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Sulfide removal and elemental sulfur recycling from a sulfide-polluted medium by *Allochromatium vinosum* strain 21D

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Summary. Phototrophic purple sulfur bacteria oxidize sulfide to elemental sulfur, which is stored as intracellular sulfur globules. The mutant *Allochromatium vinosum* strain 21D, containing an inactivated *dsrB* gene, is unable to further oxidize intracellularly stored sulfur to sulfate. This mutant was used as a biocatalyst in a biotechnological process to eliminate sulfide from synthetic wastewater and to recycle elemental sulfur as a raw material. For this purpose, the mutant was grown in an illuminated 5-liter bioreactor (30 μE/m^2/s PAR) at 30°C for 61 days in anoxic phototrophic medium. The process of sulfide removal was semi-continuous and consisted of three consecutive fed-batch sections. Sulfide was repeatedly added into the bioreactor and oxidized by the cells to sulfur. In the presence of the mutant, no unwanted sulfate was produced during sulfide removal. A maximum sulfide removal rate of 49.3 μM/h, a maximum sulfide removal efficiency of 98.7%, and 60.4% sulfur recycling were achieved. [**Int Microbiol** 2006; 9(4):253-258]

Key words: *Allochromatium vinosum* · biological sulfide removal · biological sulfur production

Introduction

Anthropogenic sulfide contamination occurs during the exploitation of resources containing hydrogen sulfide (H_2S), e.g., natural gas and crude oil; the use of H_2S in production processes, such as by tanneries; or when H_2S is an unwanted reaction product, as occurs in biogas production [14]. Sulfide is a corrosive compound with an unpleasant odor and is toxic even at very low concentrations [29]. The maximum acceptable concentration is 10 mg/m^3 [31]. Environmental protection regulations stipulate that industries are not allowed to emit sulfide in exhaust gas or wastewater. Thus, processes that eliminate sulfide from industrial waste streams are clearly necessary.

Anthropogenic sulfide can be removed from wastewaters by several different strategies. Currently, physicochemical sulfide removal technologies dominate in industry. These processes involve either air stripping or the use of various oxidizing agents, such as air, oxygen, chlorine, hypochlorite, or chlorine dioxide [30]. The most commonly used methods are further developments of the Claus process, which was invented in 1880 [8]. Many technologies are based on this process, and the general approaches can be divided into two groups: (1) processes in which 99% of the sulfide is removed, e.g., Amoco cold-bed adsorption (CBA) [7] and SNPA/Lurgi Sulfreen processes, and (2) those in which 99.9% is removed, e.g., the Shell Claus-off-gas treating (SCOT) process [24] and the Beavon- and Wellman-Lord-

processes. All of these processes are well-suited for converting natural gas with high sulfide concentrations to sulfur in continuous or discontinuous operations. As an example, Exxon Mobile produces 800,000 tons elemental sulfur/year from natural sour gas in its facilities in northern Germany [ExxonMobil (2006) Natural gas conditioning. ExxonMobil Productions Deutschland GmbH, Unternehmenskommunikation. ExxonMobil, Hannover, Germany]. However, physicochemical removal processes have several disadvantages, including high energy requirements, high chemical costs, the production of toxic products or by-products, and considerable disposal costs [2,29]. The advantages of using biotechnological sulfide removal as an alternative to physicochemical methods are its lower intrinsic capital and operating costs. Biotechnological processes generally operates at moderate temperatures and at atmospheric conditions. Furthermore, sulfide is oxidized in a single-step reaction, catalyst regeneration is automatic, and there is no production of waste [1,3].

Biological methods to oxidize sulfide to sulfur apply either aerobic chemotrophic bacteria, such as *Thiobacillus* [2,15,16,22] and heterotrophic *Xanthomonas* [5], or anaerobic phototrophic bacteria, i.e., green and purple sulfur bacteria [11]. The latter use reduced sulfur compounds as electron donors to carry out anoxygenic photosynthesis. In the case of sulfide, elemental sulfur is produced as an intermediate and sulfate as the final oxidation product [13,24]. However, it is preferable to obtain sulfur as a raw material, since sulfate requires further elimination treatment [2]. The benefit of approaches involving anaerobic phototrophic bacteria rather than aerobic chemotrophic ones (e.g., thiobacilli) is the reduction of process costs because there is no need for aeration [17]. Research on biological sulfide removal by phototrophic bacteria has involved the use of either a consortium of phototrophic bacteria [11,18,19] or green sulfur bacteria [6,8–10].

In the present work, strain 21D, a mutant of the purple sulfur bacterium *Allochromatium vinosum*, was used as a biocatalyst to convert sulfide into elemental sulfur, which was stored as intracellular sulfur globules. Due to inactivation of its *dsrB* gene, this mutant lacks the capability to further oxidize intracellularly stored sulfur, such that sulfate is not produced [25]. Regarding sulfide removal, purple sulfur bacteria offer an advantage over green sulfur bacteria in that the latter produce extracellular sulfur, which covers the inner glass walls of the photobioreactor thereby limiting light input and the sulfur yield [Jennemann GE, Bartlesville O, Geferetz D (1997) Sulfid-oxidierende Bakterien und damit durchgeführte Verfahren. Offenlegungsschrift DE 19627180 A1 Deutsches Patentamt; Borkenstein and Fischer, unpublished].

Although wild-type *A. vinosum* naturally occurs in wastewater treatment plants [20], to our knowledge neither a pure culture of a purple sulfur bacterium nor a genetically modified sulfur bacterium has been applied in wastewater treatment technology [13]. Recently, the use of genetically modified organisms for bioremediation has been critically discussed [4]. However, the unique advantage of mutant strain 21D for sulfide removal is that its intracellularly stored sulfur cannot be further oxidized to unwanted sulfate. Therefore, no further process control is required to prevent sulfate production during sulfide removal. Wastewater is a non-sterile medium, and the flow rates of untreated wastewater are too high to allow affordable sterilization [12]. However, in our experiments using sterile synthetic wastewater a pure culture of *A. vinosum* strain 21D was maintained throughout the entire treatment process. These conditions provided information about the efficiency of the system on a laboratory scale and the possibility to later apply this process on a larger scale using real wastewater.

Materials and methods

Medium and culture conditions. *Allochromatium vinosum* strain 21D [25] was kindly provided by Dr. C. Dahl, University of Bonn, Germany. The organism was cultivated in anoxic phototrophic medium as described by Imhoff [13]. The medium was supplemented with malate (10 mM) and acetate (10 mM) to obtain higher cell yields and contained kanamycin (10 μg/ml) and streptomycin (50 μg/ml).

Reactor setup. A 5-liter photobioreactor (Biostat B, B. Braun International, Melsungen, Germany) and a fresh-medium reservoir were filled with the medium described above. The reactor lid was equipped with a stirrer and contained ports to insert probes for measuring temperature, oxygen, and pH. The reactor lid also contained inlets and outlets for gases, fresh medium, Na_2S, sampling, and pH adjustment using NaOH or HCl. Further details are shown in Fig. 1A. Temperature (30°C), pH (6.9), oxygen (0), and agitation (50 rpm) were computer-controlled. Three neon tubes (Osram L36W/30 warm light) were placed in an upright position equidistantly around the bioreactor at an angle of 120° for constant illumination (Fig. 1B). This arrangement yielded a photon irradiance on the surface of the outer glass wall of 30 $\mu E/m^2/s$ PAR, as measured by a light meter (Quantitherm QRT1, Hansatech Instruments, Kings Lynn, England).

Process conditions. The sulfide removal bioprocess was semi-continuous and consisted of three successive fed-batch sections. Each of the sections was initiated by photoorganoheterotrophic growth with malate and acetate to achieve higher cell yields. Sulfide was consecutively added three times. After each sulfide addition, the culture grew photolithoheterotrophically with malate/acetate and sulfide. Each fed-batch section was terminated by a dilution. This was done by using a laboratory pump to remove 2 l of culture medium from the reactor into the settler and then replace it with 2 l of medium from the fresh-medium reservoir (Fig. 1A). After a residence time of 7 days within the settler, the cell-free supernatant was pumped into the waste container. The sedimented cells, which contained stored elemental sulfur, were dried at 80°C for 2 days to evaporate the water content and destroy the remaining living cells. The dried material was used to determine the amount of elemental sulfur produced.

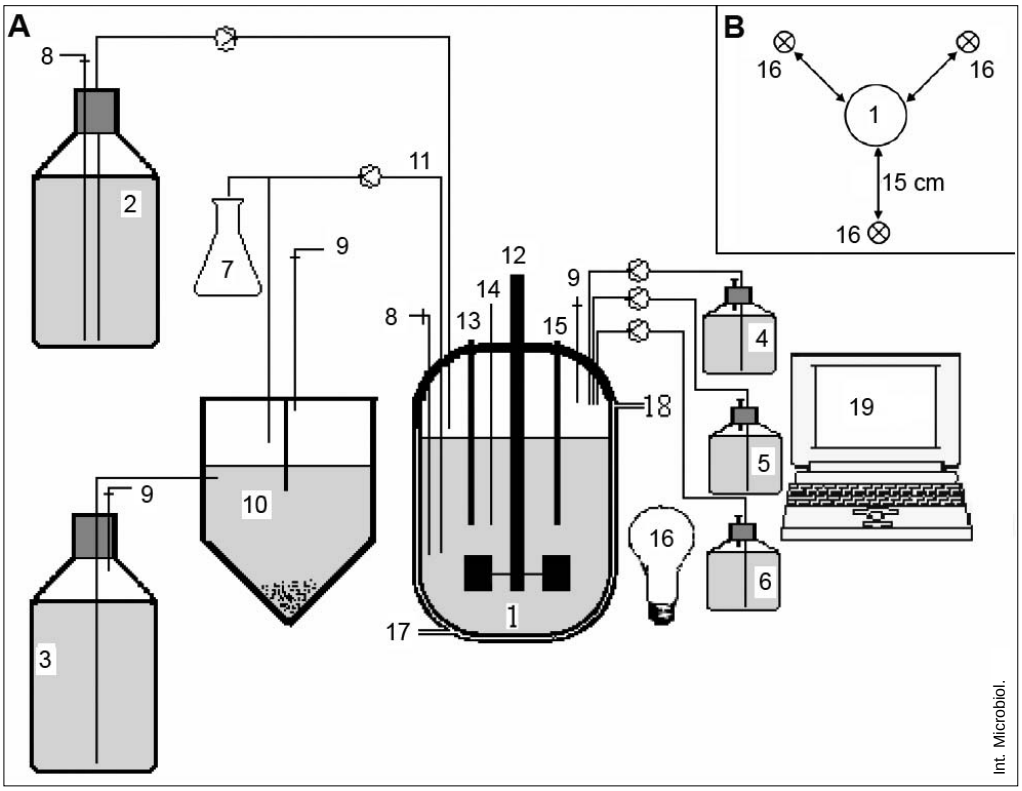

Fig. 1. Schemes of the biotechnological device used to convert sulfide into sulfur by *Allochromatium vinosum* strain 21D (**A**) and of the light source arrangement (**B**), viewed from the top. Photobioreactor (1), fresh-medium reservoir (2), waste tank (3), flask with 1 M Na_2S (4), flask with 1 M HCl (5), flask with 1 M NaOH (6), sampling flask (7), N_2/CO_2 gas inlet (8), gas outlet (9), settler (10), outlet for sampling and product (11), stirrer (12), pH probe head (13), temperature probe head (14), O_2 probe head (15), light source (16), cooling/heating liquid inlet (17), cooling/heating outlet (18), computer (19).

Analytical methods. Elemental sulfur was extracted from the cells with chloroform, separated by reversed-phase HPLC, and analyzed by UV absorption at 263 nm according to the method described by Rethmeier et al. [26]. Sulfide was determined with a bromobimane fluorescent labeling assay and separated by reversed-phase HPLC as described by Rethmeier et al. [26]. Bacterial growth was determined by the increase in bacteriochlorophyll *a* (BChl *a*), which was extracted with methanol and measured according to Stal et al. [28].

Results and Discussion

Allochromatium vinosum strain 21D was successfully used as a biocatalyst to oxidize sulfide to elemental sulfur in a semi-continuous process consisting of three consecutive fed-batch sections (Fig. 2, fed-batch sections I, II and III). The reactor was initially inoculated with 1 l of a well-grown preculture of *A. vinosum* strain 21D (BChl *a* concentration: 1.3 mg/l). Each of the three fed-batch sections contained three consecutive sulfide additions (additions 1–9). At the beginning of the experiment, cells were free of sulfur globules (Fig. 3A). The organism converted sulfide into elemental sulfur, which was deposited as numerous sulfur globules inside the cells (Fig. 3B). At the end of each fed-batch section, 2 l of medium (containing cells with sulfur globules) were withdrawn from the reactor and transferred into the settler (Fig. 1A) to determine the amount of sulfur produced (see Materials and Methods). *A. vinosum* strain 21D was able to remove sulfide with a maximum removal rate of 49.3 μM/h and a maximum sulfide removal efficiency of 98.7% (Table 1). The average sulfide removal efficiency for the total experiment was 91.7%.

Since *A. vinosum* strain 21D stored elemental sulfur inside and not outside the cells, the inner glass wall of the bioreactor remained free of sulfur. Also, no sulfate was produced due to the genetic inability of the organism to further oxidize intracellularly stored elemental sulfur. During the entire run of the bioreactor process, 871.6 mg elemental sulfur was produced from a total of 1443 mg sulfide added, which corresponds to 60.4% recycling. Pott and Dahl [25] found that only about one quarter of the added sulfide (5 mM) was converted into elemental sulfur by *A. vinosum*

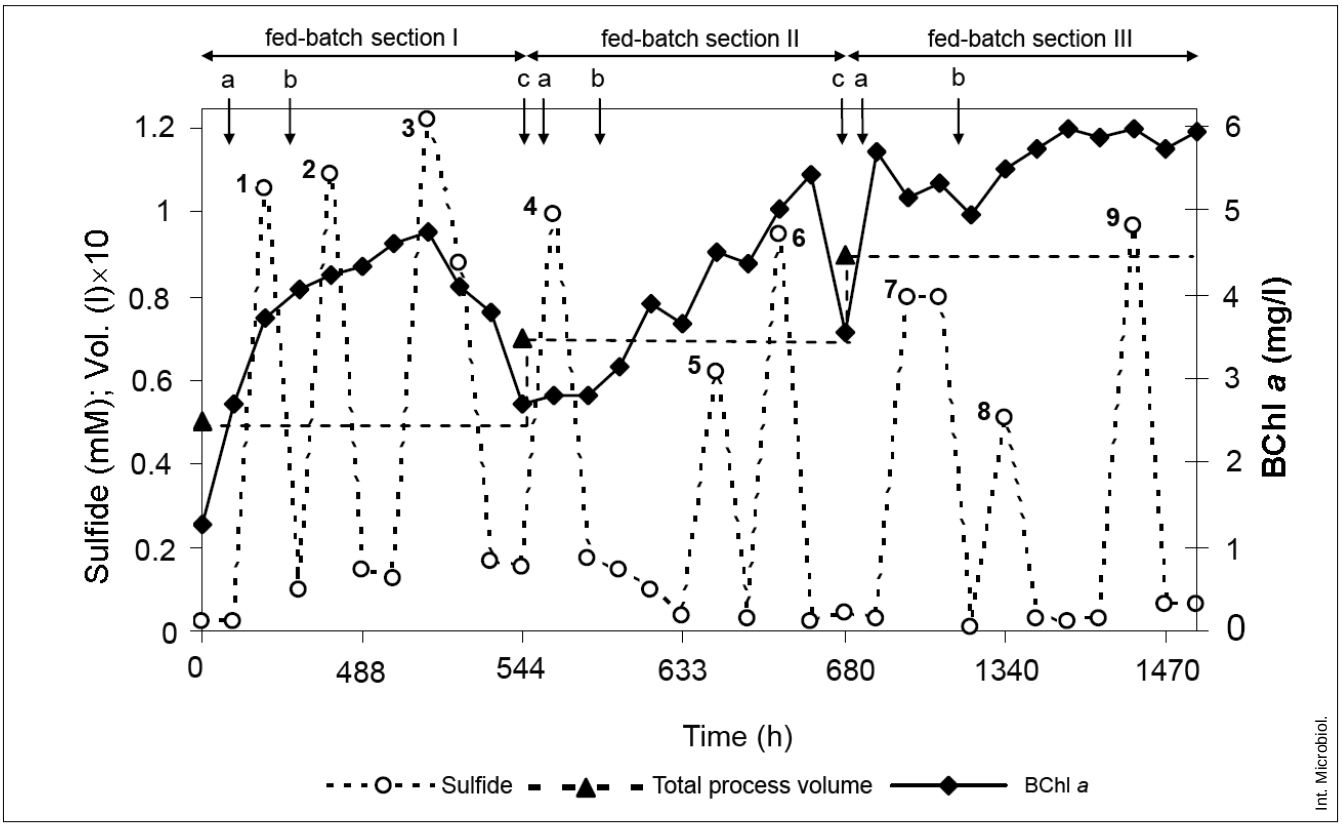

Fig. 2. Bacteriochlorophyll *a* (BChl *a)* and sulfide concentrations and total process volume during the biotechnological removal of sulfide by *Allochromatium vinosum* strain 21D. Sulfide inputs are marked with numbers (1–9). (**a**) Growth with malate and acetate (10 mM each); (**b**) growth with malate, acetate and sulfide; (**c**) dilution.

strain 21D. These results are in agreement with those of Madigan and Brock [21] for *Chloroflexus aurantiacus*, which oxidized only 70% of the added sulfide to elemental sulfur during photolithoautotrophic growth. In those reports, no explanations were offered concerning whether and which other inorganic sulfur compounds might have been produced from the added sulfide in addition to the detected elemental sulfur. Average biological sulfide removal efficiencies in biotechnologies using phototrophic organisms range from 81 to 100%, conversion efficiencies are in the range of 8–90% [9].

Kleinjan et al. [17] suggested that sulfide removal biotechnologies should use only those microorganisms that store sulfur extracellularly, due to easier separation of sulfur from biomass. However, we propose the industrial or agricultural use of a "biomass sulfur" product, i.e., without the need for prior separation of these two components. The sulfur content of purple sulfur bacteria can comprise up to 50% of the cell dry mass [27]. Dried, and therefore dead *A. vinosum* strain 21D cells, including a high percentage of sulfur, might be ground to a powder and then used, for instance, as a biofungi-

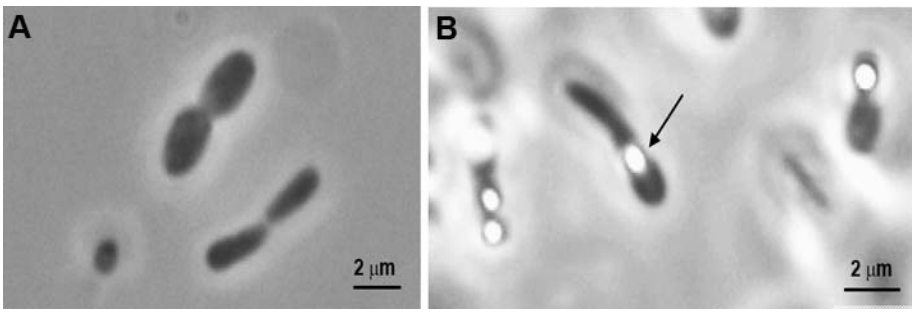

Fig. 3. Photomicrographs of sulfur-free cells of *Allochromatium vinosum* strain 21D. (**A**)**,** at the beginning of the experiment. (**B**)**,** with sulfur globules at the end of a fed-batch section. Arrow points to a sulfur globule.

Table 1. Sulfide removal rates and efficiencies of biotechnological sulfide removal by *Allochromatium vinosum* strain 21D. The process was run in a photobioreactor for 61 days (1470 h). The numbers of sulfide inputs correspond to the numbers provided in Fig. 2

No. of sulfide inputs	SRR[a] (μM/h)	SRE[b] (%)
1	2.1	90.6
2	43.1	86.7
3	49.3	86.5
4	49.2	82.7
5	25.3	95.3
6	40.5	97.6
7	1.2	98.7
8	17.3	94.2
9	12	93.3

[a]Sulfide removal rate.
[b]Sulfide removal efficiency for each sulfide addition.

cide. We assume that the remaining cell mass would not interfere with industrial or agricultural applications. To operate the photobioreactor over a period of 61 days, the entire process needed 197.6 kWh of electrical energy. Although some energy was saved because there is no need for aeration in this anaerobic system, the use of electric light as a source of photon irradiance still remains cost intensive. Whether electric energy can be replaced by sunlight to drive phototrophic sulfide removal by *A. vinosum* strain 21D should be determined.

Sulfide removal rates and efficiencies, and the conversion rate showed that the use of *A. vinosum* strain 21D is a potent phototrophic alternative to current biotechnological sulfide removal techniques and that it efficiently and effectively converts sulfide from synthetic sulfide-polluted wastewater into elemental sulfur with a high yield. Thus, both elimination of the toxic compound sulfide and production of the useful raw material elemental sulfur were achieved. Compared to other biological methods (e.g., using green sulfur bacteria) that have been tested, the above-described procedure has the following advantages: first, monitoring the process is easier as there is no need to prevent further oxidation of sulfur to sulfate; second, since no elemental sulfur is stored outside the cells, no sulfur attaches to the inner glass wall of the bioreactor. Further research can now be carried out using *A. vinosum* strain 21D on a larger scale with real wastewater or even in gas desulfurization. Biotechnological gas desulfurization by the green sulfur bacterium *Chlorobium limicola* [1] and chemolithotrophic bacteria of the *Thiobacillus* genus [16] has already been successfully applied.

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Eliminación de sulfuro y reciclado de azufre elemental de un medio contaminado con sulfuro por *Allochromatium vinosum* cepa 21D

Resumen. Las bacterias fotótrofas rojas del azufre oxidan sulfuro a azufre elemental, que se almacena en el interior de la célula en forma de glóbulos de azufre. Debido a una inactivación del gen *drsB*, el mutante *Allochromatium vinosum* 21D es incapaz de continuar la oxidación del azufre almacenado intracelularmente y formar sulfato. Este mutante se utilizó como biocatalizador en un proceso biotecnológico para eliminar sulfuro de agua residual sintética y para reciclar azufre elemental como materia prima. Con esta finalidad, se cultivó dicho mutante en un biorreactor iluminado de 5 litros (30 μE/m^2/s PAR), durante 61 días a 30°C en un medio fototrófico anóxico. El proceso de eliminación de sulfuro era semicontinuo y consistía en tres secciones consecutivas de cultivo con alimentación (*fed-batch*). El sulfuro se añadía continuamente al biorreactor y las células lo oxidaban hasta azufre. En presencia del mutante, durante la eliminación de sulfuro no había producción del sulfato no deseado. Se obtuvo una velocidad máxima de eliminación de sulfuro de 49,3 μM/h, con una eficacia máxima de 98,7% y un reciclado del 60,4% de azufre. [**Int Microbiol** 2006; 9(4):253-258]

Palabras clave: *Allochromatium vinosum* · eliminación biológica de sulfuro · producción biológica de azufre

Eliminação de sulfureto e reciclado de enxofre fundamental de um meio contaminado com sulfureto por *Allochromatium vinosum* cepa 21D

Resumo. As bactérias fototróficas vermelhas do enxofre oxidam sulfureto a enxofre fundamental, o qual se armazena em glóbulos de enxofre intracelulares. Devido a uma inactivação do gene *drsB*, o mutante *Allochromatium vinosum* 21D é incapaz continuar a oxidação do enxofre armazenado intracelularmente a sulfato. Este mutante foi utilizado como biocatalizador em um processo biotecnológico para eliminar sulfureto de água residual sintética, e para reciclar enxofre fundamental como matéria-prima. O mutante foi crescido em um biorreator iluminado de 5 litros (30 μE/m^2/s PAR), durante 61 dias a 30°C em um meio fototrófico anóxico. O processo de eliminação de sulfureto foi semi-contínuo e consistiu de três seções consecutivas com alimentação (*fed-batch*). O sulfureto era continuamente acrescentado ao biorreactor e oxidado pelas células a enxofre. A ação do mutante utilizado impediu a produção de sulfato não desejado durante a eliminação de sulfureto. Se obteve uma velocidade máxima de eliminação de sulfureto de 49,3 μM/h, uma eficiência máxima de eliminação de sulfureto de 98,7% e um reciclado do 60,4% de enxofre. [**Int Microbiol** 2006; 9(4):253-258]

Palavras chave: *Allochromatium vinosum* · eliminação biológica de sulfureto · produção biológica de enxofre