MM-A*BC did improve the growth of the strain, as it is shown in the Fig. 2. Nevertheless, the highest concentration of molasses assayed in the medium did not achieve even half of the growth level of the strain, when compared to its growth in CDM medium.

Subsequently the importance of each salt solution from CDM was tested. In Fig. 3 the results from these assays are presented. The test were done in MM with concentration of molasses of 8 g/l and in the presence of As[III] with concentration of 10 mg/l.

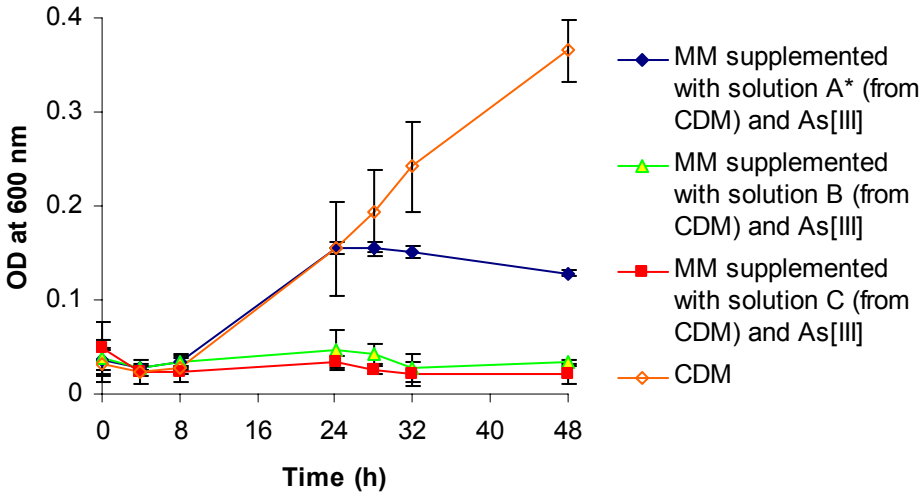

Fig. 3. Growth of the strain *C. arsenoxidans* in function of the type of salt solutions from CDM supplemented in the MM.

These assays investigated the importance of each salt solution from CDM as microelements, essential for the growth of the strain, and revealed the importance mainly of solution A*. These assays were performed in MM containing 10 g/l of molasses as a carbon-source and in the presence and in absence of As[III]. In Fig. 3 is shown an example of the results from the experiments in the presence of As[III]. The growth of the strain in these assays was not improved after the addition either of salt solutions B, or solution C from CDM. This may be due to one side of the constituents of the molasses which could lower the importance of these salts for the growth of *C. arsenoxidans* in the MM, or from another side on lower their accessibility the strain due to chemical interactions with the molasses. Another possibility is that the growth of the strain is independent from these salt solutions, when it is performed in a more favorable medium, which is not the case with this one.

3. Conclusion

From the preliminary assays of the growth of the arsenite-oxidizing strain *C. arsenoxidans* in different nutrition media, only those based on organic by-products, containing lactate - sauerkraut brine and whey, proved to be suitable for further studies. An important point for the use of organic by-products as a carbon-source for the growth of *C. arsenoxidans* was their preliminary treatment for the preparation of the media. The neutralization of whey and sauerkraut brine was very important as the *C. arsenoxidans* is a neutrophilic bacterium. Molasses as a carbon-source did not support the growth of the strain to a higher level, when compared to the growth of the strain in CDM, and therefore was not suitable for biomass production. Nevertheless, the assays performed in this medium proved the importance of salt solution A* supplementation in all media based on organic waste from food industry. Moreover, results obtained for the growth of the strain in whey based media showed that the addition of salts solutions from CDM resulted in better biomass production and enhanced growth. The use of the whey as a diary by-product, which can be used for the biomass production of different microorganisms is already studied (Lee *et al.*, 2003; Cristiani-Urbina *et al.*, 2000; Bledsoe *et al.*, 1999) and proposed for use as an inexpensive carbon-source.

The use of sauerkraut brine as a carbon-source supporting the growth of microorganisms has not yet been proposed. An important differentiation between these two products, when used as carbon-sources for *C. arsenoxidans*, is that only in media based on the sauerkraut brine the three salt solutions from CDM were not observed to be important for the growth of the strain. One possible explanation of these results and observations may be the differentiation in the chemical compounds of the sauerkraut brine and the whey.

Subsequently, the sauerkraut brine based media and the whey based media, which have shown the best ratio of biomass production were also assayed for the possible interactions with the As[III]. These measurements were done by HPLC-ICP-AES. The obtained results showed that neither sauerkraut brine based media, nor the whey based media underwent interactions with arsenite or arsenate and did not oxidize or reduce it in absence of the bacterial strain. These media also did not uptake or bind the arsenic species. Therefore, these media were suitable for the simultaneously conversion and production of biomass from *C. arsenoxidans* in order to be used for arsenic remediation purposes in wastewater treatment technology.

The fact that the addition of salts solution to sauerkraut based media was not necessary to give a high biomass and the media indifference toward the As[III], established these media as the

most favorable for the overall process. Therefore, all further assays were performed in these media and were compared with the results obtained in CDM.

The first essential contribution of this study is the proposition for the use of neutralized sauerkraut brine as a carbon-source supporting the growth of a bacterial strain, which was not assayed prior to our knowledge. The second essential contribution of these results is the possibility of simultaneous conversion of organic by-products and the oxidation of arsenic in water effluents, through the use of bacterial strain *C. arsenoxidans*. The importance of the simultaneous conversion of liquid organic waste products and the oxidation of As[III] is based on the As[V] formation, which is less toxic then the As[III], as well as the degradation of organic waste. These achievements can be used in further studies for the development of a biological or biochemical technology for treatment of arsenic polluted water.

Part II: Immobilization of *C. arsenoxidans* in Ca-alginate beads

1. Introduction

The oxidation of As[III] into As[V] by *C. aresenoxidans* was studied in shaking flasks using free cells as it was described in the Part I and using calcium –alginate-entrapped cells. The immobilization of microorganisms makes them easier to handle as biocatalysts, which is of great advantage in many industrial processes and for biodegradation. The entrapment of whole cells of *C. aresenoxidans* in Ca-alginate beads was carried out in order to establish whether the strain could be used when the cells are immobilized. This possibility for the application of the strain in water treatment is connected with the increase of the volume of water treated as well as decrease of possible release of the cells into the environment.

The influence of the main parameters and experimental systems on the oxidation of As[III] from immobilized *C. arsenoxidans*, was examined. For this purpose an experimental design technique was set up. The factorial experimental design methodology involves changing all variables from one experiment to the next. The reason for this is that the variables can influence each other, and the ideal value for one of them can depend on the values of the others.

The objective of this part of the work was to establish how the main studied parameters: the temperature, pH, the concentration of carbon-source in the medium and the concentration of As[III] interacted and affected the arsenic oxidation efficiency of *C. arsenoxidans*. Therefore, a full factorial design 2^4 scheme was developed and used to study the oxidation of As[III] in aqueous solutions. All the experiments were performed in SBM and SBM-A*BC media containing 10% (vol/vol) sauerkraut brine, with the exception of the experiments investigating the influence of carbon-source concentration as a variable. The results were analyzed statistically using the Student´s *t*-test.

Diliana D. Simeonova, Kalina Micheva, Daniel A. E. Muller, Florence Lagarde, Marie-Claire Lett, Veneta I. Groudeva and Didier Lièvremont

Arsenite oxidation in batch reactors with alginate-immobilized *Cenibacterium arsenoxidans*

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Arsenite oxidation in batch reactors with alginate-immobilized *Cenibacterium arsenoxidans*

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Abstract:

Arsenic is one of the major groundwater contaminants world-wide. It was previously demonstrated that the ß-proteobacterium *Cenibacterium arsenoxidans* has an efficient As[III] oxidation ability. The present study was conducted to evaluate the performance of alginate-encapsulated *C. arsenoxidans* in the oxidation of As[III] to As[V] in batch reactors. A two-level full factorial experimental design was applied to investigate the influence of main parameters involved in the oxidation process i.e. pH (7–8), temperature (4°C–25°C), kind of nutrient media (2%–20% sauerkraut brine), and arsenic concentration (10–100 mg L^{-1}). 100 mg L^{-1} As[III] was fully oxidized by calcium alginate immobilized cells in 1 h. It was found that the temperature as well as the kind of nutrient media used were significant parameters at a 95% confidence interval whereas only temperature was a significant parameter at a 99% confidence interval. The immobilization of the As[III] oxidizing strain in alginate beads offers a promising way to implement new treatment processes in the remediation of arsenic contaminated waters.

Keywords: Calcium alginate; immobilized bacteria; *Cenibacterium arsenoxidans*; arsenic; arsenite

INTRODUCTION

Arsenic (As) is a metalloid constitutive of more than 300 natural minerals. It is often found in association with sulphur as for example in arsenopyrite (FeAsS), realgar (AsS) and orpiment (As_2S_3). The environmental mobilization of arsenic, through the dissolution of these minerals during erosion processes, as well as through anthropogenic activities (mining, smelting, glass manufacturing, pesticides, chemical weapons…), contribute to groundwaters enrichment.

Literature reports a very large range of arsenic concentrations (0.5-10000 µg L^{-1}) in numerous aquifers distributed all over the world (Smedley and Kinniburgh, 2002). High concentrations are primarily related to natural sources i.e. geogenic arsenic of sedimentary aquifers. As a consequence, more than 100,000,000 people world wide are exposed to excessive amounts of inorganic arsenic (Chowdhury et al., 2000) whereas 13,000,000 people drink water with more than 10 µg L^{-1} in the United States (O'Connor, 2002), which corresponds in many countries to the maximal level authorized (WHO, 1996; EC, 1998; USEPA, 2001). Unquestionably, inorganic arsenic induces apoptosis, but paradoxically, arsenic appears to be a valuable chemotherapeutic agent for treating some types of cancers (Slack and Rusiniak, 2000; Bode and Dong, 2002). The toxicity of arsenic depends not only on its oxidation state but also on its chemical form i.e. inorganic forms of arsenic are the most hazardous forms whereas organic forms are generally considered to be less or even non toxic. It is known that microorganisms play an important role in arsenic transformations i.e. changes in valence or methylation which partly control arsenic speciation in the environment. The oxidation of arsenite (As[III]) to arsenate (As[V]) by heterotrophic bacterial species is considered as a detoxification mechanism since trivalent arsenic compounds are more toxic than pentavalent compounds.

Different water treatment technologies have been developed for arsenic removal. They are based on diverse processes such as coagulation, filtration, adsorption, reverse osmosis… (Bissen and Frimmel, 2003). Nevertheless, most of these technologies target As[V] and require a pre-oxidation step to transform As[III] to As[V]. This step is usually performed by using powerful chemical oxidants (ozone, chlorine, or H_2O_2) but microbial As[III] oxidation might constitute an attractive alternative strategy. *C. arsenoxidans* is a ß-proteobacterium which has already demonstrated its ability to oxidize As[III] in free suspension (Weeger et al., 1999). In this work, we studied the performances of the strain after its immobilization in a Ca-

alginate gel. Immobilization offers the advantage to stabilize the cells and may also improve their productivity by increasing their local concentration (Gikas and Livingston, 1993). Moreover, an inexpensive sauerkraut-based nutrient media was used as carbon source. Optimal conditions of oxidation in laboratory batch reactors were determined by implementing an experimental design.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study exceeded 98% purity and were used without further purification. All buffers and solutions were prepared with doubly deionized water (Milli-Q, 18 MΩ cm^{-1}, Millipore) and sterilized by membrane filtration (0.22 µm pore size) or by autoclaving at 120°C for 20 min. As[III] and As[V] solutions were prepared from 10,000 mg L^{-1} (0.13 M) stock solutions of sodium arsenite ($NaAsO_2$) and sodium arsenate ($Na_2HAsO_4.7H_2O$), respectively. Stock solutions were stored at 4°C in the dark.

Bacterial strain and growth medium

The arsenite oxidizing strain *C. arsenoxidans* was isolated from an arsenic contaminated aquatic environment and further preserved in a 20 % glycerol aqueous solution at –80°C. The strain's characteristics and growth conditions (Weeger et al., 1999) as well as its arsenite oxidase genes (Muller et al., 2003) have been previously described.

The synthetic growth medium (SB) was prepared by mixing the following three solutions :

Solution A : 0.0812 M of $MgSO_4,7H_2O$ (Sigma), 0.187 M of NH_4Cl (Merck, 99.8%), 0.07 M of Na_2SO_4 (Prolabo, 99%), 0.574 mM of K_2HPO_4 (Prolabo, 97%), 4.57 mM of $CaCl_2,2H_2O$ (Merck, 99.5%). Solution B : 4.8 mM $Fe_2SO_4,7H_2O$ (Prolabo, 99%). Solution C : 0.95 M $NaHCO_3$ (Prolabo, 99.5%). 100 mL of solution A, 2.5 mL of solution B, 10 mL of solution C were mixed and made up to 1 L with sauerkraut brine 10% (Coopchou, Vendenheim, France). Sauerkraut brine solution (10%) was used as the organic carbon source (Simeonova et al., 2004). The final pH of this complete medium was 7.2. All the solutions were prepared with doubly deionized water (Milli-Q system, Millipore) previously sterilized by autoclaving (120°C, 20 min). Solution A was sterilized by autoclaving (120°C, 20 min), and solutions B and C by filtration through a 0.45 µm pore size filter (Millipore).

Bead preparation and measurement of bead size

The calcium alginate (CA) beads were prepared as follows: sterile 0.2 M solutions of $CaCl_2$ and 3 % (w/v) calcium alginate in Milli-Q water were prepared. Calcium alginate solution

was added drop by drop through pipette tips (d=0.3 mm) into stirred $CaCl_2$ solution. The mean diameter size of beads was 2.2 ± 0.2 mm (n=1000).

Encapsulation and cell loading

C. arsenoxidans was maintained on SB agar medium at 4°C. One colony was transferred to 40 mL of chemically defined growth medium CDM (Weeger et al., 1999) supplemented with 10 mg L^{-1} of As[III] to obtain arsenic-induced cells. They were incubated for 72 h at 25°C in dark on a rotary shaker (250 rpm). *C. arsenoxidans* (OD 0.3-0.4) were harvested by centrifugation (5,000 rpm, 15 min), washed twice with Milli-Q water and resuspended in Milli-Q water. Three 200 µL aliquots were dried under vacuum until constant weight to calculate the cell mass. Adequate concentration of cells were further mixed with an aqueous solution of alginate (final concentration 3 % w/v) to yield an appropriate final cell concentration of 0.5 to 4 mg of cells (dry weight) per g of alginate bead (wet weight). Cells-alginate suspension was further used to produce beads as previously described. The cell-gel beads were maintained in the $CaCl_2$ solution for 2 h, washed with sterile distilled water and used immediately. Cell concentration was determined by absorbance at 600 nm and correlated to the cell dry weight through a calibration curve.

Cell release

Cells release from beads was investigated as follows: 2.5 mg of beads were introduced into sterile 40 mL polystyrene flasks containing 8 mL of SB broth (pH 7.2). Two different cell mass loadings (1 and 2 mg_{cells} g^{-1} beads) and two Ca-alginate concentrations (3 and 5 %) were used. After 1 h and 24 h, aliquots of liquid phase were sampled and viable cell counts (CFU ml^{-1}) were estimated by plating serial dilutions on CDM agar (Weeger et al., 1999). Plates were incubated aerobically at 25°C for 48 h. After 24 h, beads were filtered and transferred to fresh media for another 24 h period and sampling was carried out under the same conditions. The experiment was performed for 96 h.

Sorption of As[III] and As[V] onto beads

Sorption of both As[III] and As[V] by alginate beads (2.5 mg) was investigated as a function of time (contact time 1 h and 24 h) in the SB broth (pH 7.2; volume 8 mL) supplemented with different arsenic concentrations (0, 5, 10, 15, 25, 50, 75 and 100 mg L^{-1}) at 25°C. Adsorption experiments (n=4) were performed in sterile 40 mL polystyrene flasks. The concentrations of arsenic species in liquid phase were determined by HPLC–ICP–AES. The amount of arsenic sorbed by the gel was determined as the difference between the final and initial concentration. Controls with arsenic without beads were run simultaneously.

Bio-oxidation of As[III]

As[III] bio-oxidation by cell-gel beads were performed as follows: 2.5 mg of beads were introduced into sterile 40 mL polystyrene flasks containing 8 mL of SB broth supplemented with As[III] to reach a final concentration of 50 mg L^{-1}. After a contact time of 3 h, aliquots of the liquid medium were sampled for chemical analysis (HPLC–ICP–AES). The influence of encapsulated cell mass loading on bio-oxidation was evaluated by conducting experiments with beads of various cell loading (0.5-4 mg_{cells} g^{-1} beads). Experiments were made in quadruplicate. Controls with beads (without cells) and As[III] were also run.

Factorial design experiments

A factorial design was used to identify the optimal conditions and to single out the factors which have the most influence on the bio-oxidation. It was implemented by identifying four variables that could affect the bio-oxidation. These variables were pH, temperature, As[III] concentration and sauerkraut brine concentration (Table 1). We assigned for each of these variables a low (– 1) and a high (+ 1) value. These values corresponded to intervals in which the batch reactor system was expected to operate. The high level (+ 1) of each factor is far enough apart from the low level so that a significant effect, if it exists, is likely to be detected.

Using a full factorial experimental design, the number of experiments required to determine the influence of these four parameters was 16 (2^4). Experiments were performed in duplicate.

HPLC–ICP–AES

Concentrations of As[III] and As[V] in aqueous solutions were determined by HPLC-ICP-AES as previously described (Weeger et al., 1999), using an anion exchange polymeric column (PRP-X100, 250 mm X 4.1 mm i.d., particle size 10 µm, Hamilton). Arsenic compounds were eluted at 1 ml min^{-1} in phosphate buffer (pH 7.2), degassed and filtered on a 0.65 µm cellulose acetate membrane (Millipore). Detection was at 193.7 nm wavelength using an ICP–AES apparatus (JY138 ULTRACE, Jobin Yvon), coupled to an HPLC pump (LC–10 AT, Schimadzu) via a Teflon tube. The ICP-spectrometer was calibrated before each use and again after every 10 samples by running a blank and samples of known concentrations. Detection limits were 0.11 mg L^{-1} and 0.14 mg L^{-1} for As[III] and As[V], respectively. Quantification limits were 0.47 mg L^{-1} and 0.36 mg L^{-1} for As[III] and As[V], respectively.

RESULTS AND DISCUSSION

Oxidation of As[III] by CA encapsulated cells

We have previously demonstrated that free cells of *C. arsenoxidans* were able to fully oxidize 100 mg L^{-1} As[III] in less than 24 h (Weeger et al., 1999). In this work, cells of *C. arsenoxidans* were encapsulated in alginate beads to be better handled than free suspensions. Algination was chosen for immobilization as it is a rapid biocompatible process operating at ambient temperatures. Entrapment parameters such as alginate concentration, bead size or initial cell loading are known to influence kinetics of degradation or cells survival rate (Lee and Heo, 2000). In our study, alginate concentration was kept constant (3% w/v, chosen to ensure a good mechanical strength of the gel). The size of the beads is also important since the mass flux of arsenic entering the beads varies with bead radius (Gutenwik et al., 2002). As shown in Fig. 1. oxidation of As[III] by immobilized *C. arsenoxidans* is extremely rapid and significantly depends on the initial cell loading. High cell concentrations contributes to increase As[III] oxidation rate (up to 96 mg L^{-1} h^{-1}) which was ~ 3 folds more than that obtained with free cells (35 mg L^{-1} h^{-1}). Nevertheless, the maximum specific oxidation rate (155.2 ± 15.5 $mg_{As[III]}$ h^{-1} g^{-1}_{cells}) was obtained with a cell loading of 1 mg of cells per gram of alginate beads (Table 2). Diffusion coefficient of the compound in the beads (intra-gel diffusivity) varies with the local cell concentration and diffusion hindrance increase with the cell concentration and physical obstruction of the polymer chains. Thus, one could explain that diffusion of the compound in the beads could be very low and thus could lead to increase in the time required for As[III] oxidation. On the other hand, the gel matrix could behave as a protective agent for the cells against high concentration of arsenic. In our experiments, no lag phase or adaptation period was observed compared to free cells. Moreover, in a encapsulation process, cells experience shear due to mixing with the polymer solutions. In other words, initial cell loadings may not reflect perfectly the number of viable cells. Moreover, some authors have shown that the activity of the cells in the matrix is correlated to their location. The biomass close to the external surface is more active (Gikas and Livingston, 1997). Consequently, calculated rates of As[III] oxidation (Table 2) might be higher.

Cells release from alginate matrix

It is known that the gel matrix may not ensure a complete retention of the immobilized microorganism. Yahashi *et al.* (1996) observed that the immobilized-cell system was subject to cell leakage. Mechanical properties of the beads are dependent of the initial cell loadings

and growth of the strain inside the beads could also change these properties. Release of viable bacteria in the surrounding medium are also reported (Scannell et al., 2000; Klinkenberg et al., 2001) and appears for example when growth occurs in the beads. The microbial colonies reach the surface of the gel bead and this leads to the release of microorganisms. This phenomenon is not always desirable and some authors concentrated their efforts to control rates of cell release (Champagne et al., 1994; Zhou et al., 1998). In our case, permeability and integrity of alginate gel seemed to be preserved during experiments. Moreover, as shown in Fig. 2, free cells are continuously released from the immobilization matrix. These results are in agreement with those of Champagne et al. (1992) who reported the release of *Lactococcus lactis* cells (from 1.10^6 to 3.10^7 CFU mL^{-1}) independently of initial cell density within the beads or alginate concentration.

Two different cell mass loadings (1 and 2 mg_{cells} g^{-1} beads) and two Ca-alginate concentrations (3 % and 5 %) were used (Fig. 2). Data showed that the release and the viability of the cells, expressed as CFU mL^{-1} encapsulated in alginate gels is not highly influenced by the concentration of the calcium alginate solution and by the initial cell loading. One might have expected that a high cell density induce the growth of cell colonies preferentially at the periphery of the beads (Mater et al., 1999) and that the cells were more susceptible to be released. Nevertheless, we observed in our experiments that the release of cells during the first hours was limited and this phenomenon did not play a major role. High gel concentration (i.e. 3% and 5% alginate), due to their mechanical properties (i.e. mechanical strength), did not allow cell release as easily as low gel concentration (i.e. 1% alginate in which the term " cell confinement " seemed to be more appropriate). As a consequence, these high gel concentrations will be used to increase the mechanical stability of the alginate support in view of further experiments under packed-bed reactor operation.

Sorption of As[III] and As[V] on alginate beads

Alginate beads (2.5 mg) without biomass were put into As[III] or As[V] solution with concentrations ranging from 0 to 100 mg L^{-1} and equilibrated for 24 h. Results are shown in Fig. 3. Sorption behaviors of As[III] and As[V] were completely different. Sorption of As[III] onto alginate first increased with initial arsenic concentration and reached a saturation value (0.015 mg As g^{-1} beads) at 50 mg L^{-1} As[III]. In contrast, with As[V], no saturation value was observed in the range of concentration tested. Maximal sorption (0.075 mg As[V] sorbed per g of beads) was observed with the highest As[V] concentration tested i.e. 100 mg L^{-1}. The porosity of the beads was similar in all the experiments but several parameters determine

biosorption values viz. structural properties of the beads, initial concentration of metalloid ions, existence of other competing ions (phosphate for example). As other biopolymers, alginate which is an anionic sorbent has been shown to be effective at removing cationic metals i.e. Pb^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+} from solution. As[V] occurs at neutral pH (about 7) as H_2AsO4^- and $HAsO_4^{2-}$ whereas As[III] occurs within the same pH range as the neutral species H_3AsO_4. Calcium alginate beads showed no significant As[III] sorption in the whole range of concentrations tested. This low As[III] accumulation is the result of passive diffusion of the neutral species in the beads. The negatively charged As[V] species were better removed from the solution but the extent of the sorption remained low (Fig. 3). Min and Hering (1998) reported low As[V] sorption on Fe[III] doped alginate beads.

Evaluation of factors affecting As[III] oxidation

A 2^4 full factorial design (16 factor/level treatment combinations) was performed with the values of the major variables shown in Table 1. Experimental conditions (column 2 to column 5) and results of the experiments (column 6) are shown in Table 3. The results are expressed as the quantity of As[III] oxidized per gram of biomass (dry weight) per hour. Experimental runs were randomly performed to eliminate any kind of extraneous effects tied with the method used.

As could be determined from the results (Table 3), the ability of the strain to oxidize As[III] showed wide variation. As[III] oxidation varied from 25 to 145 mg As[III] g^{-1} h^{-1}. All of these As[III] oxidation rates were very high. Nevertheless, factors affect the oxidation ability of the strain in different ways. The optimal As[III] oxidation was found with the low values of pH (7) and arsenic concentration (10 mg L^{-1}) and the high values of temperature (25°C) and sauerkraut brine (20%) added in the medium (Table 4). Nevertheless, data showed that *C. arsenoxidans* had the ability to oxidize As[III] efficiently with each and every set of parameters. In applied wastewater treatment systems, these characteristics are highly desirable since conditions are continuously changing. Two variables were found to be statistically significant at $\alpha = 0.05$: temperature (T) and the concentration of sauerkraut brine (C_s) in the medium whereas at $\alpha = 0.01$, only temperature (T) was statistically significant. A new matrix was constructed with the two significant factors at $\alpha = 0.05$ and results showed that the highest As[III] oxidation rate was obtained when experiments were performed with a temperature of 25°C and a concentration of 20% of sauerkraut brine in the culture medium (Table 5).

CONCLUSIONS

C. arsenoxidans was shown previously to be a very efficient As[III] oxidizer in experiments carried out with free cells. Circumventing the drawbacks of handling a free suspension, we first report As[III] oxidation by immobilized cells in small calcium alginate beads. High concentrations (100 mg L^{-1}) of As[III] were fully oxidized by *C. arsenoxidans*. The oxidation was shown to be faster than that of free cells. A rapid exploration of selected parameters affecting the As[III] oxidation with a full factorial design showed that the most important parameter was temperature. To be most effective, the biooxidation must be performed at a temperature of 25°C ± 2°C. These results are promising and demonstrated the high potential of immobilized cells, i.e. improved biomass retention efficient and faster treatment of As[III] containing waste streams. Experiments to show that immobilized cells keep their As[III] oxidative ability during repeated batch and in continuous mode are under way.

ACKNOWLEDGEMENTS

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FIGURES AND TABLES

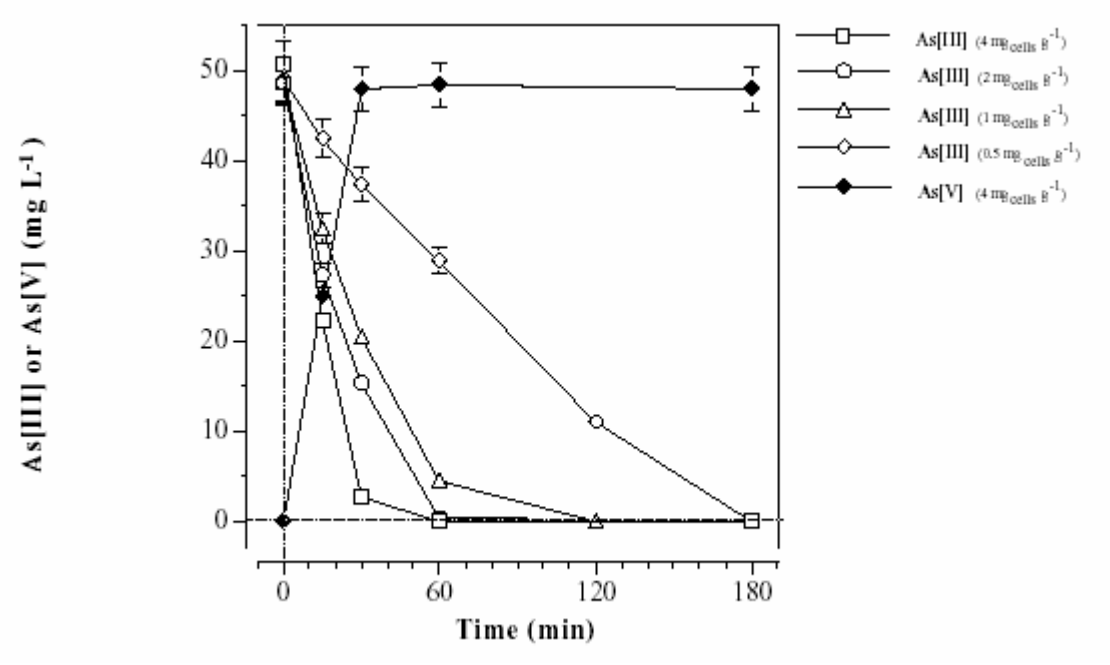

Figure 1. Disappearance of As[III] vs time (open symbols) as a function of initial cell loadings expressed as mg of cells (*Cenibacterium arsenoxidans*) per g of Ca-alginate beads. Closed symbol represents the concentration of As[V] appearing in the medium for an initial cell loading of 4 mg_{cells} g^{-1}. The As[V] curves corresponding to other cell loadings are not shown. Data are mean of two independent experiments made in duplicate and errors bars show the maximum deviation from the mean value (5 %). Initial As[III] concentration: 50 mg L^{-1}; volume of SB broth: 8 mL; beads mass loading: 2.5 mg; pH: 7.2; temperature: 25°C.

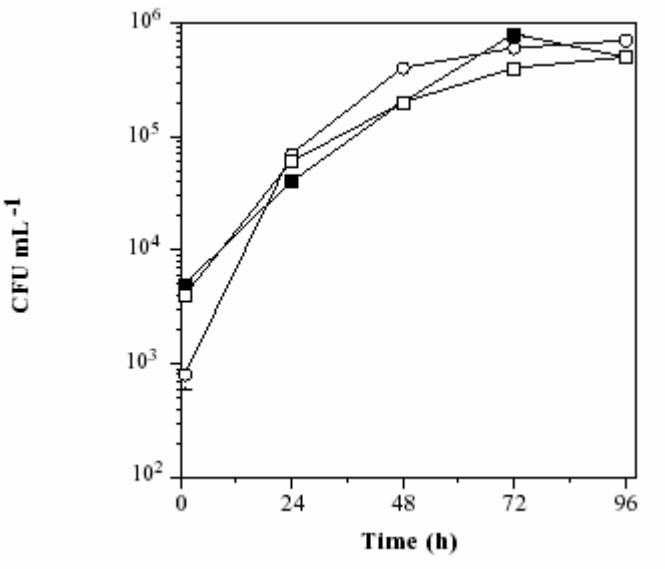

Figure 2. Release of viable cells of *C. arsenoxidans* in SB broth as a function of time. Open symbols correspond to cell release from alginate beads prepared with a cell density of 1 mg cells g^{-1} beads whereas filled symbols correspond to cell release from alginate beads prepared with a cell density of 2 mg cells g^{-1} beads. Square symbols are beads prepared with a 3 % (w/v) calcium alginate solution whereas circles are beads prepared with a 5 % (w/v) calcium alginate solution. Data are mean of two independent experiments made in duplicate and error bars show the maximum deviation from the mean value. Volume of SB broth: 8 mL; beads mass loading: 2.5 mg; pH: 7.2; Temperature: 25°C.

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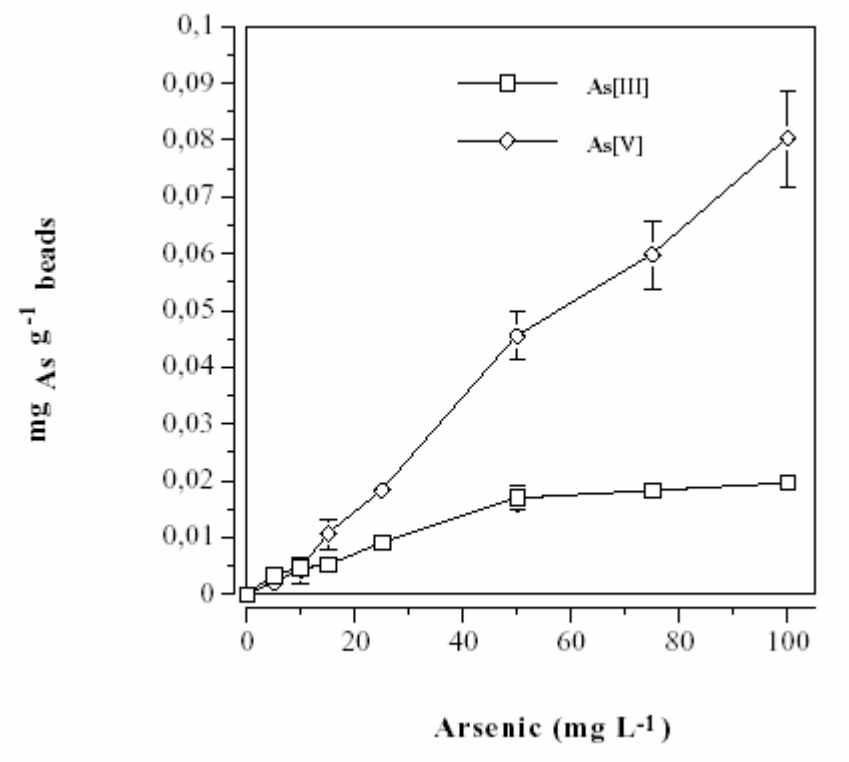

360

361 **Figure 3.** Sorption of As[III] and As[V] onto Ca-alginate beads as a function of initial As[III]
362 and As[V] concentrations in the liquid phase. Data are mean of two independent experiments
363 made in duplicate and errors bars show the standard deviation (n=4). Contact time: 24 h;
364 volume of SB broth: 8 mL; beads mass loading: 2.5 mg; pH: 7.2; temperature: 25°C.

365

366

367 **Table I.** Coded factor levels and real values for full factorial design 2^4.

Factor	Abbreviation	Low level (– 1)	High level (+ 1)	Units
pH	*pH*	7	8	–
Temperature	*T*	4	25	°C
[Sauerkraut] in medium	C_s	2	20	%
[AsIII]	C_{As}	10	100	mg L^{-1}

368

369 **Table II.** Oxidation rates as a function of cell loading.

Cell loading	As[III] oxidation rate	As[III] specific oxidation rate
$mg_{cell}/g_{CA\text{-}beads}$	mg L^{-1} h^{-1}	mg h^{-1} g^{-1}
0.5	20.3 ± 2.0	102.4 ± 10.2
1	48.5 ± 4.8	138.1 ± 13.8
2	82.4 ± 8.2	90.3 ± 9.0
4	96.5 ± 9.6	78.3 ± 7.8

370

371 **Table III.** As[III] oxidation by encapsulated *Cenibacterium arsenoxidans*. Experimental
372 design (2^4) and mean values of response.

Experiment	Factors (N°) and coded levels				As[III] oxidation
	pH(1)	*T(2)*	$C_s(3)$	$C_{As}(4)$	(mg As[III] g^{-1} h^{-1}) *
1	-1	-1	-1	-1	61.27
2	+1	-1	-1	-1	50.20
3	-1	+1	-1	-1	113.45
4	+1	+1	-1	-1	97.75
5	-1	-1	+1	-1	70.09
6	+1	-1	+1	-1	47.16
7	-1	+1	+1	-1	145.72
8	+1	+1	+1	-1	122.31
9	-1	-1	-1	+1	25.31
10	+1	-1	-1	+1	24.40
11	-1	+1	-1	+1	84.36
12	+1	+1	-1	+1	86.31
13	-1	-1	+1	+1	42.38
14	+1	-1	+1	+1	42.81
15	-1	+1	+1	+1	129.21
16	+1	+1	+1	+1	123.03

373 * mass expressed as dry weight.

374

375 **Table IV**. Effect and t-Student values.

Factors	Effect a_i	t_i
mean	79.1	
pH	-4.9	1.3
Temperature	33.7	8.6
Sauerkraut	11.2	2.9
As(III)	-9.4	2.4
Interaction (1:2)*	-0.6	0.2
Interaction (1:3)*	-1.6	0.4
Interaction (1:4)*	4.3	1.1
Interaction (2:3)*	6.1	1.6
Interaction (2:4)*	2.3	0.6
interaction (3:4)*	3.4	0.9

376 * 1, 2, 3 and 4 are the four factors as in Table I.

377 t-Student = 4.032 (df = 5) with $\alpha = 0.01$; if $t_i < 4.032$ the variable is not significant.

378 t-Student = 2.571 (df = 5) with $\alpha = 0.05$; if $t_i < 2.571$ the variable is not significant.

379

380 **Table V.** Importance of the two significant factors ($\alpha = 0.05$) on As[III] oxidation by

381 encapsulated *Cenibacterium arsenoxidans*. Mean values of response.

Experiment	Factors (N°) and coded levels		As[III] oxidation
	T(2)	C_s*(3)*	(mg As[III] g^{-1} h^{-1}) *
1	-1	-1	40.29
2	+1	-1	95.46
3	-1	+1	50.61
4	+1	+1	130.06

382 * mass expressed as dry weight.

383

384

3. Additional results

3.1. Influence of the temperature on the development of the strain

The growth of the strain was studied at 8° C and 15° C. The final concentrations of CFU/ml were equal to those obtained at 25° C. The influence of the temperature was studied in sauerkraut brine based media, containing 10% (vol/vol) of sauerkraut brine in presence and in absence of 10 mg/l of As[III].

The lag-phase on both temperatures continued to 24h, however when the strain was grown at 25°C the lag phase was 8 hours or less. The specific growth (μ) at 8° C was 0.022 and at 15° C was of 0.054 h^{-1}. These are the specific growth ratio, when the strain was grown in SBM-A*BC (10% of sauerkraut brine) in absence of arsenic. The μ parameter was calculated on the basis of OD, in the exponential phase of growth of the strain (Micheva, 2002).

The doubling time (t_d), was 31 h and 13h, respectively. However, the temperature did not influence the final concentration of cells in the stationary phase, which were 5.4 x 10^9 CFU/ml and 6.1 x 10^9 CFU/ml, at 8° and 15° C, respectively. The lowest specific growth of the strain was calculated for the CDM medium-0.03 h^{-1}, which corresponded to a doubling time of 23 h.

4. Conclusion

This is the first attempt of study of arsenite oxidizing bacterium, when the cells are immobilized. The results showed that the strain can be immobilized successfully. The important point was the achievement of an extremely high specific ratio of arsenic oxidation from immobilized cells – 138.1 ± 13.8 mg As[III]/h/l from 1 mg of immobilized cells dry weight. The As[III] oxidation rate achieved from immobilized cells was of 96 mg/l.h, and was higher than the oxidation achieved in free cells culture, which was 35 mg/l/h, when compared according to the quantity of biomass used in both sets of assays.

Although the best results were obtained at 25° C, this study showed that the *C. arsenoxidans* could be developed as a psychrophilic microorganism, and thus its application for bioremediation processes can be successful at low temperatures, i.e. during wintertime period.

The results obtained in this part of the work depicted the importance of each studied parameter for the growth of the strain. Therefore, this study revealed the possibility for the

application of the strain in a technology for preliminary oxidation of arsenic in water effluents. It also increased the possibilities for the application of the strain using immobilized cells.

Part III: Studies on the development of *C. arsenoxidans* in a simulated "open" remediation system

1. Introduction

This part of the assays was devoted to the understanding of the development of the strain in nonsterile sauerkraut brine medium. The aim was to establish whether the strain was capable of growth in a complex system, including other randomly introduced microorganisms. In the system bacteria and yeast were introduced into it from the air or water. The nutrient media used in these assays were prepared as described in Materials and Methods Chapter.

The necessity of proving the development of the *C. arsenoxidans* in an "open" system arose. Therefore, the strain was marked with GFP by using Mini-Tn*5* delivery plasmid derivatives, which are described in Materials and Methods Chapter. The GFP-tagged clones of *C. arsenoxidans*, were subjected to two selections, according to the type of plasmid used and the level of luminescence emitted. Thus, the expression of the GFP in the *C. arsenoxidans* clones was defined. The clone with highest expression of gfp was chosen and this strain was used to perform the further assays. These assays tracked the development of *C. arsenoxidans* during the simulation of an "open" system. For these assays a SBM-A*BC medium supplemented with arsenic, was used because this was the most favorable medium for the growth of the strain.

The final set of tests differentiated the microorganisms originated from the sauerkraut brine using the TTGE technique. This technique allowed the separation of bacteria from yeast as well as distinguishing the approximate number of different species presented in the sauerkraut brine. A combined protocol for total DNA extraction from bacterial and yeast cells was used. The aim was the simultaneous DNA extraction from both types, when they are presented in the same sample.

A. Construction and selection of gfp mutants of *C. arsenoxidans*

C. arsenoxidans strains were conjugated with three strains of *E. coli* each of them containing a suicide plasmid of type R6K. Each of these plasmids contained a gfp-tagged transposon. The gfp-tagged strains were then used to track the development of *C. arsenoxidans* in an "open" system. The system, in which the arsenite-oxidizer *C. arsenoxidans* grew in the presence of random microorganisms introduced into the nutrient medium from the air or the water used, was considered as an "open" system.

The *C. arsenoxidans* strains were conjugated with *E. coli* strains, each one containing one of the following transposons: mTn*5gusA-pgfp21,* mTn*5gfp-pgusA,* mTn*5gfp-km*. The construction of gfp mutants of *C. arsenoxidans* was done by D. Muller and F. Den Hartoog. For each type of transposon 192 clones were isolated. The clones were then subjected to two selections in order to establish the clones with the highest level of *gfp* protein expression. The first selection found clones emitting the most light. This selection was carried out after 48 h growth of the clones in microplates containing 150 µl of liquid CDM. The preliminary selection was done in duplicate for all strains. From the 192 clones containing the mTn*5gfp-pgusA* only 3 colonies were selected for further study. From the clones containing mTn*5gfp-km* 6 clones were selected for further studies and from the mutants containing the mTn*5gusA-pgfp21* 5 clones were chosen with the highest fluorescence expression.

The second selection found the clone with the highest expression level and most stable parameters of expression- both in liquid medium and on solid medium. Results from the comparative selection of *gfp* clones in liquid CDM are presented in Fig. 1.

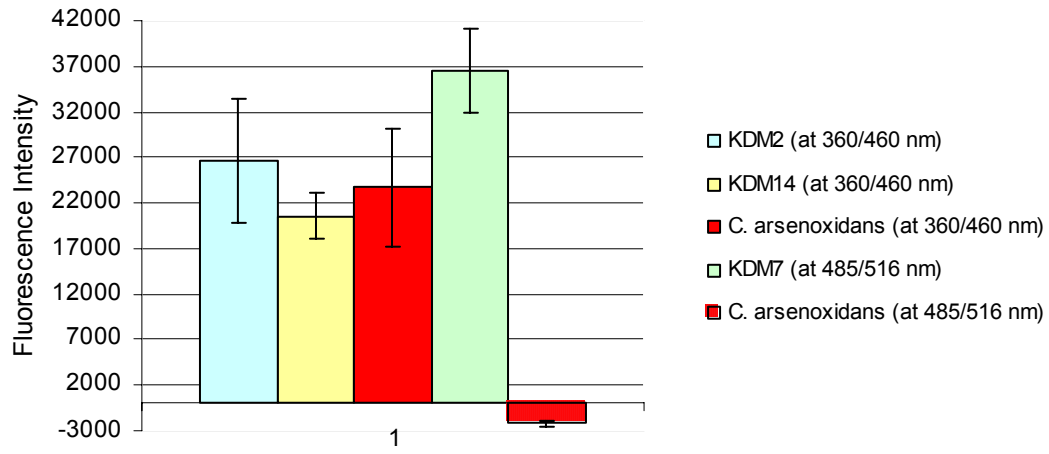

Fig. 1. Comparison of the relative fluorescence from the clones with the highest level of *gfp* expression in liquid CDM. Measurements were carried out at the 48h of growth of the cultures.

In Fig. 1 the mutants obtained from the three types of conjugations, which shown the highest level of fluorescence in liquid CDM are presented. The clone named KDM2 contained an mTn*5gfp-pgusA* transposon. The clone KDM7 had an mTn*5gfp-km* transposon and the clone KDM14 had a mTn*5gusA-pgfp21* transposon.

The highest difference in the level of fluorescence, when compared to the control sample (the wild strain of *C. arsenoxidans*) in liquid CDM, was found for the strain named KDM7. The relevant expressions of fluorescence from other type of constructions were not satisfactory, when compared to the fluorescence of the wild strain under the same conditions of measurements. The emission of light of the clones containing mTn*5gusA-pgfp21,* mTn*5gfp-pgusA* were measured at the interval of 360 to 460 nm and those with mTn*5gfp-km* were measured at 485 to 516 nm wave length, as described by Xi *et al*., (1999) and Tang *et al*., (1999). The wild type of *C. arsenoxidans*, when measured at 485 to 516 nm wave length showed absorption of the light (Fig. 1).

Therefore second fluorescence measurements were performed for all preliminary selected colonies. The measurements of fluorescence in CDM medium containing 0.3 % of agar were studied. The low concentration of agar allowed a homogenized colonization of the microplate well surface from the clones. This resulted in a lowering of the difference between the signals of the colony and the medium. These assays were done also at the 48 hours of growth of the colonies under the same conditions. The results are shown in Fig. 2.

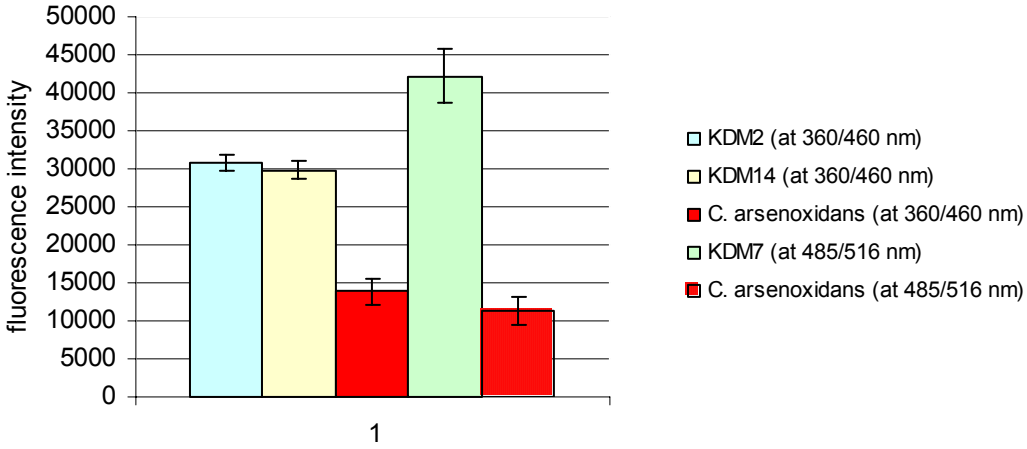

Fig. 2. Comparison of the relative fluorescence from the gfp clones with the highest level of *gfp* expression in CDM supplemented with 0.3% of agar. Measurements were carried out at the 48 h of growth of the cultures.

On the semi solid CDM containing 0.3% of agar the predicted homogenized colonization of the surface from bacteria was confirmed. Moreover the measurements of emitted light from all three types of mutants confirmed the measurements in liquid CDM. The clones containing the mTn*5gfp-km* had the highest fluorescent signals, when compared to the other two types of mutants. One of them, named KDM 7 showed the best level of fluorescence, when compared to the other 5 preliminary selected strains of the same type. Thus, this clone of *C. arsenoxidans* was selected for further use in the simulation of an "open" system. This clone has been sequenced. Through blast of obtained sequence was identified similarity to two protein conservative structures of *Ralstonia solanacearum* with 86% of identity. The database access number was NP_521280.

The gfp-tagged clone KDM7 was grown in maximum level of As[III] in the medium of 150 mg/L in order to find out whether the higher level of arsenic will have an impact on the development of the strain. These assays were done in triplicated (n=9) and was found that the strain growth was not influenced.

B. Fluorescence Microscopy

After the selection of a *gfp*-tagged mutant, several assays were conducted in sauerkraut brine medium in order to observe the growth of the *C. arsenoxidans*. The growth of the strain was observed on two types of media - the most favorable and the least favorable media for the development of the *C. arsenoxidans*. The SBM supplemented with the salt solutions from the CDM (SBM-A*BC) in absence of As[III] and in SBM without salt solutions (SBM), supplemented with 50 ppm of As[III]. The specific growth (μ_{max}) of the *C. arsenoxidans* in the SBM-A*BC in absence of As[III] was found to be $0.58.h^{-1}$ and the doubling time (t_d) was 1h and 11min. In comparison the μ_{max} of the strain in SBM in presence of As[III] was $0.11.h^{-1}$ and the t_d was of 6h and 20min. When compared these were the highest and the lowest parameters of the strain growth.

The sauerkraut brine was preliminary treated for the preparation of sterile medium. The concentration of sauerkraut brine in the assays was 10 % (vol./vol.).

Moreover, these two types of sauerkraut brine based media were selected, because the presence of salts solutions in sauerkraut could enhance the development of another bacteria and/or yeast in the simulated system. Therefore this medium was considered as the less favorable for the growth of the *C. arsenoxidans.* The second medium used, was the SBM supplemented with As[III]. This was considered as the medium less favorable for the development of other bacterial and yeast strains in the system, due to the presence of arsenite and the lack of additional salt solutions and microelements.

The observations of growth of the strain were carried out for 48 hours. At 0, 24 and 48 hour time period pictures were taken, using white light microscopy and in epifluorescence. Throughout the white light microscopy images it was possible to count the overall quantity of microorganisms present in the samples. Using the epifluorescence microscopy images the number of KDM7 cells, were counted. The bacteria and yeast presented in the sauerkraut brine media were studied for the possibility of emitting light signals. The limiting parameters for the distinction of KDM7 from other microorganisms were established. These parameters were the level of fluorescence expression and the shape of the object. The limits for separation and counting of KDM7 cells were set at 10 to 100 pixels, according to the image acquisition of the epifluorescent images. For the white light images the overall count of bacterial cells had the limits from 10 to 500 pixels. The yeasts were counted when the threshold was set at 500 pixels in the both type of images. In Fig. 3 a white light microscopy image taken is presented. In Fig. 4 the corresponding epifluorescent image of Fig.3 is

presented. Thus, the Fig. 3 and Fig. 4 form a pair of images, which represents the same field of the sample shoted in white light and in epifluorescence. The length of the images is 100µm with resolution of 6.96 pixels/µm. The difference of natural autofluorescence randomly introduced into the system bacteria and yeasts were induced in the range of 220-400 msec of excitation. The excitation time for the full development of fluorescence of KDM7 cells was in 100 msec.

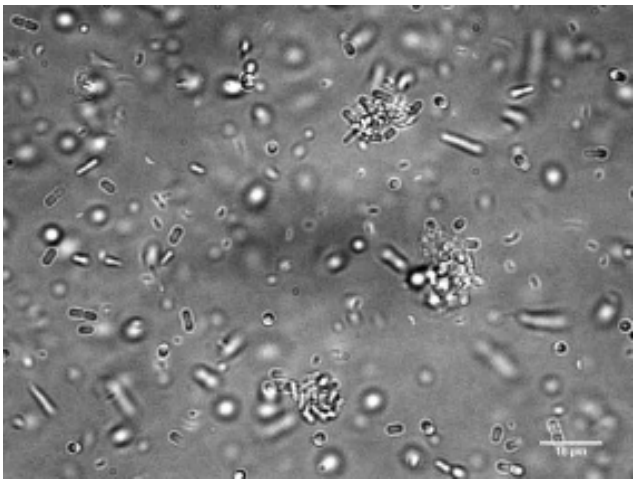

Fig. 3a. White light microscopy of sample SBM, supplemented with 50 ppm of As[III] after 48h of incubation. “Open” system image. Scale 10µm.

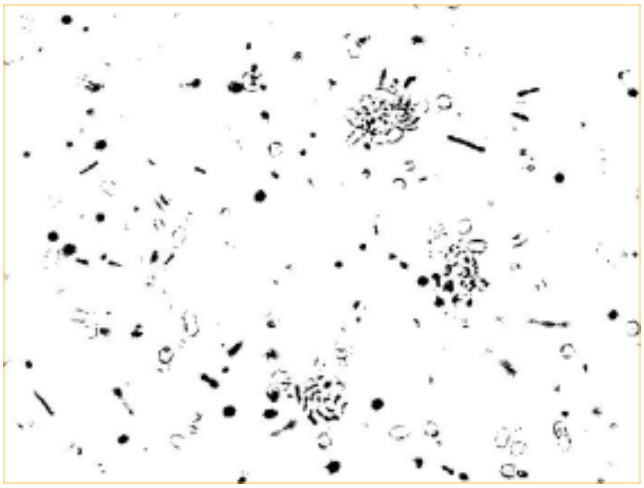

Fig. 3b. Binarized white light microscopy image with established threshold for the automatic analysis of particles. Scale 10µm.

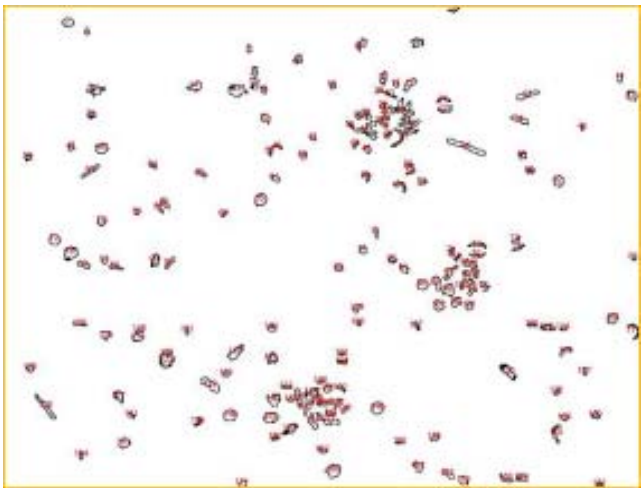

Fig. 3c. Final result of the automatic count and analysis of particles of the white light microscopy image shown in Fig.3a. Scale 10µm.

The pictures were processed in order to separate the different objects in the region of interest. The threshold of the signals was adjusted manually, and the objects were counted and analyzed using ImageJ software. In Fig. 4a, 4b and 4c the results of one epifluorescent image, is shown. The sequence of images in Fig.4 present the method for analysis of particles used, this time, when applied on epifluorescent images.

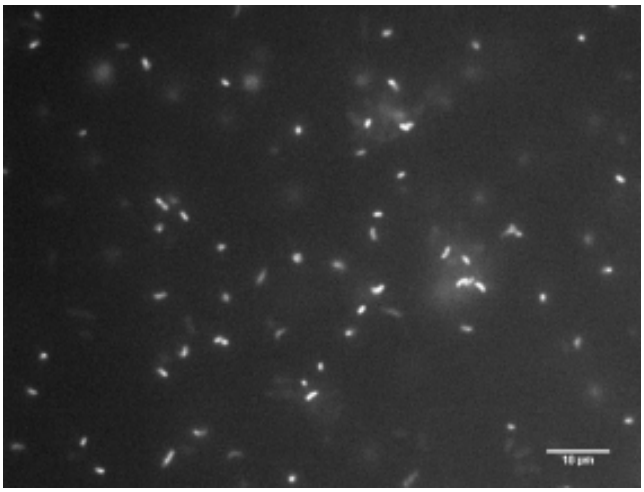

Fig. 4a. Epifluorescent image microscopy of KDM7 cells in sample SBM, supplemented with 50 ppm of As[III] after 48 h of incubation. “Open” system image. Scale 10µm.

Fig. 4a. Binarized epifluorescent microscopy image with established threshold for the automatic analysis of particles. Scale 10µm.

Fig. 4a. Final result of the automatic count and analysis of particles of the epifluorescent image shown in Fig.4a. Scale 10µm.

The objects in the images were counted automatically and the result imported and analyzed. A total number of 85 white light images and 77 fluorescent images were processed for the three points of the assay. The results are presented in Fig. 5.

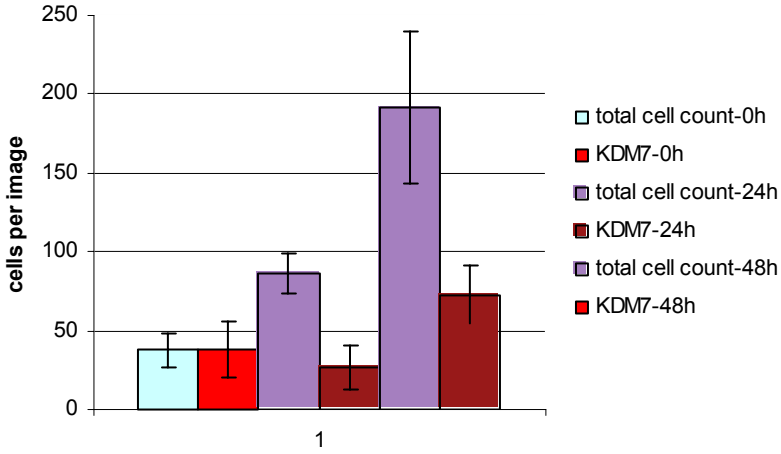

Fig. 5. Comparative development of all microorganisms in the SBM, supplemented with 50ppm of As[III] and the KDM7.

In addition the microorganisms in 9 white light images and 9 epifluorescent images were counted manually. These were chosen randomly from a pool of images taken after 0, 24 and 48 hours of growth of the cultures. The manual count of the microorganisms gave an approximate deviation of 10 to 40% to the estimated automatic count of the same images. These deviations of the results were due to the quality of the pictures as well as the different parameters each scientist who counted the microorganisms manually used.

The number of cells per image in the beginning of the assays is equal, because the media was prepared with sterile sauerkraut brine, and pretreated as described previously. The introduction of random microorganisms into the system was possible through the use of unsterile water during preparation of the medium and during the agitation of the cultures, which were not sealed with caps. Therefore, after the inoculation with the KDM7 cells in the beginning of the experiments, they presented the majority of microorganisms in the media. The results obtained showed increase in the total cells number of KDM7 at the end of experiment, which proved its adaptation to the system. Although the growth of the strain was slightly suppressed by the presence of other microorganisms in the system it remained the dominant microorganism after 48 h in the culture. The presence of As[III] in the medium, did not prevent the growth of other microorganisms and thus demonstrated an increase in the total

cell number. During the assays the optical density of the samples at fixed wave length λ= 600 nm were measured. The results are presented in Fig. 6.

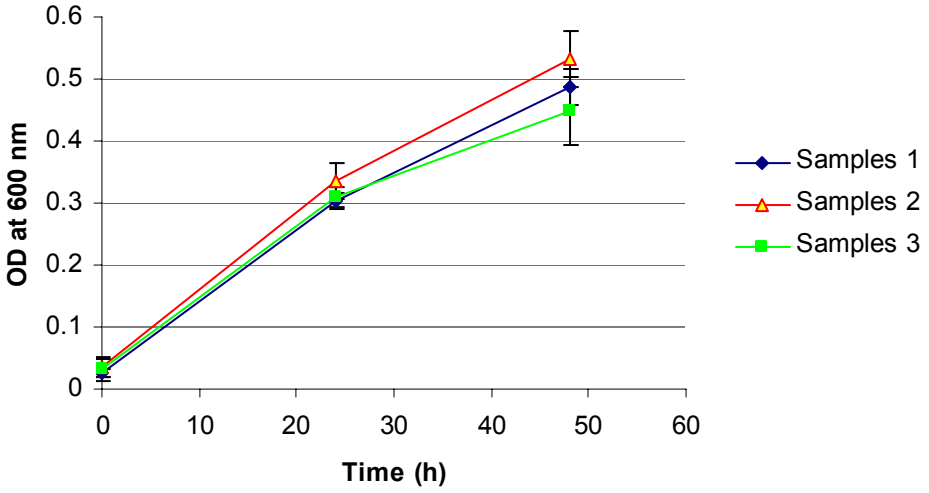

Fig. 6. Optical density of microbial consortia in SBM, supplemented with 50 ppm of As[III].

Assay 1, Assay 2, Assay 3 represent the three different sets of cultures used in the study. Each set was done in triplicate. The optical density was measured against a control of SBM media, supplemented with As[III] in the same quantity, but uninoculated with KDM7 cells.

Growth studies of *C. arsenoxidans* in this medium, when the concentration of sauerkraut brine was 10% (vol./vol) show that after 48 hour of growth as a pure culture the strain achieved optical density was of 0.43 to 0.45. This corresponds of 4.8 to 5.1 x 10^8 CFU per milliliter. Both sets of assays were started with an initial optical density of 0.02, which correspond to 3.8 x 10^7 CFU/ml of *C. arsenoxidans*. Therefore the growth of the strain in the assays simulating an "open" system is lowered, when compared to the growth in a pure culture. Nevertheless *C. arsenoxidans* showed good capabilities of adaptation and thus to keep a relatively high level of cells in the system.

The assays conducted in SBM- A*BC medium in absence of arsenic were performed in the same manner. After the 24h a flock formation was observed, because of which the measurements of the optical density of the cultures were not possible.

The flock formation phenomenon influenced the quality of pictures taken and thus the final count of microorganisms. In the 48 h of culture growth samples the formation of sediment flocks were observed at the bottom of the tubes, which was impossible to suspend. This

resulted in the shape of microorganisms on the white light pictures being almost inseparable through image processing. The flock formation made the measurement of the optical density impossible too. The flock formation also had a high negative impact on the processing of epifluorescent images. The fluorescent cells of KDM7 were included into the flock formed, and thus the overall fluorescent signal had increased, covering the whole flock. This led to the decrease of the contrast between the different objects and an increase of background noise. Therefore, the separation and count of the different cells in this particular medium were not possible through this method.

C. Microbial diversity in sauerkraut brine

Here the preliminary results from the assays aimed at the identification of microorganisms in the sauerkraut brine are presented. These assays led to a better understanding of the microorganism consortia originated and provided from the sauerkraut brine. The internal interaction between these microorganisms during the fermentation process of the sauerkraut could provide a better application of the sauerkraut brine as a complex carbon-source supporting the growth of *C. arsenoxidans* on a treatment technology.

The first step for identification of the microbial diversity in the sauerkraut brine was the extraction of total DNA from bacteria and yeast. This was done according to the protocols described in the Chapter Materials and Methods. The results are presented in Fig. 7.

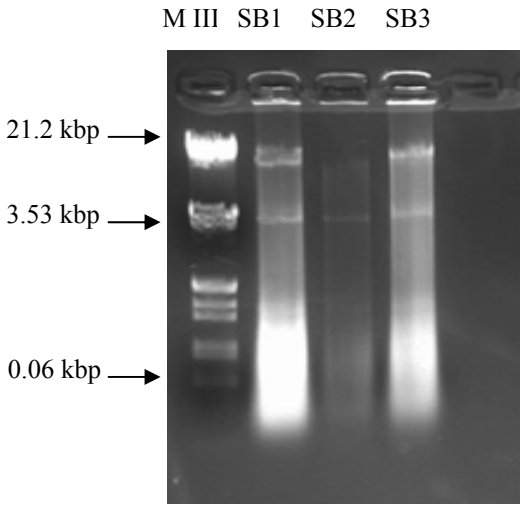

Fig. 7. Analysis of the extraction of genomic DNA of bacteria and yeast originated from sauerkraut brine in 1% agarose gel electrophoresis.

The samples shown in Fig. 7 are as follows: SB1 - extraction of bacterial genomic DNA according to the protocol of Promega; SB2 - extraction of yeast genomic DNA according to the protocol of Promega; SB3 –genomic DNA, obtained according to the protocol for simultaneous extraction of bacterial and yeast DNA, developed in this study. After the extraction the DNA was subjected to the amplification with specific primers pairs.

The separation of bacterial and yeast microorganisms DNA were performed using the TGGE technique. Therefore, the DNA fragments of interest should have length of 200 to 500 bp approximately. This is the length of DNA fragments estimated that allows the best separation of different species, when using this technique. The chosen primer pair for the amplifications of bacterial rDNA gave fragments of about 200 bp length. The primer pair used was W34/W49 and for the separation on the polyacrylamide gels the reverse primer W49 with a GC clamp on its 5' end was used. This primer pair amplified the 16S rDNA from V3 region, which is found between positions 533-520 on *E. coli* chromosome. All primers used in this study are described in table 2 of Material and Methods Chapter. The results obtained after the amplification of 16 S rDNA with this pair of oligonucleotides are presented in Fig. 8.

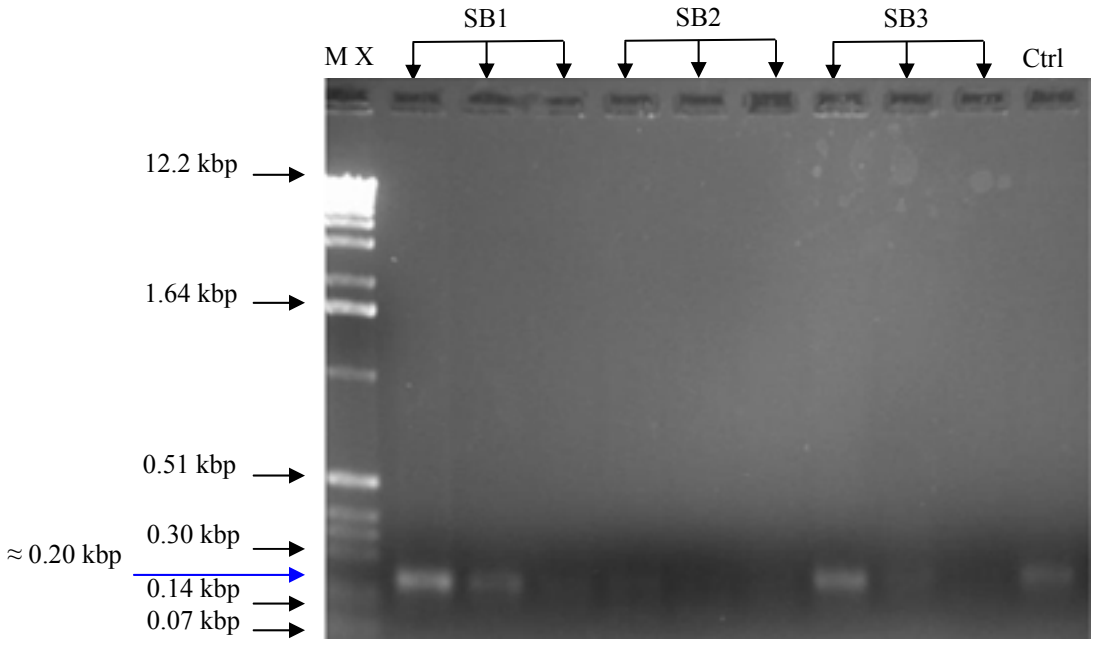

Fig. 8. Agarose gel electrophoresis of PCR fragments products obtained after amplification with W34/W49GC primers pair from bacterial 16S rDNA.

The three samples SB1, SB2 and SB3 correspond to the three different extractions of total DNA from sauerkraut brine. They were amplified with the W34/W49GC primers pair. This allowed the verification of the extraction protocol developed in this study, concerning the extraction of bacterial DNA. As shown on the Fig. 8, only the extraction protocols specific for the extraction of bacterial DNA had a positive result and bands appeared on the gel. The mix protocol for extraction of total bacterial and yeast DNA from sauerkraut brine samples had only one band after the amplification with the primers designed for amplification of bacterial DNA. The three lines of loading for each sample represent three different quantities of DNA used in the PCR reaction. Line 1 for each sample shows the amplification product from PCR reaction with DNA in the quantity obtained ready in the extraction procedure. Line 2 represents the quantity of 10^{-1} of DNA used in the PCR and line 3 corresponded to a dilution of 10^{-2} of DNA. The electrophoresis was performed on 2% agarose gels. The control (Ctrl) was a strain *Ralstonia solanacearum* from the laboratory strain collection.

For the identification of yeast different approaches were used. The first one consisted of two steps - an amplification of a big fragment of 18 S DNA, which contained several variable regions and having length of about 1500 bp, and a subsequent nested amplification of the first PCR products with another primer pair. The second primers pair amplified fragments of about 500 bp lengths. For that approach the primers pair EF4f/ EF3r was used first and subsequently the primers pair NS2f/ Fung5r. The oligonucleotide Fung5r was tested with two different GC clamps. The PCR products obtained through this approach are presented in Fig. 9.

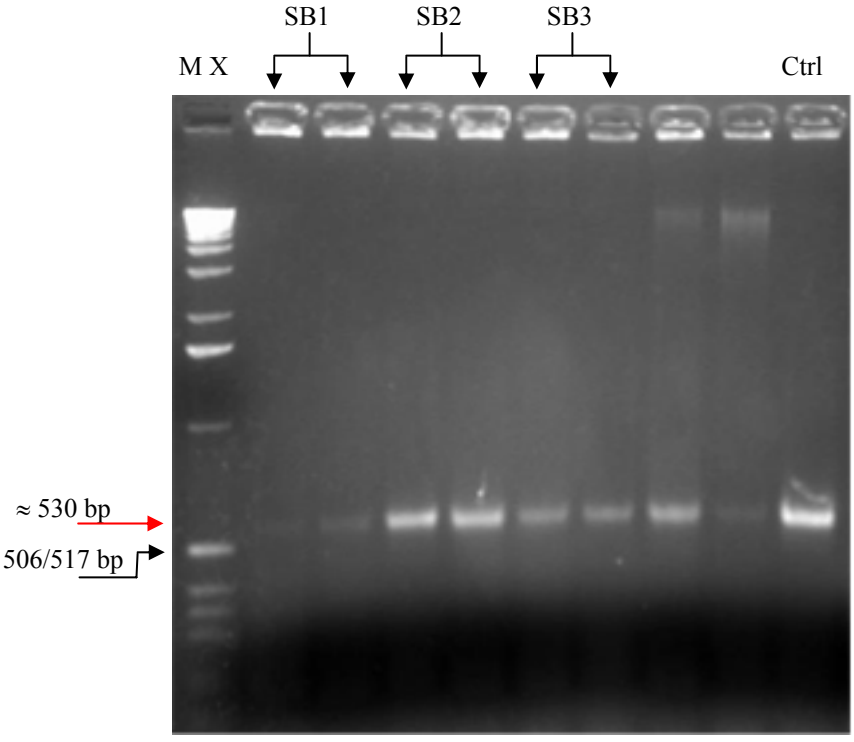

Fig.9. PCR products obtained after amplification of 18S rDNA fragments from yeast for TGGE (primers EF4f/Fung 5rGC)

The DNA obtained using the three types of extraction protocols was also subjected to verification for the specificity of the type of extracted DNA – bacterial or yeasts. This was done using PCR amplification of fragments with primers designed for yeast. The amplicons having the expected fragment length are shown with the red arrow in Fig. 9. The sample SB1 did not have a positive result in the amplification with yeast designed primers pair. This can be seen as an internal control for the specificity of the type of DNA extracted. In contrast with that the samples SB2 (Promega protocol for extraction of yeast DNA) and SB3 (mix protocol from this study) had bands with the expected fragment length on the gels. As in the case of the bacterial primers pair the results were positives for the protocol developed in this study. The separation of the amplified fragments was done on 2 % agarose gels. The control strain used for in the PCR reactions was SSC 228 *Saccharomyces cerevisiae*. Therefore, the mix protocol developed in this study could be used for simultaneous extraction of DNA from samples containing yeast and bacteria.

The second approach used for yeast separation and subsequent identification was the direct amplification of a fragment with 425 amplicons length through the primers pair of FF2/ FR1 (FR1-GC). These amplicons contained the V5 region of 18 S rDNA and the parts of the constituent flanking fragments surrounding it. The PCR products were loaded onto polyacrylamide gels and run in different temperatures and time intervals in order to obtain the best resolution of the fragments and to obtain separate bands for each microorganism. The assays were run separately for the bacterial and yeasts PCR products. In the beginning of the assays for each type of microorganisms the gels were run with a large temperature and time intervals. Using this assay was possible to establish the shorter temperature intervals where more than one microorganism were grouped and thus to reorganize the separating parameters of the technique and to obtain the best possible resolution for each microorganism presented. The initial parameters of assays for bacteria were in the temperature interval between 48 to 68° C. The gels were run for 7 h at 100 V. Subsequently the parameters were changed to two temperature intervals. The first one was from 50 to 54° C and the second one was from 57 to 60° C. The gels were run for 16 hours at 70 V. For each temperature interval, two separate bands were obtained.

The PCR fragments obtained after the amplification of the yeasts DNA were treated in the same way. In Fig. 10 a short range temperature gradient gel - from 40.6° to 43°C is presented.

Running time was 6 h at 100 V. The concentration of urea was 5 M and the concentration of polyacrylamide-13%, for the gel showed in the Fig. 10.

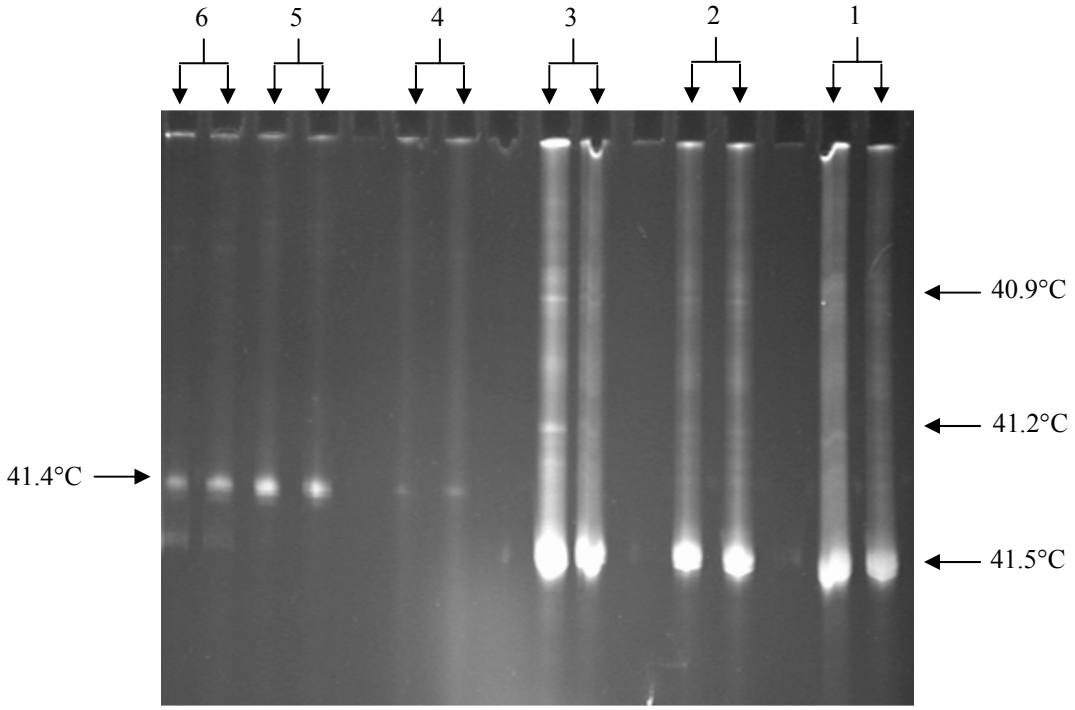

Fig. 10. TTGE of DNA fragments obtained from samples SB1, SB2 and SB3 after PCR amplification with EF4f/Fung 5rGC and FF2/ FR1-GC primers specific for yeasts.

The samples loaded are as follows:1) SB1 fragments obtained with FF2/FR1GC; 2) SB2 fragments obtained with FF2/FR1GC; 3) SB3 fragments obtained with FF2/FR1GC; 4) SB1 fragments obtained with EF4f/Fung5rGC; 5) SB2 fragments obtained with EF4f/Fung5rGC; 6) SB3 fragments obtained with EF4f/Fung5rGC.

The DNA amplified with primers pair FF2/FR1GC (samples 2 and 3) resulted in 3 bands on the gel. This corresponded to the amplification of the DNA obtained through the extraction protocols specific for yeast and the mix protocol. Only one band was obtained after the amplification of DNA with EF4f/Fung5rGC pair (samples 5 and 6), which also corresponded to an amplification of DNA fragments from the same extraction products. The bands were then excised and stored for further purification and reamplification of the fragments.

2. Conclusion

This part of the work led toward the definition of the possible interaction and development of strain *C. arsenoxidans* in an "open" system. The first part of the studies simulated a real "open" system, in which the development of *gfp*-tagged *C. arsenoxidans* strain was observed through epifluorescent images, while the overall number of microorganisms in the same system was evidenced through white light image microscopy. The main point of these experiments was the tracking of *C. arsenoxidans* development, when other microorganisms were presented in the system. Through this assays it was possible to establish the average representation of *C. arsenoxidans* cells, when compared to the global development of random microorganisms.

Two types of sauerkraut brine based medium were selected, because the presence of salts solutions in sauerkraut did not improve the growth of *C. arsenoxidans*, but it may enhance the development of other microorganisms in the system. The SBM-A*BC medium in absence of arsenic was considered less favorable for the growth of the *C. arsenoxidans*, because of the lack of arsenic, which can play the role of suppressor for the development of other microorganisms in the medium. The second type medium-the SBM supplemented with arsenic, was considered as the medium less favorable for the development of other bacterial and yeast strains in the system.

The observations over the development of the random microorganisms and KDM7 cells in the two media used resulted in the finding of flock formation in the SBM-A*BC medium and in the absence of arsenic. This process was not observed in SBM supplemented with 50 mg/l of As[III].

The interest of these assays is the possibility of distinction of an aimed microorganism having special properties as a GFP, or stained with fluorescent dyes, from consortia of microorganisms. Such results cannot be obtained through classical methods in microbiology. The method of image cell counts can be influenced through the quality of the images. Therefore, the most important parameter for the image processing is the quality of the images captured.

The second part of assays aimed at the identification of the microorganisms originated from the sauerkraut brine. These were the preliminary studies, which led toward to the identification of the microorganisms originated from the sauerkraut brine. This information

could help the better application of the sauerkraut brine, after a simple neutralization as a carbon-source supporting the growth of the arsenite-oxidizer *C. arsenoxidans*. During these assays a new protocol for simultaneous extraction of rDNA from bacteria and yeast was tested. This protocol had positive results when specific primers for amplification of bacterial 16 S or yeasts 18 S rDNA fragments, respectively were used. The number of fragments obtained after amplification of DNA with primers having GC-clamp obtained through the protocols described from Promega and the newly developed protocol were equal, when were separated on polyacrylamide gels. The TGGE technique used allowed the separation of bacteria and yeasts and resulted in the finding of 3 bacterial species and 4 yeast species. The final identification of these microorganisms is still to be done.

The overall results showed that the sauerkraut brine can be used successfully as a nutrient medium for the development of *C. arsenoxidans*, only after neutralization even when the system is "open" for randomly introduced microorganisms from the environment. Thus, these preliminary studies resulted in a high level growth performance of the *C. arsenoxidans*, when the strain had to grow in presence of other microorganisms.

Part IV: Method for screening of arsenic-transforming bacteria

1. Introduction

The microplate screening assay was developed as a tool, to facilitate the screening of arsenic transforming bacteria. This method provides easy evaluation of abilities of different bacterial strains possessing oxidation or reduction ability toward arsenic species, prior to the complete chemical analysis of each strain of interest.

All strains used in the development of this method were also subjected to chemical analysis. Bacterial oxidation and reduction of arsenic As[III] and As[V] respectively in the culture supernatants were tested using the HPLC-ICP-AES technique. An alternative application of this method was developed for bacterial colonies plated in Petri dishes. The method was successfully used when As[III] was added into CDM and the oxidation of the arsenic from the arsenate-oxidizing strains were evidenced through a brown-red color formation.

Therefore the limits of the color formation were established using the chemical reactions between either As[III] or As[V] and $AgNO_3$ solution in Tris-HCl or Tris-maleate. The intensity of the color was also established as a function of the As[V] and As[III] content. The final stage of the assays was to standardize the colors of precipitates formed according to the CIE system. Color measurements of the precipitates were made according to the tristimulus coordinate system *CIE L*a*b** scale (Commission Internationale de l'Eclairage, *L*a*b** color system) which expresses any color as three parameters: *L** (lightness), *a** and *b** (chromaticity coordinates). The *L** reference value varies from 0 (black) to 100 (white), *a** values from –60 (green) to +60 (red), *b** values from –60 (blue) to +60 (yellow). The decision for the choice of the *CIE* system was evaluated after screening for different color systems. The *CIE* system is the most abundant and well known color system and also serves as the basis for some recently developed systems for color definition (http://hyperphysics.phy-astr.gsu.edu/hbase/vision/cie.html).

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3. Additional results

Some additional results obtained during the assays for the method establishment, which were not published in the paper are presented. These results are separated in 3 parts:

- Definition of the color formation
- Chromameter measurements of the colors
- Application of the screening assay in Petri dishes

3.1. Definition of color formation.

The reaction between either As[III] and As[V] and $AgNO_3$ solution in Tris-HCl or Tris-maleate resulted in the formation of precipitates, colored from brownish-red to yellow, respectively as a function of their As[V] and As[III] content.

To define the correlation between the intensity of the colors and concentrations of solutions used, 3 different concentrations of $AgNO_3$ and Tris-HCl buffer (pH 7.50) - 0.1 M, 0.2 M, 0.5 M, latter one supplemented with As[III] or As[V] ions, to 50 mg L^{-1} concentration were tested. The $AgNO_3$ solution and Tris-HCl buffer containing the arsenic were mixed volume to volume.

The correlation between the intensity of the colors and concentrations of used solutions are shown on the Figure 1.

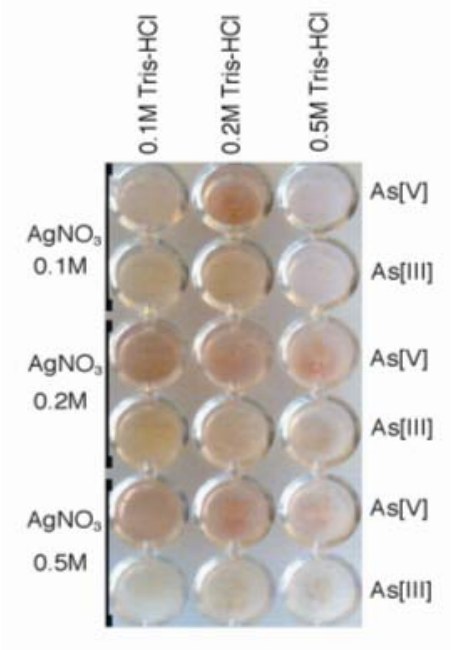

Fig. 1. Influence of different concentrations of $AgNO_3$ and Tris-HCl on the intensity of precipitate color. Initial concentration of As[III] or As[V] ions in each well is 50 mg L^{-1}. (Digital image was processed in PhotoShop 5.0).

The best contrast between the two basic colors of precipitates (a light yellow silver-*ortho*arsenite and a light brown-red silver-*ortho*arsenate) and their intensity was highest when 0.1 M $AgNO_3$ and 0.2 M Tris-HCl were mixed in a volume to volume (100 µl) (Fig. 1). These conditions were used in all subsequent experiments

3.2. Chromameter measurements of the colors.

Color measurements of the precipitates according to the coordinate system *CIE L*a*b** scale which expresses any color as three parameters were done with a CR100 Minolta Chromameter at 60 s after the formation of the precipitate.

The results of color measurement are shown in Figure 2 according to the *CIE L*a*b** system.

The additional measurements of the precipitate colors at 90 s indicated a decrease in color luminosity (e.g. *L**).

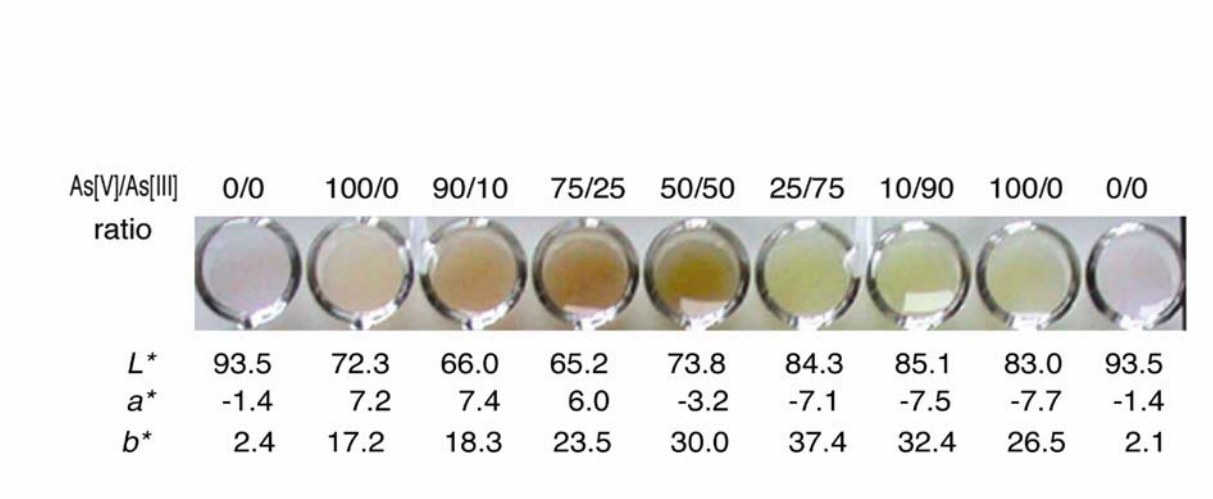

Fig. 2. Determination of precipitate color as a function of As[V]/As[III] ratio (%)and their Chromameter values (*L**, *a**, *b*)*, according to *CIE* system. Per well: 100 µl 0.2 M Tris-HCl buffer, 100 µl 0.1 M $AgNO_3$. Initial concentrations of As - 50 mg L^{-1}. (Digital image was processed in PhotoShop 5.0).

The color corresponding of 100% As[III] (well 8 in the Fig.2) and the color corresponding of 100% of As[V] (well 2 in the Fig.2) had the lowest color intensity when compared to the

white color of the Tris-HCl and $AgNO_3$ precipitate (wells 1 and 9 in Fig.2) and the color corresponding of 50/50 As [III]/As[V] (well 5 in Fig.2) . The explanation of this phenomenon is still to be elucidated.

3.3. Application of the screening assay in Petri dishes

The application of this method in Petri dishes was successful only when it was tested using CDM. After the addition of $AgNO_3$ this medium allowed the color formation of both basic colors-the brownish-red which indicated the oxidation of As[III] into As[V] around the oxidizing colonies and the yellow of non transformed As[III]. The results of this application of the method are presented in Fig. 3.

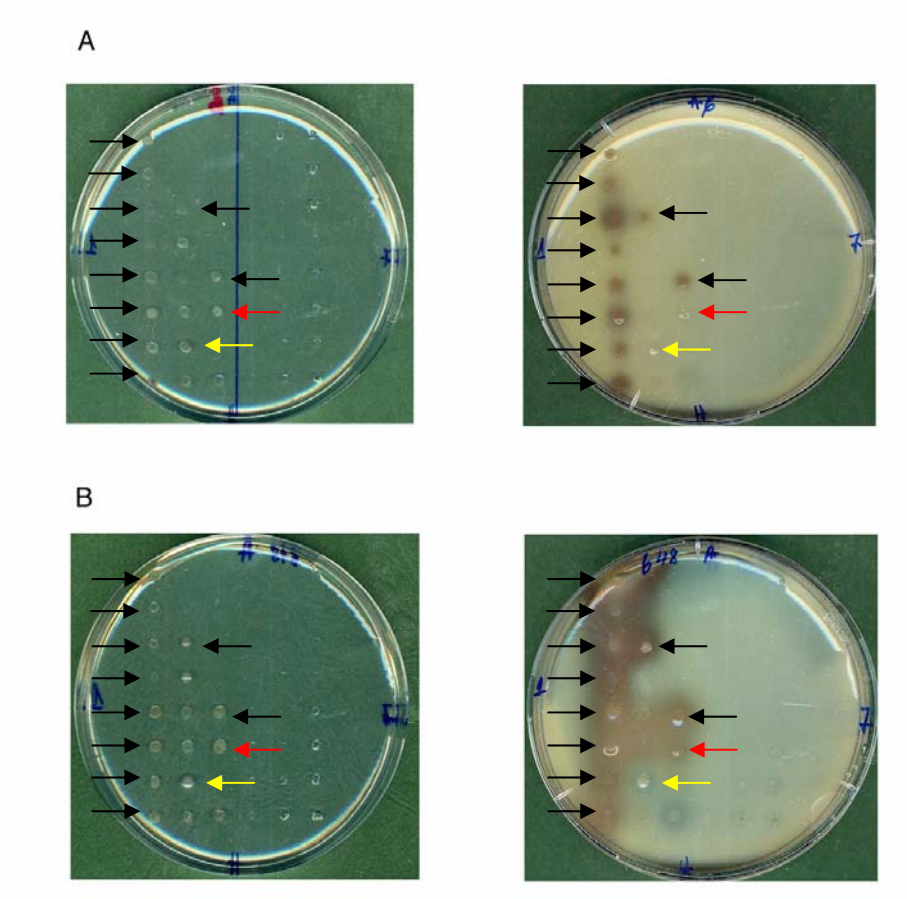

Fig.3. Silver nitrate screening method in CDM agar plates supplemented with 100 ppm of As[III]. A) 24 h cultures B) 48 h cultures.

The bacterial strains shown in Fig. 3 are from the culture collection of arsenite-oxidizing and arsenate-reducing bacteria in the laboratory. On Figure 3 two Petri dishes containing the same strains are presented. The Petri dishes in Fig. 3A were cultivated 24h prior the addition of $AgNO_3$ solution. The Petri dishes on Fig. 3B were cultivated for 48h prior the addition of $AgNO_3$ solution. The black arrows point out the strains which had arsenite – oxidizing ability. As can be seen in Fig. 3 the colonies had developed a brown color after supplementation of $AgNO_3$ solution. These strains showed arsenite – oxidizing ability after 24 h. The red arrow points out a colony, which showed an arsenite – oxidizing ability after 48 h of growth and the arrow in yellow points out a strain, which have no arsenite – oxidation ability, but high resistance toward the As[III]. The CDM was supplemented with 100 ppm of As[III].

4. Conclusion

Best color formation was observed when the following concentrations of silver nitrate and Tris-HCl were used: 1) 0.1 M Tris-HCl and 0.2 M silver nitrate solution, and 2) 0.2 M Tris-HCl and 0.1 M silver nitrate. The lower limits of color formation as a function of pH of the buffer were: pH 5.8 for As[V] and pH 6.0 for As[III], in Tris-maleate buffer. The highest level of color formation tested was at pH 8.4 for both types of arsenic species.

To extend the possibilities of the application of this assay, further studies of color formation in acid environment could be developed. Duquesne, (2004) successfully used the color formation between the silver nitrate and As[III] and As[V] in an acid pH range. This was carried out in the specific nutrient medium, used for the growth of the bacterial strains studied. The reported colors were: gray for the As[III] species and yellow-orange for As[V] species, but still the method has not been standardized in acid pH levels.

Chromameter measurements were done in order to standardize the colors and therefore to diminishing the personal appreciation of the precipitate colors as much as possible. Further development of the assay toward automatic measurements of colors is also possible. In this way the possible misreading of the colors formed, will have less influence upon the final results. Thus, such kind of standardization or automatic measurements of the colors, can be developed in a future studies. Nevertheless, the assay in its present form has the advantages of rapid and easily performed screening for arsenic-transforming bacterial strains.

CHAPTER IV

MATERIALS AND METHODS

1. Bacterial strains

The strains from the bacterial strain collection of the Laboratoire de Microbiologie et de Génétique (group of Professor Lett) and the newly isolated strains of the laboratory of General and Industrial Microbiology (group of Assoc. Professor Groudeva), which were used in this study are presented in Table 1.

Table. 1.

Strain	**Sample**	**Reference**	**Identification (16S rDNA)**
S1, S8, S11-S16	Soil sample 1 (sewage sludge), Salsigne, France	J.-B. Bucher, (1999)	Unidentified strains
S2, S3	idem	Same work	*Pseudomonas* sp. Q37-87
S4-S6, S9, S10	idem	Same work	*P. stutzeri*
S7	idem	Same work	*Acinetobacter calcoaceticus*
S73,S83	idem	Delbecque	Unidentified strains
S82	idem	Same work	*P. stutzeri*
S84	idem	Same work	*Pseudomonas* sp. 5A
S17-S19	Soil sample 2 (gypsum, containing arsenic), Salsigne, France	J.-B. Bucher, 1999	*P. stutzeri*
S20	idem	Same work	Unidentified strain
S21	Soil sample 3 (Calcium oxide), Salsigne, France	Same work	Unidentified strain
S22	idem	Same work	*R. solanacearum*
S76	idem	A. Delbecque	Unidentified strain
S24	Soil sample 4 (Champ Magné), Salsigne, France	J.-B. Bucher, 1999	*P. fluorescens*
S25	idem	Same work	*P. corrugata*

Strain	Sample	Reference	Identification (16S rDNA)
S26	idem	Same work	*Pseudomonas* sp.
S27	idem	Same work	*P. stutzeri*
S28, S33	idem	Same work	*Acinetobacter calcoaceticus*
S31	idem	Same work	*Nocardioides simplex*
S77-S79, S87	idem	A. Delbecque	*P. stutzeri*
S88	idem	Same work	Unidentified strain
S34	Soil sample 5 (concentrated agglomerations), Salsigne, France	J.-B. Bucher, 1999	*P. tolaasii*
S35	idem	Same work	*Pseudomonas* sp
S36	Soil sample 6 (soil under monitoring), Salsigne, France	Same work	*P. stutzeri*
S38	idem	Same work	*Pseudomonas* sp.
S39	idem	Same work	*P. corrugata*
S40-S43	idem	Same work	Unidentified strains
S44	idem	Same work	*Pseudomonas* sp. Q37-87
S80	idem	A. Delbecque	*P. fluorescens*
S81, S89	idem	Same work	Unidentified strain
S47, S48	Soil sample 7 (pile of wastes, left from cyanic treatment), Salsigne, France	J.-B. Bucher, 1999	Unidentified strains
S55, S56 S58-S60	Soil sample 8 (soil from Rouyre), Salsigne, France	Same work	Unidentified strains
S57	idem	Same work	*Acinetobacter calcoaceticus*
S64	Soil sample 9 (Château le Caunette soil)	Same work	*Pseudomonas* sp. Q37-87

Strain	Sample	Reference	Identification (16S rDNA)
SM1 SM2	Soil sample As75 from mining site Astano, Swiss.	This study	*Ralstonia eutropha* kT1
SM3, SM4	idem	This study	*Ralstonia eutropha*
SM5	Soil sample As81, from mining site Astano, Swiss	This study	*P. putida*
SM7	idem	This study	*P. orysihabitans*
SM9	idem	This study	*Comamonas testosteroni*
SM11	idem	This study	*P. stutzeri*
SM12 SM23	idem	This study	*Alcaligenes* sp.
SM22	Soil sample (place ”C”) from a desolated agricultural site, next to the smelt factory KZM Plovdiv area, Bulgaria	This study	*Stenotrophomonas maltophilia*
SM26	Soil sample (place “A”) at 10cm distance from the iron fence of KZM Plovdiv factory, Bulgaria	This study	*P. putida*
SM27	Soil sample (place “B”), next to the canal for evacuation of wastewater out off KZM Plovdiv, Bulgaria	This study	*P. fluorescens*
ULPAs1	Sample of water purification plant in Germany	Weeger, 1999	*Cenibacterium arsenoxidans*

Table.1. Name and origin of bacterial strains used.

2. Plasmids

All the three plasmids used for the transformation of ULPAs1 were from the type R6K (suicidal).These plasmids were gfp-tagged ones.

mTn*5gusA-pgfp21* - from Faculty of Agricultural and Applied Biological Sciences, Katholieke Universitait Leuven, Belgium (Xi *et al*., 1999)

mTn*5gfp-pgusA* – kindly supplied by the author from Faculty of Agricultural and Applied Biological Sciences, Katholieke Universitait Leuven, Belgium (*Xi et al*., 1999)

mTn*5gfp-km*- kindly supplied by the author (Tang *et al*., 1999)

3. Nutrient Media

3.1. Chemically defined medium (CDM)

Stock solutions

Solution A :	Solution B :	Solution C :
Na-lactate: 50 g	$FeSO_4$: 160 mg	$NaHCO_3$: 4 g
$MgSO_4$. 7H2O : 20 g	100 mL deionazed H_2O	50 mL deionazed H_2O.
NH_4Cl : 10 g		
Na_2SO_4 :10 g		
K_2HPO_4 : 100 mg		
$CaCl_2$. $2H_2O$: 672 mg		

Solution A - sterilized by autoclave for 15 min at 120°C;

Solutions B and C - sterilized by filtration through membrane filter of 0,22 μm pore-size, Nalgène.

Preparation of the medium: 1 liter of the medium was prepared from

100 mL of Solution A;

2,5 mL of Solution B;

10 mL of Solution C

Made up to 1L with sterile double deionazed H_2O. Final pH of this medium - 7.2

3.2. Solid CDM

The preparation of the solid CDM was the same, as CDM broth except that the necessary quantity of agar to obtain a final concentration of 20 $g.L^{-1}$, was added to Solution 1 before autoclaving.

3.3. Sauerkraut brine medium (SBM)

The sauerkraut brine was kindly supplied by the producer Coopchou, Vendenheim (France) and had a natural pH of 3.5 - 3.7.

The preliminary treatment of sauerkraut brine for the preparation of nutrient media was, as follows:

filtration through a paper filter, centrifugation at 6000 rpm for 20 min, heating twice at 90°C in a water bath - for 15 min and allowed to settle for 24h at 4°C. A second filtration through a paper filter, dilution 1:5 (vol./vol.) with sterile ddH_2O and final filtration through 0.20µm pore-size membrane (Nalgene, France), were performed.

The Sauerkraut brine medium was prepared in two variants:

- with salts solutions from CDM, except Na-lactate.
- without salts solutions from CDM

The final pH of SBM was adjusted to 7.2, with 1 M NaOH.

3.4. Whey medium (WM)

The whey based medium used commercially available products from the french cheese industry (fromage blanc Rians and Auchan). The initial whey pH was 4.0 - 4.3.
The preliminary treatment of the whey for the preparation of nutrition medium followed the protocol described below:
Filtration through paper filter, dilution with double deionised water 1:1,5 (vol./vol.), neutralization with 1M NaOH up to pH 7.2, a second filtration through paper filter, followed by sterile filtration through 0.20μm pore-size membrane.
The whey medium was prepared in two variants:

- with salts solutions from CDM, except Na-lactate.
- without salts solutions from CDM

3.5. Molasses medium

The molasses concentrate was supplied by Les Sucreries et Raffineries d'Erstein, France (http://www.sucre-erstein.com/cadres/presentation.html). The used medium contained the same salts solutions as CDM (in the same concentrations), except that molasses replaced the Na-lactate as a sole carbon source.
A stock solution of molasses with 20g.L^{-1} initial concentration was prepared and sterilized through filtration. The final medium was prepared by the addition of the molasses into the salt solution.

3.6. Nutrient Medium "DV"

This medium is the Desulfovibrio medium described in Handbook of Microbilogical Media by Ronald M. Atlas (1997).

3.7.Nutrient Medium “Dc&Ds”

This medium is based on the DSMZ media 197 (http:// dsmz.de/media/med197.htm) and 198 (http:// dsmz.de/media/med198.htm). The preparation and the use of the medium was under aerobic conditions.

3.8. Nutrient Medium “Dtm”

This medium is based on the DSMZ medium 682, which consisted of DSMZ medium 197, with lowered concentration of sodium sulfate to 0,7 g/l and “seven vitamins solutions” of 503 DSMZ medium. In “Dtm” nutrient medium the 3,4,5-trimetoxabenzoate as a carbon source in medium 682, was replaced with Na-lactate (2 g/l). The preparation of the nutrient medium was under aerobic conditions (http:// dsmz.de/media/med682.htm).

3.9. Nutrient Medium “DvB”

Solutions

Solution A:	Solution B:	Solution D :
Na-lactate: 3.5 g	Na-thioglicolate: 0.1 g	$FeSO_4.7H_2O$
$MgSO_4$, $7H_2O$: 2.0 g	10 ml ddH_2O	10 ml ddH_2O
NH_4Cl: 1.0 g		
KH_2PO_4: 0.5 g	Solution C:	
$CaSO_4$: 1.0 g	Ascorbic acid: 0.1 g	
Yeast extract: 1.0 g	10 ml ddH_2O	
970 ml ddH_2O		

The medium is based on the DSMZ medium 63, which was slightly modified.

Preparation of the medium: All solutions were autoclaved at 121° C for 15 min. Solutions B, C and D were added into solution A, afterward. The pH 7.5 of the medium was adjusted with NaOH. During the preparation, the medium was shaken continuously to keep the formed gray precipitate suspended (http:// dsmz.de/media/med063.htm).

3.10. Nutrient Medium "BDtm"

Stock solutions

Solution A :	Solution B :
Na-lactate: 35 g	$(NH_4)Fe(SO_4)2.6H_2O$: 5 g
$MgSO_4$, $7H_2O$: 20 g	100 mL deionazed H_2O
NH_4Cl : 10 g	
KH_2PO_4 : 5 g	
$CaSO_4.2H_2O$: 1 g	

Solution A, was prepared in 1L. Both solutions were sterilized by autoclave at 121°C for 20 min.

Preparation of the medium: 1 liter of the medium was prepared from

100 mL of Solution A;

10 mL of Solution B;

Made up to 1L with sterile double deionazed H_2O.

4. Molecular biology techniques

4.1. Extraction of total DNA

The extractions of genomic bacterial DNA from pure cultures were done according to the protocol of Promega, with the kit supplied by the producer Promega Corporation, USA.

The extractions of genomic DNA from yeast were according to the protocol of Promega. The enzyme lyticase was substituted with the enzyme zymolase with concentration of 5 mg/ml.

A protocol for simultaneous extraction of bacterial and yeast genomic DNA, based on the Promega's protocols, was developed and tested. In this protocol the enzymes lysosim with 10mg/ml concentration and zymolase with concentration of 5 mg/ml were used. The first incubation at 37°C was fixed at 30min.

4.2.Amplification of DNA fragments through PCR

Table. 2. Oligonucleotides used in this study, with primers binding sites and targeted genes.

Name of the oligonucleotide	5' → 3' sequence	Targeted gene	Reference
fD1	AGAGTTTGATCCTGGCTCAG	16S rDNA (*E. coli* 8-23)	Devereux and Willis, 1995
rD1	AAGGAGGTGATCCAGCC	16s rDNA (*E. coli* 1541-1525)	Devereux and Willis, 1995
W34	TTACCGCGGCTGCTGGCAC	16S rDNA, V3 region (*E. coli* 533-520)	Cho and Kim, 2000
W49	ACGGTCCAGACTCCTACGGG	16S rDNA, V3 region (*E. coli* 330-348)	Cho and Kim, 2000
W49-GC	cgcccgccgcgccccgcgcccggcccgccgcccccgccc cACGGTCCAGACTCCTACGGG	16S rDNA, V3 region (*E. coli* 330-348)	Cho and Kim, 2000
FF2	GGTTCTATTTTGTTGGTTTCTA	18S rDNA, V5 region	Zhou *et al*., 2000
FR1	CTCTCAATCTGTCAATCCTTATT	18S rDNA, V5 region (1664-1649)	Zhou *et al*., 2000
FR1-GC	cgcccgccgcgcgcggcgggcggggcgggggcacggg gggCTCTCAATCTGTCAATCCTTATT	18S rDNA, V5 region (1664-1649)	Zhou *et al*., 2000
EF4f	GGAAGGG(G/A)TGTATTTATTA	18S rDNA (195-214)	Smith, 1999; van Elsas, 2000
EF3r	TCCTCTAAATGACCAGTTTG	18S rDNA (1747-1727)	Smith, 1999 van Elsas, 2000
NS2f	GGCTGCTGGCACCAGAACTTGC	18S rDNA (578-557)	Smith, 1999 van Elsas, 2000
Fung5r	GTAAAGTCCTGGTTCCC	18S rDNA (747-729)	Smith, 1999 van Elsas, 2000
Fung5r-GC	cgcccgccgcgcgcggcgggcggggcgggggcacggg gggGTAAAAGTCCTGGTTCCC	18S rDNA (747-729)	Smith, 1999 van Elsas, 2000
Fung5r-GC/DL	cgcccgccgcgccccgcgcccggcccgccgcccccgccc cGTAAAAGTCCTGGTTCCC	18S rDNA (747-729)	Smith, 1999 van Elsas, 2000

PCR reaction mixtures for amplification of bacterial DNA fragments and the program used are shown in table 3. The annealing temperature was changed according to the different pairs

of used primers. The extension time was in function of the length of the amplified fragment as was the number of cycles. The GC-clamps used in this study are presented in table 2 in small characters, attached prior to the primer.

Table.3.

Each rection contained:		The PCR thermal cycling scheme		
PCR buffer without $MgCl_2$ (x 10):	2,45µl	Initial denaturation:		95°C/1’
$MgCl_2$ (25 mM):	1,53 µl		Denaturation:	94°C/40’’
dNTPs (20 mM):	0,30 µl	39 cycles	Annealing:	55°C/40’’
Taq Polymerase (5 U/µl):	0,10 µl		Extension:	72°C/1’ 50’’
ddH_2O:	19,62 µl	Final extension:		72°C/15’
Primer (F/R) (20µM):	0,5 µl			
DNA matrix (40ng/µl):	0,5 µl			
Final volume:	25µl			

The PCR reaction mixtures for amplification of fungal DNA fragments and the program used are shown in table 4.

Table.4.

Each rection contained:		The PCR thermal cycling scheme		
PCR buffer without $MgCl_2$ (x 10):	5,00µl	Initial denaturation:		95°C/1’
$MgCl_2$ (25 mM):	5,00 µl		Denaturation:	94°C/1’
dNTPs (20 mM):	0,50 µl	36 cycles	Annealing:	48°C/1’
Taq Polymerase (5 U/µl):	0,20 µl		Extension:	72°C/2’
ddH_2O:	33,30 µl	Final extension:		72°C/10’
Primer (F/R) (20µM):	2,00 µl			
DNA matrix (40ng/µl):	2,00 µl			
Final volume:	50µl			

4.3. Purification of DNA fragments for sequencing

Each PCR-amplified fragment of 16S bacterial or 18S fungal rDNA was purified prior to sequencing, according to the protocol of Amersham for the MicroSpin^{TM} Columns (Amersham Pharmacia Biotech,Inc., USA).

4.4. Temperature gradient gel electrophoresis (TGGE)

This technique is used for the separation of DNA fragments of the same length, but with different sequences. The separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA fragment in polyacrylamide gels with a linear temperature gradient. The melting of DNA proceeds in "melting domains", which are the stretches of base pairs with an identical melting temperature (*Tm*). When a domain with a lowest melting temperature, reaches its melting temperature at a particular position in the temperature gradient gel, a transition of a helical to a partially melted molecule occurs and the migration of this molecule stops. The sequence variations in "melting domains" cause the differentiation between the final positions of the fragments. This method can separate DNA fragments up to 500 bp. The percentage of the fragments separation is nearly 100%, due to the attachment of a GC rich sequence (GC-clamp) to one of the sides of the DNA fragment. This GC-clamp prevents the two DNA strands from complete dissociation and its length can vary between 30 and 50 nucleotides. The GC-clamps used in this study are presented in table 2 in small characters.

Each PCR-amplified fragment (10µl) was mixed with 3 µl of loading buffer (0,05g l^{-3} bromophenol blue, 0,05g l^{-3} xylene cyanol, 4 ml of 100% glycerol, diluted with ddH_2O to final volume of 10ml) and loaded onto the gel. The polyacrylamide gels (200 x 160 x 1 mm^3) contained 8 to 13% of acrylamide/bis, 5 to 7 M urea, 1.25 x TAE, 0.1% TEMED, 0.30ml of ammonium persulfate of 10% solution.

TGGE were performed at 60, 70, 90 and 100V for 6 and 14 h in BioRad apparatus (Bio-Rad, Richmond,CA, USA). The range of tested temperatures gradients varied from 41,0°C to 65°C in total and was in function of the urea quantity and voltage.

5. Biochemical techniques

Measurements of lactic acid isomers through the method of Gutmann

To assay the isomers of lactic acid in the SBM, WM and CDM a 0.1 ml portion of each media supernatant was taken for the determination of D-(-)- and L-(+)-lactic acid by using D- lactate dehydrogenase and L-lactate dehydrogenase (Boehringer Mannhaim), (Gutmann and Wahlefeld, 1974)

. The 0.1 ml portion of supernatant was mixed with a solution containing 0.3 ml of Glycine-Hydrazine buffer (pH 9.2), 0.6 ml of ddH_2O, 1.5 mg of NAD and 7.5 µl of L- or D-dehydrogenase. The prepared samples were incubated for 10 min at ambient temperature (22-25°C) and then were measured at λ_{340} with a Uvikon 930 spectrophotometer (Kontron Instruments). In the control (blank) the supernatant from the samples was replaced with ddH_2O. The quantities of both forms were determined prior to inoculation with *C. arsenoxidans* of the three media and after 72 h of growth of the strain.

6. Microscopy techniques

Principles of Luminescence (Fluorescence and Phosphorescence)

The fluorescence is a form of luminescence, which is a consequence of the absorption of a photon from molecules as flurophores, fluorochromes, or a fluorescent clamp. This reaction is a part of the luminescence reactions assembly, which consist of: photoluminescence (fluorescence and phosphorescence) and other types of luminescence (chimiluminescence, bioluminescence, thermoluminescence, electroluminescence, etc.). The photoluminescence is based on the excitation and light emission due to the photon energy. The essential differentiation between the different types of luminescence is the origin of the excitation energy used.

The fluorescence is characterized as emission of photons due to the absorbency of light. The emission is due to the transformation of one molecule (at electron level) from excited level (S_1) back to its normal energy level (S_0).

The phosphorescence is the emission of light after the end of light excitation of a given molecule. This reaction differs from the fluorescence by the way of lowering the excitation

energy. In this case the average life of the molecule in the S_1 energy level is much longer, when compared to the fluorescence.

A preliminary selection of all 576 gfp mutants, from the three conjugations of *C. arsenoxidans* and the donor strains of *E.coli* S17 λ*pir* (containing one of the 3 different plasmids used), was performed on a Synergy HT (Bio-Tek). The software used was KC4 supplied with the apparatus from Bio-Tek.

The tracking of *gfp* mutant of the strain *C. arsenoxidans* named KDM7, were performed in a nonsterile sauerkraut brine medium in presence and absence of As[III] with concentration of 50 ppm.

A quantity of 600 µl of each cell suspension was powered in a well and the cells were excited with UV light for 20 sec. The image acquisition was performed with a Nikon 2000 Eclipse epifluorescence microscope, equipped with a CCD camera by which gray scale images were captured. Quantification of the digitized fluorescent signals from KDM7 cells, was accomplished through the image analysis software package ImageJ (http://rsb.info.nih.gov/ij/). The count of KDM7 cells emission signals was treated separately from the images captured with white light. Thus, to obtain the results from both types of images two different protocols were developed and used.

The fluorescent images were transferred from 16 to 8 bit gray scale images and the threshold to remove any remaining background noise was established manually. Then the custom designed plugin "analyze particles" was used, by which the following parameters were established and measured:

- the numbers of particles for each image
- the area of each particle measured;
- the mean gray value;
- the standard deviation of mean gray value;
- mode
- Min and max gay value for each particle.

The function "area" resulted in the definition of the max size of KDM7 particles. The "mode" feature gave the intensity of light emission of most represented emitting point and their standard deviation. Thus the percentage of KDM7 cells was found out from the whole quantity of fluorescent cell captured in the images.

Second protocol for object count was developed concerning the images captured with white light. These image processing's were assayed in order to perform a statistically valid method

for count of objects with different shapes and meaning in the gray scale. The following protocol was used:

- all images were transferred into 8 bit resolution
- the threshold was adjusted manually, which allows separating particles with gray-scale pixels below or above a fixed level. Additionally the remaining pixels were not fixed to the background of the picture.
- a second binarisation and adjustment of the pictures threshold were done
- analyses were done automatically, using the process ”analyze particles”

The results obtained, represented the overall quantity of cell per image, including those possessing the fluorescence and those which were not fluorescent.

7. Immobilization of bacteria

For the immobilization of the *C. arsenoxidans* cells in Na-alginate beads 0.3g of Sodium alginate was dissolved in 15 ml of ddH_2O. A second solution of 0.5g of $CaCl_2.2H_2O$ in 75 ml of ddH_2O, was prepared and a 72h of precultured *C. arsenoxidans* cells in a CDM were used. Another preculture from *C. arsenoxidans* cells induced with As[III] were prepared prior to the encapsulation in the alginate beads. The induced cells were prepared in sauerkraut brine medium, supplemented with salts solutions and 10ppm of As[III]. The cell suspensions with an optical density of 0.3-0.4 at 600 nm were used for immobilization and the final concentration of the alginate was 3-5%. The 200ml of the preculture were centrifuged at 5000rpm, washed twice and finally suspended in 20ml of sterile ddH_2O. For the entrapment of the cells 3 ml of the previously prepared cell culture was added and mixed into the Na-alginate solution, which was sterilized prior to use at 121°C for 15 min.

The beads formation followed the protocol:

The Na-alginate cell suspension was passed dropwise into the $CaCl_2.2H_2O$ solution through a needle with 0.3 mm diameter; the receiver solution was shaken constantly with the forming beads in it; drops were formed at 10cm distance above the receiver; after formation the beads were left for 2 to 3h in the $CaCl_2.2H_2O$ and washed subsequently with a fresh 0.2 M $CaCl_2.2H_2O$ solution.

8. Chemical analysis (HPLC-ICP-AES)

Arsenic compounds were eluted with a phosphate buffer (7.2 < pH < 7.4) composed of an mixture of equal volumes of the two following solutions: Solution 1 was prepared by adding 10 mM sodium monohydrogenphosphate ($Na_2HPO_4.12H_2O$) solution into 10 mM sodium dihydrogenphosphate ($NaH_2PO_4.2H_2O$) solution up to pH 6.3. Solution 2 was 0.1M $Na_2HPO_4.12\ H_2O$ (pH 7.8).

Concentrations of As[V] and As[III] in culture supernatants were determined by HPLC-ICP-AES using an anion exchange polymeric column (PRP-X100, 250 mm X 4.1 mm i.d., particle size 10 µm, Hamilton). Arsenic compounds were eluted at 1 ml min^{-1} in phosphate buffer, degassed and filtered on a 0.65 µm cellulose acetate membrane (Millipore). Detection was at 193.7 nm wavelength using an ICP–AES apparatus (JY138 ULTRACE, Jobin Yvon), coupled to an HPLC pump (LC – 10 AT, Schimadzu) via a Teflon tube. The ICP-spectrometer was calibrated before each use and again after every 10 samples by running a blank and samples of known concentrations. Detection limits were 0.11 mg L^{-1} and 0.14 mg L^{-1} for As[III] and As[V], respectively. Quantification limits were 0.47 mg L^{-1} and 0.36 mg L^{-1} for As[III] and As[V], respectively.

9. Physical and chemical parameters of the nutrient media

Measurements of the main physico-chemical parameters of SBM and WM were made in the Centre d'Analyse et de Recherche (CAR, Strasbourg, France). The methods used were carried out as the Norme Française Technique (NFT) and are as follows:

Chemical oxygen demand (COD) (NFT 90 - 101),

Biological oxygen demand in 5 days (BOD_5) (NFT 90 - 103),

Ammonium azote (NFT 90 - 015),

Kjeldahl azote NTK (NFT 90 - 110),

Nitrates (NFT 90 – 012)

Nitrites (NFT 90 - 013).

CHAPTER V

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CHAPTER VI

APPENDIX