Biotechnology Advances 33 (2015) 1246–1259

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Sulfur-oxidizing bacteria in environmental technology

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ARTICLE INFO

Available online 19 February 2015

Keywords:
Hydrogen sulfide
Biogas
Biodesulfurization
Autotrophic denitrification
Sulfur-oxidizing bacteria

ABSTRACT

Hydrogen sulfide is widely known as the most undesirable component of biogas that caused not only serious sensoric and toxic problems, but also corrosion of concrete and steel structures. Many agricultural and industrial waste used in biogas production, may contain a large amount of substances that serve as direct precursors to the formation of sulfide sulfur—sources of hydrogen sulfide in the biogas. Biological desulfurization methods are currently promoted to abiotic methods because they are less expensive and do not produce undesirable materials which must be disposed of. The final products of oxidation of sulfides are no longer hazardous. Biological removal of sulfide from a liquid or gaseous phase is based on the activity of sulfur-oxidizing bacteria. They need an oxidizing agent such as an acceptor of electrons released during the oxidation of sulfides—atmospheric oxygen or oxidized forms of nitrogen. Different genera of sulfur-oxidizing bacteria and their technological application are discussed.

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http://dx.doi.org/10.1016/j.biotechadv.2015.02.007
0734-9750/

1. Introduction

Sulfur compounds of anthropogenic origin significantly pollute the environment and their strongly negative impact in many ways is well known—acid rain, odor problems from polluted surface water bodies, wastewater treatment plants, sewerage, landfills, corrosion of steel and concrete structures, leaching and migration of heavy metals and acidic components of sediments etc. Considering sulfur pollution, serious problems are caused mainly by sulfur in reduced form as sulfides due to their odor and toxic characteristics. Unlike the interest and efforts focused to biological elimination of nitrogen and phosphorous, biological treatment of sulfur pollution was devoted relatively little attention. This is being changed recently and many new biotechnologies have been developed based on knowledge and comprehension of the biogeochemical cycle of sulfur in nature. Sulfur-oxidizing bacteria play a key role in these technologies.

1.1. Sources of sulfide sulfur in environment

Sulfur is frequently present in all organisms and occurs in organic compounds such as amino acids, proteins, enzymes, antibiotics and fats. It is present in organic substances with catalytic, structural and regulatory functions. On the contrary, a biological function of inorganic sulfur compounds is limited. These compounds either serve as a source of sulfur for assimilation and synthesis of organic compounds, or they are used as electron acceptors or donors in dissimilation processes. Assimilatory sulfate reduction is common in prokaryotes, plants and fungi. Dissimilation pathways are used mainly by Eubacteria and Archaebacteria.

Hydrogen sulfide and its ionic forms (HS^-, S^{2-}) are rarely present in natural waters with the exception of special sulfurous mineral springs. Its presence in surface waters is caused mainly by a lack of dissolved oxygen and an associated anaerobic biological decomposition of organic substances containing sulfur or by reduction of thiosulfates, polythionates, elemental sulfur, sulfites and sulfates by sulfate-reducing bacteria (SRB). In the natural environment with a low redox potential the most common inorganic sulfur compounds, but also sulfur incorporated in organic substances, may be reduced to sulfides.

Compounds containing sulfur are particularly undesirable components of input to anaerobic fermentation technology of waste organic matter, where biogas is a final product. Sulfur compounds in any oxidized form are under anaerobic conditions inevitably reduced to sulfides, which depending on the fermentation conditions can pass to biogas as gaseous hydrogen sulfide. This biogas component causes then a problem when biogas is used for energy production. Hydrogen sulfide is very corrosive to some parts of gas motors and its combustion produces SO_x emission. Therefore, it is necessary to remove this undesirable component from biogas before its utilization. Currently, this fact is reflected a greater extent at present, because biogas that is the product of anaerobic fermentation of organic wastes is classified as an alternative energy source.

Sulfide pollution, which is to be treated, comes from different sources, e.g. from anaerobic treatment of organic wastes containing sulfur incorporated in proteins (pig manure, …) (Deng et al., 2006), from sulfides directly produced in wastewater and wastes for instance by tanneries or by a production of rayon viscose. In the case of anaerobic treatment of industrial wastewater from textile (Kabdasli et al., 1995), paper (Janssen et al., 2009), breweries or pharmaceutical industry (Rodríguez Martinez et al., 2005), the problem is high concentration of oxidized forms of sulfur, mainly sulfates and sulfites. Mine water also contains a considerable amount of sulfates (Choudhary and Sheoran, 2012). Sulfides can thus be released during anaerobic treatment of these wastes and wastewater either by sulfate-reducing bacteria by direct reduction of sulfates or by activity of other anaerobic bacteria that break down and subsequently decompose the sulfur proteins.

1.1.2. Sulfate-reducing bacteria as producers of sulfide sulfur

SRB need for sulfate reduction a suitable electron donor, whose choice is based on its acquisition costs (van Houten et al., 1994) and its capabilities to reduce sulfates under given conditions without forming secondary pollutants. Thermodynamic and kinetic parameters of reactions are also important in electron donor selection, affecting a relationship between SRB and other anaerobic fermentation bacteria. Organic matter, which is formed as an intermediate product of anaerobic fermentation or which is added to a system as an external substrate, is usually used as electron donor. An energy yield of reduction reactions of SRB is generally more advantageous than the thermodynamics of reactions of other anaerobic bacteria. Therefore SRB thus decrease usable potential of organic substrate for production of biogas in anaerobic fermentation, consuming its significant proportion (Choi and Rim, 1991). Reduction of sulfates is predominant to reduction of carbon dioxide and production of methane. The significance of this competition increases with decline of COD/SO_4^{2-} ratio, i.e. it depends on the concentration of sulfates (Omil et al., 1998).

Since SRB have different affinity to the individual organic substrates (H_2 > propionate > other organic matter), they compete for substrate with different trophic groups of the anaerobic species (Chen et al., 2008b). Competition between SRB and rapidly growing fermentative and acidogenic bacteria practically does not take place because SRB fail to break down more complex organic substances. Only exceptions are a few representatives of SRB, which are capable of metabolizing sugars and amino acids (Hansen, 1993).

A suitable electron donor is hydrogen for which SRB compete with the hydrogenotrophic methanogens and homoacetogens. In the case of a non-limiting concentration of sulfates, hydrogen is entirely consumed by the hydrogenotrophic SRB, whose reaction is energetically more suitable than reaction of hydrogenotrophic methanogens (Omil et al., 1996). Hydrogen concentration decreases below certain useful threshold concentration for hydrogenotrophic methanogens, which explains their high inhibition in the presence of high concentrations of sulfates; hydrogen concentration limit for hydrogenotrophic SRB is higher (Colleran et al., 1995; Oude Elfering et al., 1994; Oude Elferink et al., 1998). Temperature plays an important role in the competition for hydrogen between the hydrogenotrophic methanogens and SRB. While the SRB are dominant under mesophilic conditions, methanogens are dominant under thermophilic conditions (Colleran and Pender, 2002).

Hydrogenotrophic SRB behave similarly in the presence of formic acid (De Smul and Verstraete, 1999). In the presence of methanol, sulfates may be reduced only by several types of SRB of the genus *Desulfotomaculum*, *Desulfovibrio* or *Desulfobacterium*. However their growth rate is very low at mesophilic temperature in comparison with methanogens and homoacetogens. It was found out that 90% of methanol was at this temperature consumed by hydrogenotrophic methanogens and only 10% by the SRB (Weijma et al., 2003), while under thermophilic conditions SRB dominate (Weijma et al., 2000). Ethanol is also a significant electron donor in sulfate reduction, which efficiency is possible to achieve 80% in the presence of ethanol even at high sulfate concentrations (Kalyuzhnyi et al., 1997). Volatile fatty acids, especially acetic, propionic and butyric acids, which are produced during acidogenesis and acetogenesis as intermediate products of anaerobic degradation of organic matter, are also important for sulfate reduction. Acetic acid in a continuously stirred reactor was preferentially utilized by acetotrophic SRB that outgrow methanogens due to thermodynamics of theirs reactions (Gupta et al., 1994). SRB behavior in reactors with immobilized biomass is not quite clear. Some studies reported that all acetic acid is used by acetotrophic methanogens, even at high concentrations of sulfates (Hoeks et al., 1984), while other sources state that SRB have a clear dominance over methanogens (Omil et al., 1996). In addition to the growth kinetics, the competition between SRB and acetotrophic methanogens is affected by many other factors referred summarily in literature (Lens et al., 2000). The rate of propionic acid and butyric acid consumption is highly dependent on the concentration of sulfates and their ratio to the COD. At higher sulfate concentrations ($COD/SO_4^{2-} < 1.7$) their utilization by SRB is more efficient than that of syntrophic acetogens; in the case of $COD/SO_4^{2-} > 2.7$ syntrophic methanogens outcompete. In the range of COD/SO_4^{2-} from 1.7 to 2.7 the competition for substrate takes place in which the portion of SRB in anaerobic culture plays a major role (Choi and Rim, 1991; Colleran et al., 1995). In the case of butyric acid syntrophic acetogens may compete with SRB even in the case of an excess of sulfate. If sulfate concentration is a limiting factor, the syntrophic acetogen activity is dominant (Visser et al., 1993). SRB can also use as a substrate for the reduction of sulfates several more complex organic substances, such as ethyl benzene (Kniemeyer et al., 2003), phenol and benzoic acid (Fang et al., 1997).

1.2. Inhibitory properties of sulfides

SRB activity may be inhibited by produced sulfides, if they occur in undissociated form (Moosa and Harrison, 2006). Produced undissociated sulfides can in liquid phase depending on pH inhibit also activities of other bacterial species in anaerobic fermentation, particularly syntrophic acetogenic bacteria and methanogenic bacteria (Wiemann et al., 1998). Sulfides may as well react with heavy metals, which are important components of the functional enzymes, e.g. Fe cytochromes, thus negatively affecting their activity (Chen et al., 2010). The undissociated form of dissolved hydrogen sulfide is toxic because it can diffuse through a cell membrane and inhibit cell activity inside. Inhibition manifests in denaturation of native proteins by formation of sulfide or disulfide cross-links between polypeptide chains, which interferes with various linking co-enzymes and sulfides as well as the sulfur assimilation metabolism (Chen et al., 2008b).

Because sulfides are toxic to microorganisms in undissociated state, the sulfide toxicity is associated with an occurrence of individual dissociation forms of sulfide sulfur and hence with the pH value. The pK_a value for this balance is round 7 and it depends on temperature; a higher temperature decreases pK_a value. Such any change in pH impacts the proportion between undissociated form (H_2S) and dissociated form (HS^-) (McCartney and Oleszkiewicz, 1991). Increase of pH from 7 to 8 reduces the proportion of undissociated form from 50% to 9% and hence the proportion of H_2S that escapes to a gas phase. The optimal value for anaerobic bacteria, especially for sensitive methanogens, however, is around pH 7.

The sulfide inhibitory concentration IC_{50} is in the range of 30 to 250 mg/L and depends on the form of bacterial species. Bacteria in biofilm reactors tolerate much higher sulfide concentration than biomass in a suspension (Lens et al., 1998); moreover their immobilization on carriers prevents their washout from the reactor (Omil et al., 1996). According to literature data (Parkin et al., 1990), the inhibitory concentration of dissolved dissociated sulfides is 100 – 800 mg/L, while for the undissociated form of H_2S it is in the range of 50 – 400 mg/L. The toxic effect of the undissociated form of sulfide sulfur affects various groups of anaerobic species in different ways. The least affected are acidogenic bacteria, followed by syntrophic acetogens whose concentration limit is compared with SRB, and methanogens are the most sensitive (O'Flaherty et al., 1998).

1.3. Hydrogen sulfide in biogas

Concentration of undissociated hydrogen sulfide dissolved in a liquid according to pH is in equilibrium with gaseous hydrogen sulfide concentration depending also on pressure, temperature and total concentration of sulfide sulfur. Concentration of hydrogen sulfide in biogas can reach very significant values ranging from 500 ppmv to 20,000 ppmv (2%). In these concentrations it causes not only serious sensoric and toxic problems, but also corrodes the concrete (Okabe et al., 2007) and steel structures in biogas plants (Zhang et al., 2008), damages pipes and pumps (Burgess et al., 2001) and significantly shortens the service life of the gas motors of cogeneration units, where it debases the lubricating oil (Gayh et al., 2010). The burning of biogas with higher concentrations of hydrogen sulfide causes also problems with excessive SO_x emissions. Besides the inhibitory effect directly in the fermentation process these are main reasons why it is necessary to remove hydrogen sulfide from biogas prior to its use for energy production.

1.3.1. Removal of hydrogen sulfide from biogas

There are many different methods for a removal of hydrogen sulfide from biogas depending on the type of fermentation and amount of sulfur compounds in input substrate. A simple method is technological suppression of undissociated sulfides directly in fermentation medium by raising pH or reducing of total sulfide concentration by precipitation with added iron salts. Another possibility is the use of sulfur-oxidizing bacteria existing in micro niches in fermentation medium or in a gas space of a fermenter. This method of micro-aeration is based on very small doses of air or oxygen fed directly into the gas space of the fermenter or to fermentation mixture, where sulfur-oxidizing bacteria (SOB) present in heterogeneous bacterial population are activated. This method is suitable for lower concentrations of hydrogen sulfide and its objective is oxidation of sulfides only to elemental sulfur, which can be removed together with output fermented material.

At high concentrations of hydrogen sulfide in biogas and high demands on the quality of biogas internal desulfurization methods are not sufficiently effective and it is necessary to remove the hydrogen sulfide from biogas externally. Chemical and physiochemical methods of precipitation, washing or absorption have already been widely used and successfully applied for a long time. However, they are always associated with high operating costs on energy and chemicals and sometimes with the production of wastes that must be further treated. Moreover, certain aggressive chemicals used cause a rapid corrosion of the equipment and exhausted catalysts, adsorbents or chemicals must be periodically regenerated or replaced (Henshaw and Zhu, 2001).

Biological methods are less expensive, do not require expensive chemicals and do not cause secondary environmental pollution (Sublette and Sylvester, 1987). It is only necessary to wash gaseous hydrogen sulfide from the biogas to water, bring it into contact with SOB culture and assure nutrient demand. However, it is also possible to utilize favorable properties of sulfur-oxidizing bacteria for external desulfurization. The final products of the biooxidation of sulfides are not further dangerous; elemental sulfur can be removed from the system completely due to its low solubility in water and used in industry or as a fertilizer (Beristain-Cardoso et al., 2008). Sulfates can be discharged directly into surface water.

Hydrogen sulfide is captured from biogas to an aqueous medium by washing or scrubbing. Composition and pH of the used medium depend on the type of population of SOB, which may be acidophilic or alkalophilic. Due to the better and the faster absorption of hydrogen sulfide in the alkaline medium, systems with higher pH are predominant (Krischan et al., 2012). The aqueous medium with captured sulfides is fed to the bioreactor with SOB culture or washing and biooxidation procedures are coupled in the same reactor (Syed et al., 2006).

Direct dosing of air or oxygen as oxidizing agent into the liquid phase is used by methods, where the sulfur-oxidizing bacteria are cultivated either in a suspension culture or as in the form of biofilms on some carriers. Packed bed biofilters, bioscrubbers or trickling filters are used, which are filled with a variety of media—charcoal, compost, ceramics, lava, turf, and synthetic materials (Fortuny et al., 2008; Li et al., 2008; Midha et al., 2012). These methods are convenient even for very high concentrations of hydrogen sulfide in the biogas up to 20,000 ppmv. These methods are viable alternatives to chemical processes and their applicability has been proved in industrial scale—Thiopaq® or Sulfothane^{TM} processes (SULFOTHANE, THIOPAQ). It is apparent that the adaptation ability of sulfur-oxidizing bacteria to high concentrations of hydrogen sulfide brings the potential for broad application of biological desulfurization of biogas or other gases containing hydrogen sulfide.

2. Microbiology of biological oxidation of sulfur compounds

Biological removal of sulfides is based on the activities of photoautotrophic or chemolithotrophic sulfur-oxidizing bacteria. Photoautotrophic SOB obtain energy for their metabolism from light energy, and chemolithotrophic SOB directly from the oxidizing reactions, where oxygen (aerobic microbial species) or nitrates or nitrites (anoxic microbial species) serve as acceptor of electrons released during the oxidation of sulfides. From a technological point of view, for the biological removal of sulfides, the most appropriate are chemolithotrophic sulfur-oxidizing bacteria (*Thiobacillus*, *Sulfolobus*, *Thermothrix*, *Beggiatoa* and *Thiothrix*), also known as colorless sulfur-oxidizing bacteria. They are particularly suitable for their highest rate of sulfide oxidation, modest nutritional requirements and extremely high affinity for sulfides and oxygen. These properties allow them to successfully compete with chemical oxidation of sulfides in both the natural environment and bioreactors with a limited supply of oxygen (Janssen et al., 1998).

2.1. Phototrophic sulfur-oxidizing bacteria

As the name implies, phototrophic organisms for a proper chemical reaction process require light as an energy source (Tang et al., 2009). Hydrogen sulfide is oxidized to elemental sulfur, while CO_2 is reduced and incorporated to organic compounds. The intensity of an applied light energy is dependent on sulfide concentration and is expressed by the so-called van Niel curve (Cork et al., 1985). This curve represents an amount of light energy that must be supplied for sulfides at a given concentration to be oxidized to elemental sulfur (Eq. (1)). If sulfide concentration is increased at a given light intensity, they accumulate in the photoreactor; if on the contrary their concentration is lower, they are oxidized to sulfates (Eq. (2)) (Syed et al., 2006). The hydrogen source for the photosynthesis of these bacteria is hydrogen sulfide (Eq. (1)) (Janssen et al., 1999) or molecular hydrogen, but not water. For this simple reason, the bacteria thus do not release oxygen into the atmosphere, and it is therefore better to consider the process as chemosynthesis.

$$2\,H_2S + CO_2 + \text{light} \rightarrow 2\,S^0 + CH_2O + H_2O \qquad \Delta G^o = 75.36\ \text{kJ mol}^{-1} \tag{1}$$

$$H_2S + 2\,CO_2 + 2\,H_2O + \text{light} \rightarrow SO_4^{2-} + 2\,CH_2O + H_2O \tag{2}$$

The main problem associated with the use of phototrophic sulfur-oxidizing bacteria consists in their slow growth rate, and therefore the light source must be sufficiently strong, which not only requires considerable investment, but also operating costs (Oh et al., 1998). The growth of bacteria need not necessarily be lithotrophic, if suitable organic material is present in medium; bacteria can use it as a source of carbon, energy or nitrogen. For photoautotrophic bacteria growth it is also interesting that they use two types of reactions. Under condition of light, they directly convert light energy to chemical energy and in the dark they reduce carbon dioxide to organic compounds using the energy stored in the form of ATP (Madigan and Martinko, 2006).

Among phototrophic bacteria the green sulfur-oxidizing bacteria (GSB) and purple sulfur-oxidizing bacteria (PSB) are the most frequent, which in addition to light and carbon dioxide as a source for the formation of new cell material also require inorganic nutrients (ammonium salts, chlorides, phosphates, sulfates or some trace elements).

Green sulfur-oxidizing bacteria oxidize sulfides through elementary sulfur to sulfates. It should also be noted that GSB in comparison with other phototrophic organisms can perform its function in much lower light intensity conditions (Tang et al., 2009). GSB are able to perform photosynthesis in anaerobic conditions, but do not grow in darkness. They include such species as *Chlorobium*, *Chloroherpeton*, *Prosthecochloris*, *Pelodictyon* and *Ancalochloris*. The most significant is *Chlorobium limicola*, *thiosulfatophilium* form, which has its optimal light absorption spectrum in the range of 350–850 nm, the optimum being 760 nm (Stanier et al., 1986).

Purple sulfur-oxidizing bacteria (*Allochromatium*, *Chromatium*, *Thioalkalicoccus*, *Thiorodococcus*, *Thiococcus*, *Thiocystys*, *Thiospirillum* …) store produced sulfur in the form of spherical particles within the cells. Subsequently, oxidation takes place and produced sulfates are released from the cell. PSB are also capable of utilization of organic compounds, and they are therefore considered as facultative photolithotrophic bacteria. There are also such species of sulfur-oxidizing bacteria (*Ectothiorhodospira*, *Halorhodospira*, *Thiorhodospira*), which are capable of producing sulfur outside the cell (Tang et al., 2009).

The bacteriochlorophyll of PSB has a composition close to chlorophyll-*a*; in addition they also contain red and yellow carotenoids.

2.2. Chemolithotrophic sulfur-oxidizing bacteria

Chemolithotrophic -oxidizing bacteria known as colorless sulfur-oxidizing bacteria use the energy of reduced inorganic sulfur compounds (hydrogen sulfide, thiosulfates, sulfites, elemental sulfur), but in some cases also from organic sulfur compounds (e.g. methanethiol, dimethyl sulfide, dimethyl disulfide) (Cattaneo et al., 2003). Carbon dioxide is generally used as a carbon source for the construction of new cell material. Colorless sulfur-oxidizing bacteria are Gram-negative, and in terms of the growth requirements, they differ greatly even in the different

Table 1
Different conditions for several representatives of *Thiobacillus sp.* (Syed et al., 2006).

	Thiobacillus ferrooxidans	*Thiobacillus thiooxidans*	*Thiobacillus novellus*	*Thiobacillus thioparus*	*Thiobacillus denitrificans*
Optimal pH	1.3–4.5	2.0–3.5	7.0	7.5	7.0–7.5
Optimal temperature °C	30–35	28–30	30	28	28–32

species of one genus (Table 1). Their optimum temperatures are from 4 to 90 °C and pH values are in the range of 1 – 9. Most of studied chemolithotrophic bacteria thrive best in mesophilic or thermophilic conditions (*Thiobacillus*, *Sulfolobus*, *Thermothrix*, ...) (Tang et al., 2009).

Chemolithotrophic-oxidizing bacteria consist of two morphologically and taxonomically distinct groups; they are small short rods of the genus *Thiobacillus* (*Thiobacillus thioparus*, *Thiobacillus denitrificans or Thiobacillus thiooxidans*) and long filamentous bacteria of the genera *Beggiatoa* and *Thiothrix*. The group of long filamentous bacteria oxidizes H_2S to S^0, which they store inside the cell, and it can be subsequently oxidized to sulfates. When supply of hydrogen sulfide is adequate, sulfur is stored inside the cell in the form of black droplets, but in case of hydrogen sulfide shortage or surplus of the oxidizing agent, S^0 is further oxidized to soluble sulfates able to pass through the cell walls (Schmidt et al., 1987).

Chemolithotrophic sulfur-oxidizing bacteria are classified by carbon and energy source into four groups (Tang et al., 2009).

Obligate chemolithotrophic sulfur-oxidizing bacteria use carbon dioxide as a source of carbon and different inorganic sulfur compounds to obtain energy (some species of *Thiobacillus*, *Thiomicrospira*).

Facultative chemolithotrophic sulfur-oxidizing bacteria also use CO_2 to build cells and inorganic material for acquisition of energy, but in addition they are able to use heterotrophically organic compounds from which they utilize C and simultaneously extract energy from the energy-rich bonds (*Paracoccus denitrificans*, several species of *Thiobacillus*, *Thermotrix* or *Sulfolobus*).

Chemolithotrophic sulfur-oxidizing bacteria are able to gain energy by oxidation of reduced sulfur compounds, but cannot bind CO_2 (several species of *Beggiatoa* and *Thiobacillus*).

Chemoorganotrophic sulfur-oxidizing bacteria oxidize sulfur compounds without gaining energy from reactions (*Thiobacterium*, *Thiothrix*).

Classification of different trophic groups of chemolithotrophic sulfur-oxidizing bacteria by carbon and energy source is presented in Table 2.

The most common and most studied genus is *Thiobacillus sp.* Many species are acidophilic with optimal pH 1–6 (*T. thiooxidans* pH 2.0–3.5 or *Thiobacillus ferrooxidans* pH 1.3–4.5). *T. thiooxidans* can also grow in extreme conditions of pH < 1. *Thiobacillus denitrificans* and *Thiobacillus novellus* are neutrophilic and their range of optimal pH is 6–8. *T. thioparus* grows in the broad range of pH 5 – 9 and its optimum is 7.5 at 28 °C. Apart from the sulfides, it can also oxidize thiosulfates (Vlasceanu et al., 1997). *Thioalkalispira micro-aerophila* is an alkalophilic microorganism with optimum pH 10.

The temperature requirements of the various sulfur-oxidizing bacteria are also different. Mostly, they are mesophilic or thermophilic, but some bacteria (*Thermothrix azorensis*) have an optimum growth temperature of 76 to 86 °C (Odintsova et al., 1996; Syed et al., 2006). Some sulfur-oxidizing bacteria are mixotrophic i.e. they can oxidize both reduced inorganic sulfur compounds and organic sulfur compounds (*T. novellus*, *Pseudomonas acidovorans*, *Pseudomonas putida*, *Beggiatoa sp.* and *Thiothrix sp.* (Oyarzún et al., 2003)). Most of the representatives of the genus *Thiobacillus sp.* are strictly aerobic (*T. thiooxidans*, *T. novellus*, *T. thioparus*); on the contrary *T. denitrificans* and *Thiobacillus ferroxidans* are facultatively anaerobic; some bacterial genera are tolerant to micro-aerobic conditions (*Thiothrix nivea*, *T. micro-aerophila*) (Syed et al., 2006) or *Aneurinibacillus aneurinilyticus* (Chen et al., 2010).

Table 2
Overview of trophic groups of sulfur-oxidizing bacteria.

Energy source	Carbon source	Full name
Solar photo -	Organic - organo	Photoorganotroph
	Inorganic - litho	Photolithotroph
Chemical compounds chemo -	Organic - organo	Chemoorganotroph
	Inorganic - litho	Chemolithotroph

Some species of the genus *Thiobacillus* also differ in terms of capacity to convert sulfide sulfur to elemental sulfur. While *Thiobacillus neapolitanus* is capable of converting only 50% of input sulfides to elemental sulfur, *Thiobacillus* sp. strain W5 has a maximum elemental sulfur production capacity of 90% (Visser et al., 1997).

Some species of the genus *Thiobacillus* were reclassified in 2000 into the genera *Acidithiobacillus* and *Halothiobacillus* (Kelly and Wood, 2000b). In both cases, they are γ-proteobacteria. *Acidithiobacillus* gen. nov. They grow as Gram-negative obligate acidophilic rods (optimum pH < 4), gaining energy for growth from the oxidation of reduced forms of sulfur or iron ions, and some species also utilize hydrogen. These are mesophilic and thermophilic species. An example is *Acidithiobacillus thiooxidans* earlier known as *T. thiooxidans.* This bacterium grows on elemental sulfur, thiosulfate or tetrathionate. It cannot oxidize iron, but in co-culture with *Leptospirillum ferrooxidans* it can utilize sulfide sulfur from pyrite. Other members of the genus *Acidithiobacillus* are *Acidithiobacillus caldus*, *Acidithiobacillus albertensis* and *Acidithiobacillus ferrooxidans* (earlier *T. ferrooxidans*). *A. ferrooxidans* is an acidophilic, obligate chemolithotrophic bacterium that grows with energy derived from the oxidation of ferrous to ferric ions or metabolizes other metal sulfides. The ability of this organism to oxidize Fe^{2+} to Fe^{3+} is the basis for the removal of sulfide sulfur, which reacts with Fe^{3+} to form elemental sulfur and Fe^{2+} that is in turn oxidized to Fe^{3+} by *A. ferrooxidans*. The entire closed cycle is shown in Eqs. (3) and (4) (Giro et al., 2006).

$$H_2S + Fe_2(SO_4)_3 \rightarrow S^0\downarrow + 2\,FeSO_4 + H_2SO_4 \tag{3}$$

$$2\,FeSO_4 + H_2SO_4 + 0.5\,O_2 \rightarrow Fe_2(SO_4)_3 + H_2O \tag{4}$$

Bacteria able to oxidize Fe^{2+} to Fe^{3+} for the purpose of removing sulfide sulfur include *L. ferrooxidans* (Madigan and Martinko, 2006) and *Sulpholobus acidocaldarius* (Brock and Gustafson, 1976).

Halothiobacillus gen. nov. forms rods, which get energy by the oxidation of reduced forms of sulfur; however, they are incapable of oxidizing ferrous ions. They are tolerant to high salt concentrations and some species even require them for their growth (*Halothiobacillus halophilus*). Their optimum growth temperature is 30 – 40 °C. Further members of this genus are *Halothiobacillus neapolitanus* (earlier *Thiobacilluis neapolitanus*) and *Halothiobacillus hydrothermalis*.

2.3. Autotrophic denitrifying sulfur-oxidizing bacteria

Certain species of sulfur bacteria—autotrophic denitrifying sulfur-oxidizing bacteria—use oxidized forms of nitrogen (nitrates or nitrites) as electron acceptors. Biological oxidation of sulfides in the presence of nitrates or nitrites becomes a further useful method for removal of reduced forms of sulfur from polluted gases and waters. This desulfurization method is based on the activity of bacteria, which include mainly *T. denitrificans*, *Thiomicrospira denitrificans*, *Thiobacillus versutus*, *Thiosphaera pantotropha* and *P. denitrificans*. These bacteria can use a variety of inorganic reduced forms of sulfur (S^{2-}, S^0, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}) for their chemolithotrophic growth with simultaneous reduction of nitrate or nitrite to nitrogen gas. The final products of sulfide oxidation are elemental sulfur or sulfates and nitrogen gas or nitrites as intermediate (Tang et al., 2009).

P. denitrificans ranks among α-proteobacteria. It is Gram-negative spherical bacterium, named *Micrococcus denitrificans* by its discoverer M. Beijerinck in 1908 and renamed in 1969 as it is known to date. It is bacterium preferring aerobic conditions and can grow as chemoheterotroph on monocarbon organic compounds such as methanol or methylamine, which it oxidizes to CO_2, and also as chemolithotroph that uses reduced forms of sulfur and hydrogen as electron donors in denitrification (Baker et al., 1998).

T. denitrificans belongs to β-proteobacteria. During oxidation of sulfides and thiosulfates both oxidized form of nitrogen and Fe^{2+} in pyrites (FeS_2 and FeS) can be exploited. Unlike other chemolithotrophic SOB it is a facultative anaerobe. In an aerobic environment, it is capable of utilizing thiosulfates and thiocyanates, and in an anaerobic environment it is capable of utilizing additionally sulfides and elemental sulfur. Optimal growth conditions are at pH 6.9 and at a temperature of 29.5 °C, while the optimum pH for denitrification is 6.85 and temperature 32.8 °C (Kelly and Wood, 2000a). It has a very good immobilization capability on suitable carriers (granular activated carbon) in a relatively short time (Ma et al., 2006).

T. denitrificans ATCC 33889 was reclassified in 2007 as *Sulfurimonas denitrificans DSM 1251* (Takai et al., 2006). It ranks among ε-proteobacteria and is capable of oxidizing sulfides, thiosulfates and elemental sulfur, while both nitrates and oxygen are used as the electron acceptor. The optimum growth temperature is 26 °C.

T. thioparus ranks among the few members of autotrophic denitrifiers reducing nitrates to nitrites by the sulfide oxidation (Tang et al., 2009). Although autotrophic denitrification bacteria are chemolithotrophic, there exist many denitrifying bacteria capable of adaptation to autotrophic as well as heterotrophic and mixotrophic growth (*P. versatus*, *P. denitrificans*, *Beggiatoa sp.*) (Matin, 1978).

However, all experience from studies with pure cultures cannot be transferred exactly to real conditions due to the fact that mixed culture of microorganisms is used in practice, in which chemolithotrophic denitrifiers can be dominant. Because autotrophic bacteria use inorganic substances such as CO_2 as a source of carbon, external organic carbon sources are not necessary. Hence, in the case of technological application of autotrophic denitrification in wastewater treatment, it brings substantial decrease in operating costs and, moreover, the production of excess activated sludge is two to three-fold lower (Chung et al., 1996; Kleerebezem and Mendez, 2002; Sublette and Sylvester, 1987; Visser et al., 1997).

The presence of some organic substances, e.g. acetic acid serving as a further carbon source, may increase the total denitrification rate (Cardoso et al., 2006). An however, using mixed culture enriched with *Thiomicrospira* CVO, found out that in the presence of acetic acid, first chemolithotrophic denitrification took place and it was followed by heterotrophic nitrate reduction only after depletion of the sulfides (An et al., 2010).

3. Mechanisms for oxidation of reduced sulfur compounds

3.1. Abiotic oxidation of reduced sulfur compounds

Chemical oxidation of sulfide takes place in parallel with biological oxidation and can positively affect the biological activity of SOB by decreasing inhibitory concentration of sulfides. Therefore it is particularly welcome during oxidation of high concentrations of sulfides. It proceeds spontaneously in aqueous alkaline medium in the presence of supplied oxygen and leads to the formation of following intermediate products—elemental sulfur, polysulfides, sulfites and thiosulfates. Among the factors influencing stoichiometry and kinetics of chemical oxidation of sulfides is the molar ratio of sulfides and oxygen, pH, temperature, ionic strength and the presence of catalysts or inhibitors (Chen and Morris, 1972; Nielsen et al., 2004).

The chemical oxidation of sulfides proceeds almost immediately at pH > 6 by reaction of elemental sulfur with the added sulfides, producing polysulfides (Eqs. (5) and (6)), which are characterized by blue–green color. Eventually sulfides react directly with oxygen mainly into pentasulfides (Eq. (7)) (Chen and Morris, 1972).

$$HS^- + S_8 \rightarrow H^+ + S_9{}^{2-} \tag{5}$$

$$S_9{}^{2-} + HS^- \rightarrow 2\,S_5{}^{2-} + H^+ \tag{6}$$

$$HS^- + 2\,O_2 \rightarrow S_5{}^{2-} + 3\,OH^- + H_2O \tag{7}$$

Pentasulfides are further chemically oxidized, if there is a sufficient amount of oxygen, primarily to thiosulfates (Eq. (8)) or sulfites (Eq. (9))

$$S_5{}^{2-} + 3\,O_2 + 3\,OH^- \rightarrow 2,5\,S_2O_3{}^{2-} + 1,5\,H_2O \tag{8}$$

$$S_5{}^{2-} + 5,5\,O_2 + 8\,OH^- \rightarrow 5\,SO_3{}^{2-} + 4\,H_2O \tag{9}$$

Sulfites are chemically stable for 1 day in a strongly alkaline medium (pH 10). Thiosulfates are also relatively stable for 2–3 days in an aerated aqueous abiotic medium. However, they are energetically highly advantageous substrate for the sulfur-oxidizing bacteria, which can oxidize them to sulfates according to the amount of oxidizing agent supplied (Eq. (10)). Therefore, their removal from the system is caused by biological oxidation (Chen and Morris, 1972).

$$S_2O_3{}^{2-} + H_2O + 2\,O_2 \rightarrow 2\,SO_4{}^{2-} + 2\,H^+ \quad \Delta G^o = -818.3\ \text{kJ/reaction} \tag{10}$$

Chemical oxidation of sulfides to sulfates at pH > 9 can be expected in the longer term because the formation of sulfates needs higher activation energy from the enzymatic reaction. In the normal course of chemical oxidation, i.e. in the time of hours, it is possible to expect only the formation of pentasulfides, thiosulfates and sulfites (Chen and Morris, 1972).

From the above-stated, it follows that chemical oxidation of sulfides is an integral part of desulfurization besides biological process that may benefit from it particularly in high concentrations of sulfides. Suitable energetically favorable substrates for sulfur-oxidizing bacteria are formed, while at the same time toxic effects of the sulfides on bacteria are reduced (González-Sánchez and Revah, 2007). However, biological oxidation of sulfides generally prevails over abiotic oxidation (Yavuz et al., 2007).

3.2. Biological oxidation of sulfide sulfur with oxygen

During the biological oxidation of hydrogen sulfide, just like in the case of the chemical oxidation, the first proceeds formation of polysulfides and their subsequent oxidation to elemental sulfur S^0 and sulfite with oxygen as the main electron acceptor, which are reduced to H_2O. The oxidation of elemental sulfur to sulfate occurs in two ways. More often, the reaction runs via enzyme cytochrome c with concurrent formation of ATP. Another way may be use of adenosine phosphosulfate reductase (Díaz et al., 2010).

The biological oxidation of sulfides in the presence of oxygen is described in Eqs. (11)–(13) (Tang et al., 2009).

$$H_2S + 0.5\,O_2 \rightarrow S^0 + H_2O \qquad \Delta G^o = -209.4\ \text{kJ/reaction} \tag{11}$$

$$S^0 + 1.5\,O_2 + H_2O \rightarrow SO_4{}^{2-} + 2\,H^+ \quad \Delta G^o = -587.1\ \text{kJ/reaction} \tag{12}$$

$$H_2S + 2\,O_2 \rightarrow SO_4{}^{2-} + 2\,H^+ \quad \Delta G^o = -798.2\ \text{kJ/reaction} \tag{13}$$

Monitoring of the kinetics of sulfide removal by a suspension of SOB biomass confirmed that the final product of the oxidation of sulfides depends on the proportion of dissolved oxygen and concentration of sulfide sulfur. If the oxygen dose is adequately controlled, chemolithotrophic sulfur-oxidizing bacteria are capable of oxidizing sulfur compounds to oxidation state 0 (Tang et al., 2009). Due to low air flow rate (0.7 – 0.9 $m^3/(m^3\ d)$) there was reached a sharp drop in the concentration of H_2S in biogas while concentrations of sulfates in output were not recorded. This suggests the formation of elemental sulfur inside the reactor (van der Zee et al., 2007). The further higher dosing of oxygen allows oxidation of formed elemental sulfur up to sulfates. However, in practice, maintaining a constant oxygen/sulfide ratio is relatively difficult because mostly concentration of sulfides changes over time. Maintenance of optimal oxidation–reduction potential may more precisely control the oxidation of sulfides (Janssen et al., 1998).

Thiosulfates and polysulfides as intermediate products of the oxidation of sulfides are formed at high sulfides load and extreme pH conditions. Thiosulfates can also be formed by chemical oxidation (autoxidation) of sulfides and polysulfides in alkaline conditions, and may further be oxidized to sulfates by bacterial activity (Steudel, 1996).

Since the biooxidation of sulfide sulfur consists of a sequence of successive oxidation reactions, it is clear that sulfur-oxidizing bacteria are capable of oxidizing all reduced sulfur compounds, which are the intermediate products of complete oxidation of sulfides.

In the case of removal of some organic sulfur compounds, it was found that these compounds are converted by sulfur-oxidizing bacteria to hydrogen sulfide, which is further oxidized as sulfides. This operation is carried out mainly by the bacteria of the genera *Hypnomicrobium* and *Thiobacillus*. Studies with the bacteria *T. novellus* proved that, for instance, dimethyl disulfide and dimethyl sulfide are first transformed to methanethiol, which is further oxidized into sulfide. In the treatment of a mixture of these compounds decomposition rates and efficiency decrease in the order: hydrogen sulfide (100%), methanethiol (100%), dimethyl disulfide (87%) and dimethyl sulfide (73%), whereas dimethyl sulfide is slowly degradable (Cha et al., 1999). *Pseudomonas* sp. disposes of similar metabolic pathways as *Thiobacillus sp.* However, it cannot utilize methanethiol and dimethyl disulfide as a carbon source, but only as a source of sulfur (Ito et al., 2007).

3.2.1. Effect of oxidizing agent and sulfide sulfur ratio

Many studies are focused on this issue. Alcantara et al. in their experiments found out that at ratios of the molar concentration of dissolved oxygen and sulfides 0.53, 1.1 and 3.5, sulfides were quite rapidly oxidized to elemental sulfur and thiosulfate. Oxidation up to sulfates took place only after additional dose of oxygen and only at the highest molar ratio of 3.5. Another study, that used microbial consortium with dominated *Thiobacillus sp.*, concluded that for the molar ratio of oxygen and sulfide from 0.5 to 1.5, sulfides are partially oxidized to elemental sulfur, and only the higher ratio 1.5 – 2.0 ensures the complete sulfide oxidation to sulfates. When the molar ratio of oxygen and sulfide is below 0.5, the sulfide oxidation rate is very low (Alcantara et al., 2004). The same fact was confirmed by the Janssen study (Janssen et al., 2009), which proved that the molar ratio of oxygen to sulfides, if we want to oxidize sulfides only to elemental sulfur, should be in the range of 0.6 to 1.

Elemental sulfur is thus produced with a limited supply of an oxidizing agent or high concentration of sulfides in the medium, i.e. at the lower molar concentration ratio of oxygen and sulfides. Unlike chemical hydrophobic sulfur, biologically produced elemental sulfur is hydrophilic in character and can be dispersed in water (Janssen et al., 1999). This results in the formation of foam, which has a constant height at low concentration of elemental sulfur and low air flow rate but is unstable. At higher elemental sulfur content and higher air supply, stable foam is created with a fast growing layer. This foam may occur in case of higher concentration of sulfur and higher concentration of supplied oxygen, when the local concentration of sulfur near to the liquid/gas surface is close to its critical concentration, which leads to the formation of a stable network structure of the S_8 molecules. This creates a surface film that prevents breaking of air bubbles that rose to the liquid level, and these contribute to the increase of the height of the foam. This problem occurs particularly in bioreactors working with suspended biomass. The anti-foaming effects may be exhibited by polysulfides, as they are capable of changing the surface properties of biologically produced sulfur, or that they may be chemically oxidized to hydrophobic elemental sulfur (Kleinjan et al., 2006).

Bacteria of *Thiothrix sp.* or *Beggiatoa sp.* in conditions of excess sulfides or oxygen deficiency accumulate elemental sulfur in the cells; after exhaustion of sulfides they continue with the oxidation of sulfur to sulfates (Schmidt et al., 1987). If we want to remove sulfur from the system and use it (e.g. as fertilizer), the most suitable type of bacteria is *Thiobacillus*, which excretes the produced sulfur outside the cell in the form of small particles able to agglomerate into aggregates reaching sizes of up to 3 mm.

3.2.2. Mechanisms of biooxidation of thiosulfates

Biooxidation of thiosulfates by bacteria *T. thioparus* in the first step creates tetrathionates (Eq. (14)), which are further metabolized via tri- and dithionates to sulfates. Present elemental sulfur was formed probably non-biologically (Vishniak, 1952).

$$S_2O_3{}^{2-} + 0.25\,O_2 + 0.5\,H_2O \rightarrow 0.5\,S_4O_6{}^{2-} + OH^- \qquad (14)$$

Mechanisms of thiosulfate biooxidation run by splitting of thiosulfates into sulfides and sulfites and their subsequent oxidation to sulfates (*Thiocystis violacea*, *Thiocapsa roseopersicina*) (Dahl and Trueper, 1989; Kampf and Pfennig, 1980). The conversion of thiosulfate to sulfate (Eq. (10)) by a multi-enzyme system (*P. versatus*) was studied in Cobley and Cox (1983), Lu and Kelly (1983) and Oh et al. (1998). The oxidation of thiosulfate to tetrathionate can be carried out by the bacterium *T. novellus* SRM, cultivated on substrate containing $Na_2S_2O_3$. During the oxidation of $S_2O_3^{2-}$ to $S_4O_6^{2-}$ and subsequently to SO_4^{2-} *T. novellus* gained the energy for growth. A decrease in pH was observed, which was caused by the formation of acidic intermediate products (Cha et al., 1999).

3.3. Biological oxidation of sulfides with oxidized forms of nitrogen—autotrophic denitrification

The biological oxidation of sulfides with the oxidized forms of nitrogen is a process known as autotrophic denitrification. The composition of the final products of this process and the rate of removal of both ions depend on the N/S molar ratio, loading rate, ratio of sulfides and nitrates and on the initial concentration of sulfides.

3.3.1. Influence of initial concentration of sulfides

The biooxidation of sulfides with nitrates as acceptors of electrons released during the oxidation of sulfides proceeds in a similar way as with oxygen, i.e. in two stages. However, a lower rate of nitrate removal was observed in the second phase. This means that the oxidation of sulfur to sulfates requires less nitrates as electron acceptor, than the oxidation of sulfide to elemental sulfur. Thiosulfates as intermediate products of the oxidation of sulfides are present only at the beginning of oxidation, and only in small concentrations. The sulfide removal rates in the presence of nitrates at a concentration of 10 mM were observed at sulfide concentrations of 2.1, 6.3, 10.7 and 16.3 mM. Rate increased linearly with initial sulfide concentrations up to 10.7 mM. Further increase in the concentration of sulfides to 16.3 mM resulted in a significant decrease in the rate of their removal and increase of the lag phase, which was caused by the inhibitory effect of sulfides on autotrophic denitrifying bacteria present (An et al., 2010).

Cardoso also presented similar results, and confirmed that increase of the initial concentration from 2.5 to 10 mM reduced the removal rate of both sulfides and nitrates 21 times (Cardoso et al., 2006).

Chen et al. determined the inhibition limit of sulfides for granulated biomass of heterotrophic and autotrophic denitrifiers under anaerobic conditions as 200 mg/L (Chen et al., 2008a). Later, they however successfully isolated the new consortium H7 comprising *Penibacillus sp.* and micro-aerophilic *A. aneurinilyticus*, which were in micro-aerobic conditions (dissolved oxygen concentration 0.4 – 0.8 mg/L) capable of effective removal of sulfides at a concentration of 280 mg/L in the presence of nitrates and acetic acid. Nitrates were completely reduced, but as the significant product of this reduction was nitrites, whose concentration however decreased with increasing sulfide doses (Chen et al., 2010). From the above-stated, it follows that the concentration of sulfides significantly influences process of autotrophic denitrification and it is therefore necessary to control it efficiently.

3.3.2. Influence of molar ratio N/S

The final product of the oxidation of any reduced form of sulfur depends on the molar ratio N/S and type of reduced sulfur form used, which is clear from Eqs. (15)–(19) (An et al., 2010; Cardoso et al., 2006; Gadekar et al., 2006) as well as from Eqs. (20)–(22) (Jing et al., 2008).

$$S^{2-} + 0.4\,NO_3^- + 2.4\,H^+ \rightarrow S^0 + 0.2\,N_2 + 1.2\,H_2O$$
$$\Delta G^o = -191.0\ \text{kJ/reaction} \qquad (15)$$

$$S^o + 1.2\,NO_3^- + 0.4\,H_2O \rightarrow SO_4^{2-} + 0.6\,N_2 + 0.8\,H^+$$
$$\Delta G^o = -547.6\ \text{kJ/reaction} \qquad (16)$$

$$S^{2-} + 1.6\,NO_3^- + 1.6\,H^+ \rightarrow SO_4^{2-} + 0.8\,N_2 + 0.8\,H_2O$$
$$\Delta G^o = -743.9\ \text{kJ/reaction} \qquad (17)$$

$$S^{2-} + 4\,NO_3^- \rightarrow SO_4^{2-} + 4\,NO_2^-$$
$$\Delta G^o = -501.4\ \text{kJ/reaction} \qquad (18)$$

$$S^{2-} + NO_3^- + 2\,H^+ \rightarrow S^0 + NO_2^- + H_2O$$
$$\Delta G^o = -130.4\ \text{kJ/reaction} \qquad (19)$$

$$5\,HS^- + 8\,NO_3^- + 3\,H^+ \rightarrow 5\,SO_4^{2-} + 4\,N_2 + 4\,H_2O$$
$$\Delta G^o = -3848\ \text{kJ/mol} \qquad (20)$$

$$5\,HS^- + 5\,NO_3^- + 5\,H^+ \rightarrow 2.5\,S^0 + 2.5\,SO_4^{2-} + 2.5\,N_2^- + 5\,H_2O$$
$$\Delta G^o = -2564\ \text{kJ/mol} \qquad (21)$$

$$5\,HS^- + 2\,NO_3^- + 7\,H^+ \rightarrow 5\,S^0 + N_2 + 6\,H_2O$$
$$\Delta G^o = -1264\ \text{kJ/mol} \qquad (22)$$

Eq. (17) represents the stoichiometric molar ratio N/S = 1.6 at which complete oxidation of sulfides to sulfates and complete reduction of nitrates to nitrogen gas proceed. In the presence of excess nitrates, where the molar ratio N/S = 4 (Eq. (18)), although the sulfides are completely converted to sulfates, the reduction of nitrates, however, ends on at the intermediate product of this reaction—nitrites. Even less favorable reaction in terms of energy takes place in the presence of insufficient amount of nitrates when the system is overloaded with sulfides and the molar ratio N/S < 1.6. When the molar ratio N/S = 1, both the incomplete oxidation of sulfides, which ends at elemental sulfur and the reduction of nitrates ending at nitrites occur (Gommers et al., 1988; Reyes-Avila et al., 2004) (Eq. (19)). This reaction also has the lowest energy yield expressed as a Gibbs energy change. In this case the presence of elemental sulfur was derived from the white–yellow turbidity of liquid (Krishnakumar and Manilal, 1999), eventually from the balance of removed nitrate and hydrogen sulfide (0.46) and the ratio of the resulting nitrogen gas and removed hydrogen sulfide (0.19). The incomplete oxidation of sulfides to elemental sulfur may explain this inequality (Cardoso et al., 2006).

Study with different molar rations N/S demonstrated that at the highest molar ratio used, i.e. N/S = 1, all sulfides were oxidized to sulfates although this ratio was lower than corresponded to stoichiometry; at N/S ratio = 0.32 only 4.4% of the sulfides were converted to sulfates during oxidation (An et al., 2010).

If nitrates are in excess as compared with the stoichiometry, the final products of their reduction are nitrites (Eqs. (18),(19),(25)), which may also serve as electron acceptor (Eqs. (23),(24),(26)) (An et al., 2010; Mahmood et al., 2007a).

$$S^{2-} + 0.67\,NO_2^- + 2.67\,H^+ \rightarrow S^0 + 0.33\,N_2 + 1.33\,H_2O$$
$$\Delta G^o = -240.3\ \text{kJ/reaction} \qquad (23)$$

$$S^{2-} + 2.67\,NO_2^- + 2.67\,H^+ \rightarrow SO_4^{2-} + 1.33\,N_2 + 1.33\,H_2O$$
$$\Delta G^o = -920.3\ \text{kJ/reaction} \qquad (24)$$

$$S^0 + 3\,NO_3^- + H_2O \rightarrow SO_4^{2-} + 3\,NO_2^- + 2\,H^+ \qquad (25)$$

$$S^0 + 2\,NO_2^- \rightarrow SO_4^{2-} + N_2 \qquad (26)$$

It is thus clear that by selection and control of the suitable molar ratio N/S, it is possible to influence the final products of the oxidation of sulfides and denitrification.

3.3.3. Autotrophic denitrification with thiosulfates

In the presence of nitrates, the complete oxidation of thiosulfates can also take place (Eq. (27)) (Manconi et al., 2007).

$$S_2O_3^{2-} + 1.6\,NO_3^- + 0.2\,H_2O \rightarrow 2\,SO_4^{2-} + 0.8\,N_2 + 0.4\,H^+$$
$$\Delta G^o = -765.7\ \text{kJ/reaction} \qquad (27)$$

Thiosulfates as electron donor for the chemolithotrophic denitrification bacteria are more advantageous than elemental sulfur or sulfides. They require the same amount of nitrates as sulfides (see Eqs. (17) and (27)), but are not toxic in higher concentrations for denitrifiers such as sulfides (Sorensen et al., 1980). Nevertheless, high concentrations of sulfides do not influence their oxidation to elemental sulfur as much as the reduction of nitrates to nitrites (Cardoso et al., 2006). The contribution to disadvantages of elemental sulfur in comparison with thiosulfates is also the limited availability of solid elemental sulfur for bacteria, as the main factor influencing the biooxidation of elemental sulfur is its specific surface (Koenig and Liu, 2001). Thiosulfates are the best donor of electrons usable by sulfur-oxidizing bacteria; elemental sulfur is the least advantageous.

3.4. Influence of changes in pH and loading rate of nitrogen and sulfur

The influence of the molar ratio N/S has already been described. Nevertheless, even when retaining this ratio, the process may be influenced by sudden change of the loading of both ions. If this shock loading takes a longer time, bacteria may acclimatize and the process may gradually come back to the initial efficiency of removal of both ions. Jing et al. tested the shock change of pH and of load of sulfide and nitrate on the culture of the anaerobic reactor in wastewater treatment plant. Culture was adapted over a period of 4 months to sulfides (520 mg/L) and nitrates (92 mg/L), the effluent concentration was 0.51 mg/L sulfides and 8.92 mg/L nitrates, whereas the nitrites were not detected. The concentrations of both ions in influent to the reactor were increased to 2, 2.5 and 3-fold; the initial value and stepwise increase in pH was from 7.0 to 8.0, 9.0 and 10.0, while maintaining constant all parameters of the continuously operated reactor—HRT 4 h with a recirculation ratio of 2.5 – 3. It was found that bacteria were more sensitive to the shock change in pH than to the load change of both ions, which resulted in an increase of the concentration of sulfides in effluent. At pH 10.0, this was 31-fold as compared with the initial state. The value of pH thus proved to be important in maintaining of process efficiency. However, the efficiency of the reactor was more quickly restored upon shock change of pH than upon shock change of the concentrations of both ions, but the time required for return to normal operation was proportional to the extent of the shock change (Jing et al., 2008).

4. Technological applications of biological oxidation of sulfides

4.1. Biological desulfurization of biogas

Given that hydrogen sulfide may reach a wide range of concentrations in biogas from 500 ppmv to 20,000 ppmv, it is necessary to have such biological methods, which are capable of removing it under different conditions. Biofilters, biowashers and bioscrubbers are being used long-term for the removal of commonly found concentrations of hydrogen sulfide from gases. However, for high concentrations of hydrogen sulfide in gases only a few types of operationally applicable devices have been developed for the present specifically for the desulfurization of biogas—Thiopaq® Process (Paques, The Netherlands) (THIOPAQ), Sulfothane ™ (Biothane, USA) (SULFOTHANE).

For an assembly of desulfurization equipment, forms of cultivation of sulfur-oxidizing bacteria are important; it can be the suspension form or the form of biofilm on a suitable carrier. The way of hydrogen sulfide capture is also significant and can be realized in separate unit or simultaneously with biooxidation.

4.1.1. Bioreactors for desulfurization using phototrophic bacteria

Utilization of anaerobic phototrophic bacteria could be suitable for removal of H_2S from biogas, particularly for elimination of potential security risks coming from mixture of CH_4 with O_2. Moreover, if the input of light to the reactor is well-regulated and controlled, then the oxidation product of H_2S is mainly elemental sulfur, which is non-toxic and non-corrosive. The light source in this case is a key factor in terms of cost and is a major limiting factor to broader application of phototrophic degradation processes. In case of usage of economically advantageous light energy, it is possible to expect the emergence of phototrophic systems that could compete with currently preferred physiochemical methods. In the glass photobioreactor, it is possible to use natural daylight, but artificial light sources (white light, IR) are used more often. Green sulfur bacteria are preferred for the decomposition of H_2S to elemental sulfur IR light with a wavelength of 700–900 nm. For determination of the best light source, the light-emitting LEDs with a maximum wavelength of 710 nm, reflector bulbs and a combination of LED lights and fluorescent lights were compared. The LED diodes proved to be the best light source and also their combination with fluorescent emitter (Kim and Chang, 1991b).

A typical reactor intended for the decomposition of H_2S using phototrophic bacteria is an agitated tank, continuously or semi-continuously operated for the gas phase and cyclically operated for the liquid phase with nutrients. The bacteria can be suspended in a liquid phase or immobilized on different carriers. By comparison of the bioconversion of H_2S to elemental sulfur using *Chlorobium thiosulfatophilum* cultivated in suspension or immobilized in alginate carrier, it was found that the fixed cells consumed 30% less light energy than the free cells at the removal rate of H_2S 68 mg/(L h) (Kim and Chang, 1991a).

A further possibility for cultivation of phototrophic microorganisms is usage of tubular photoreactors that are operating continuously. The tubes can be both horizontal and vertical; spiral arrangement can extend the retention time in the reactor. The material of tubes is transparent for light but impermeable to oxygen. The bacteria grow in biofilm on the inner walls of tubes. Quite interesting results were obtained already in the 80 s of the last century in a reactor containing 12.8 m long Tygon tube with an inner diameter of 3.2 mm. In this arrangement 95% H_2S was removed with a retention time of 24.6 min and a loading rate of 67 mg/(L h) (Kobayashi et al., 1983). Better results were obtained by Henshaw and Zhu with loading rates from 111 to 286 mg/(L h); the efficiency of conversion of sulfides to elemental sulfur reached 92–95% (Henshaw and Zhu, 2001).

The system combining the advantages of phototrophs and chemotrophs was examined for desulfurization of biogas. Two separate biofilters were used; one was colonized by bacteria *C. limicola* and the other by *T. denitrificans*. The bioreactor containing phototrophic bacteria was used during the daytime at natural sunlight and at night the flow of biogas was diverted to the reactor with chemotrophs. The advantage of this arrangement is also operational flexibility of the system, where it is possible to remove elemental sulfur from the biofilter chamber or do its maintenance at the time when the second bioreactor is in operation (Syed et al., 2006).

4.1.2. Micro-aeration

In many biogas plants with the moderate concentration of hydrogen sulfide, micro-aeration is used for desulfurization. Micro-aeration is based on the controlled supply of limited amount of air directly to the gas space of anaerobic reactors or to the fermentation mixture and potentially present sulfur-oxidizing bacteria can oxidize hydrogen sulfide only to elemental sulfur, which is removed with suspended solids of digestate. The air or oxygen dose must be controlled to ensure only the oxidation of hydrogen sulfide, but not to reduce the yield of methane. An appropriate parameter, which can be used to characterize the micro-aerobic environment is oxidation–reduction potential (ORP). However, the range of ORP values, which would characterize the micro-aerobic environment, significantly differs in the case of some authors (Jenicek et al., 2010). The method is a suitable alternative to chemical methods mainly due to a simple application and low operating and investment costs and is especially suitable for lower concentrations of hydrogen sulfide in biogas. Desulfurization process proceeds directly in the anaerobic reactor and there is no need for any additional bioreactors.

Oxygen dosing into the anaerobic reactor is only partially consumed by the sulfur-oxidizing bacteria for the oxidation of hydrogen sulfide; also facultative anaerobic bacteria are able to use it, and some little amount remained in biogas. Anaerobic bacteria can tolerate the presence of very low oxygen concentrations both in a granular sludge (Kato et al., 1994), and suspended culture (Estrada-Vázquez et al., 2003). Using micro-aeration, it is possible to reduce hydrogen sulfide in biogas to less than 500 mg/Nm^3 without reducing efficiency of anaerobic fermentation (Fdz-Polanco et al., 2009). Tests of methanogenic activity (TMA) confirmed that there is no decline in the activity of the individual functional groups of anaerobic bacteria. As micro-aeration decreases the sulfide concentration, better decomposition of organic matter occurs, which was confirmed by reduction of the VSS/TSS ratio and by decrease of concentration of dissolved organic substances, ammonia and phosphates. Ability of micro-aeration to desulfurize biogas with a lower content of hydrogen sulfide is known; nevertheless, some laboratory experiments have demonstrated capability of sulfur-oxidizing bacteria to remove even very high concentrations of hydrogen sulfide (around 7.5 g/m^3) (Jenicek et al., 2010), but full-scale experience is lacking. By comparison of two reactors operating one in anaerobic and another in micro-aerobic conditions, it was found that micro-aeration affects the behavior of anaerobic bacteria consortium. The chemical composition of two anaerobic sludge samples does not differ; however, the micro-aerobic culture showed better dewaterability and low foaming potential and in liquid phase lower concentration of COD of 33% was found (Jenicek et al., 2014).

Attempts to replace oxygen in the anaerobic reactor nitrates, which should alleviate the disadvantages of the oxygen use in an anaerobic environment in the production of biogas, have proven to be unsuccessful. Delivered nitrates were denitrified preferably present heterotrophic bacteria, whose thermodynamic reaction was energetically more favorable than reaction of SOB (Díaz et al., 2010).

4.1.3. External bioreactor with suspension culture of sulfur-oxidizing bacteria

The first step in the external desulfurization of biogas is absorption of hydrogen sulfide to some liquid, preferentially alkaline liquid (Eq. (28)). The absorption liquid is highly buffered HCO_3^- and CO_3^{2-} (Eqs. (29) and (30)), and therefore no pH fluctuations occur during the consumption of NaOH. The liquid needs to content also nutrients for SOB as it represents for them cultivation medium (Krischan et al., 2012).

$$H_2S + Na^+ + OH^- \rightarrow HS^- + Na^+ + H_2O \tag{28}$$

$$H_2S + Na^+ + HCO_3{}^- \rightarrow HS^- + Na^+ + CO_2 + H_2O \tag{29}$$

$$H_2S + 2\,Na^+ + CO_3{}^{2-} \rightarrow HS^- + HCO_3{}^- + 2\,Na^+ \tag{30}$$

The liquid from the absorber is dosed to the bioreactor supplied by air and sulfides are oxidized to elemental sulfur (Eq. (31)). The suspension of biomass goes into a lamellar settler, where the settled sulfur is sucked out from the bottom and sulfur-oxidizing bacteria suspension returns to the bioreactor (Krishnakumar et al., 2005).

$$HS^- + 0.5\,O_2 \rightarrow S^0 + OH^- \tag{31}$$

If oxygen is sometimes supplied in excess due to fluctuation of the sulfide concentration, sulfates are formed with associated undesirable acidification of the system (Eq. (32)).

$$HS^- + 2\,O_2 \rightarrow SO_4{}^{2-} + H^+ \tag{32}$$

It is subsequently necessary to neutralize medium and balance the concentration of Na^+ ions. On industrial scale, it is technically not possible to avoid oxidation of part of the sulfide sulfur to sulfates associated with acidification. This is also enhanced by abiotic oxidation, which leads to the formation of thiosulfates. Therefore, part of the absorbent liquid must be regenerated with NaOH (Elkanzi, 2009).

The absorption step is not only applied in the case of a suspension bioreactor, but is necessary also in the case of other type of SOB cultivation. In separated absorption and oxidation units, treated biogas is advantageously not contaminated with nitrogen and non-consumed oxygen from supply of air, but the investment costs are higher. Therefore in some cases absorption of hydrogen sulfide and biooxidation with air takes place in one combined unit; the system is more sensitive to precise control of air dosing.

4.1.4. External bioreactor—biofilter

Operation of bioreactor with suspended biomass may result in washout of bacteria from the reactor and in some cases also in forming of undesirable foam. For this reason, it is suitable to use biomass immobilized on various types of carriers. Depending on the type of carrier bioreactor can be operated as biofilter or as fluidized bed bioreactor (Krishnakumar et al., 2005; Potivichayanon et al., 2006).

Stationary carriers are used for immobilization of SOB in biofilter. The absorption of hydrogen sulfide can be realized in a separated unit or coupled with oxidation in biofilters (Namgung et al., 2012; Panza and Belgiorno, 2010). Biofilters can be operated as submerged filters or as trickling filters. Biogas is dosed together with oxygen or air to the bottom of biofilter; absorbent liquid containing nutrients and non-consumed sulfides are recycled back to the top of biofilter. In this system hydrogen sulfide is absorbed into liquid and oxidized either subsequently or simultaneously by the immobilized bacterial culture (Koe and Yang, 2000; Syed et al., 2006).

The common requirements on a suitable carrier for bacterial immobilization are sufficiently large specific surface area, porosity of the material, low pressure losses, low cost, light weight, high ability to retain water (the moisture contents of the packing should be between 40% and 60%) and the ability to absorb gas odor (Cho et al., 2000). When selecting a suitable carrier, it is also important that produced elemental sulfur is properly separated from the culture and can be separated from the system. Many materials suitable for this application are discussed in literature: porous ceramics (Lee et al., 2006), lava (Cho et al., 2000), polyurethane foam, coconut fiber (Filho et al., 2010), peat and compost (Chung et al., 1996), Raschig rings or different shaped plastic. Frequently used material is granular activated carbon. This has the additional advantage apart from the immobilization of the culture; it can balance sudden increase of the hydrogen sulfide load by its adsorption and subsequent release at lower loading (Duan et al., 2005; Jiang et al., 2008). Further advantages of activated carbon are good mechanical resistance, large specific surface area for the immobilization of bacteria and possibility of better control of the process as regards the distribution of gas, fluctuations in the pressure and pH values and apart from hydrogen sulfide, it is also capable of capturing other pollutants (Sagastume and Adalberto, 2003).

4.1.5. External desulfurization—fluidized bed reactor with biomass carrier

Biomass in fluidized bed reactors is immobilized on small carrier particles that are fluidized by liquid up-flow. Krishnakumar et al. tested a fluidized bed reactor with internal circulation, in which the carrier constituted 1 mm particles of extruded low density polyethylene (990 kg/m^3) with porcelain clay. *T. denitrificans* was dominant in immobilized biomass. The interesting reactor design enables to keep the active biofilm without clogging and excellent transfer hydrogen sulfide from biogas to liquid and biofilm. The tubular reactor had another tube inside and fine bubble aerator was installed in its bottom. The aeration caused up-flow of liquid in the inner tube and return flow in the outer space. The flow was adequate to fluidize carrier particles. The reactor was monitored using ORP (−350 to −400 mV) and pH (pH 8) sensors. The produced sulfur settled at the bottom of the reactor and could be removed from settling space in the bottom and the possibility of its further oxidation to sulfates or formation of polysulfides was limited. The sulfide load was gradually increased from 2.1 kg/(m^3 d) to 20.5 kg/(m^3 d) by increasing of the influent at a constant concentration of sulfides. Up to a sulfide load of 19 kg/(m^3 d), the efficiency was almost 100% and 80% of the removed sulfides were oxidized to elemental sulfur and only 3% to sulfates. Above sulfide load of 22 kg/(m^3 d), the efficiency declined, probably due to the toxicity of sulfides. At a load of 30 kg/(m^3 d), efficiency dropped to 90%, the concentration of thiosulfates increased (to 15–8%) and polysulfides started to appear in the liquid. At low loads of sulfides to biomass and uncontrolled air supply, the predominant proportion of the oxidized sulfur is present in the form of sulfates and at high biomass load, the biological activity of the sulfur-oxidizing bacteria decreases, and the proportion of the chemical oxidation and formation of polysulfides and thiosulfates increases. *T. denitrificans* disposes with adaptability to high sulfide loads and is considered as an excellent candidate for desulfurizing bioreactors. Fluidized bed bioreactor was proven to operate at much higher loads than the previously known reactors, because the sulfur produced is efficiently removed from the system, and it is advantageous for the accumulation of sulfur-oxidizing bacteria in the biofilm (Krishnakumar et al., 2005).

4.2. Simultaneous denitrification and oxidation of sulfides

Biological oxidation of sulfur and reduction of oxidized forms of nitrogen are both processes used in technologies of environment protection. From the above it follows that in some cases it is useful to combine these technologies in wastewater treatment plants. Denitrification is inevitable technology needed for reaching very strict limit of nitrogen in treated wastewater effluent. Removal of hydrogen sulfide from biogas is also necessary and mutual coupling of both processes is always beneficial. Autotrophic denitrification is therefore a suitable alternative or supplement of heterotrophic denitrification in the case of an insufficient quantity of biodegradable organic substrate. The main objective of the technology application may be autotrophic denitrification in wastewater treatment line, where the organic substrate for nitrogen removal is totally or partially replaced by sulfide sulfur and efficiency of denitrification is emphasized. The other objective may be focused on removal of sulfides, which are subjected to biological oxidation in the presence of nitrates as electron acceptors and stress is put on efficiency of sulfide removal.

4.2.1. Autotrophic denitrification

Autotrophic denitrification is not yet a widespread technology in wastewater treatment plants; it is still in phase of research and pilot scale application. Several applications of autotrophic denitrification have been implemented on the treatment of industrial wastewater. Pure culture of *T. denitrificans* or enriched methanogenic culture mostly in immobilized form was examined. The applicability of simultaneous denitrification and oxidation of sulfides is represented by the following newest experiences and results of research papers.

Manconi compared autotrophic denitrification using thiosulfates and sulfides as an electron donor in three systems—with pure culture of *T. denitrificans*, with activated sludge and activated sludge enriched with pure culture of *T. denitrificans*. Experiments were carried out at different molar ratios N/S. Mixed reactor with volume of 2 L was operated at 30 °C and pH 7.5 ± 0.1. From comparison of denitrification rates in activated sludge and enriched culture of *T. denitrificans*, it was apparent that activated sludge did not contain a significant amount of autotrophic denitrifiers and in enriched culture *T. denitrificans* is responsible for denitrification. Enriched activated sludge after adaptation to sulfides already from the beginning of the operation reduced nitrates directly to gaseous nitrogen, without accumulation of nitrites as intermediate products of denitrification. Complete oxidation of thiosulfates with only activated sludge was observed during the first 22 days of operation of the reactor, when the molar ratio N/S was 1.2 and nitrate load was 10 mmol/(L d). The efficiency of nitrate removal in this period was 90%, but nitrites were formed as by-products. Reducing the molar ratio N/S from 1.2 to 1.0 resulted in the complete disappearance of nitrites; the nitrates were removed completely while low concentrations of thiosulfates remained in the effluent. Even after the nitrate load was gradually increased to 29 and 35 mmol/(L d), the denitrification efficiency remained at 100%. When using sulfides as an electron donor, the average nitrogen removal efficiency exceeded 90% at low molar ratio N/S in the range 0.5 to 0.9. The nitrate load was increased from 1.43 to 11.87 mmol/(L d). After the molar ratio N/S increased to 1.1, low concentrations of nitrite appeared at effluent and nitrogen removal efficiency dropped to 60%. Further increase of the molar ratio N/S to 1.8 caused limitation of sulfide at nitrate load of 16.65 mmol/(L d), and not only nitrites, but also nitrates started accumulate as a consequence of lack of electron donors for the reduction of the oxidized forms of nitrogen (Manconi et al., 2007).

The significant effect of the molar ratio N/S to the composition of intermediate products and final process efficiency was confirmed. In all cases sulfide oxidation efficiency reached 100%, even in the case of sulfide overdose with respect to the stoichiometry (1.6). If the molar ratio N/S = 0.6, the sulfides were oxidized to sulfates without the presence of intermediate products. For lower molar ratio N/S values with excessive amount of sulfides, reduced intermediate products were formed (the milky appearance in the reactor indicated the presence of elemental sulfur). Determination of the optimum molar ratio N/S is important not only for maintenance of conditions with maximum denitrification rate, but also due to the fact that at values above this optimum accumulation of nitrites was observed. These nitrites can subsequently have a highly negative effect on the activity of bacteria in the reactor. A pure culture of *T. denitrificans* is strongly inhibited, when the concentration of nitrites is above 100 mg/L. In reactor with sulfides as electron donor nitrite concentration of 96 mg/L caused washout of biomass from reactor. On the other hand, excess sulfides also can be inhibitory not only for *T. denitrificans*, but also for other bacteria (Gommers et al., 1988).

Another study of anaerobic mixed culture gradually adapted to sulfides and nitrates on the contrary showed particular resistance to high concentrations of nitrites, which are referred to be toxic at higher concentrations to autotrophic denitrifiers. This culture preferred nitrites as electron acceptors, i.e. electrons released during oxidation of sulfides are used much more efficient during nitrite reduction than at nitrate reduction. Reactor using nitrites tolerated a very high load, 1920 mg/L of sulfides and 2265 mg/L of N-NO_2^-, unlike the reactor using nitrates as the electron acceptor, which tolerated only 580 mg/L of sulfides and 140 mg/L of N-NO_3^- (Mahmood et al., 2007a). In the study of denitrifying bacterium *Pseudomonas stutzeri* it was found that under anaerobic conditions, nitrites during the transfer of electrons from the sulfide oxidation trigger the synthesis of the cytochrome a2-c, which increases the resistance of bacterium to high concentrations of nitrite (Kodama, 1970).

Can-Dogan accomplished series of experiments with real wastewater containing both nitrates and sulfides, which was obtained from wastewater treatment plant treating industrial wastewater. Activated sludge adapted for 52 days to nitrates and sulfides was used as a source of bacteria. Due to proper adaptation, significant effects of hydraulic retention time, sulfide load or the molar ratio NO_3^-/S^{2-} on efficiency of removal of sulfides did not appear. The presence of nitrates ensured the efficiency of sulfides removal at 90% even under volumetric loading of 2.004 kg/(m^3 d). However the molar ratio NO_3^-/S^{2-} had a significant impact on the nitrate removal efficiency. Given the molar ratio $NO_3^-/S^{2-} > 4.5$, nitrogen removal efficiency was 25% at nitrate loading rate of 1.039 kg/(m^3 d); decrease of the molar ratio NO_3^-/S^{2-} to 0.7 increased the efficiency of nitrate removal up to 98% while maintaining the sulfide removal efficiency without accumulation of nitrites. In this case, the final product of sulfides oxidation was elemental sulfur in the form of yellow solid particles. If sulfide oxidation was not limited by lack of nitrates and nitrates and sulfides were in stoichiometric ratio, complete oxidation of sulfides to sulfates occurred. Effective combination of hydrogen sulfide removal from biogas and denitrification of wastewater under the optimal conditions at the aerobic wastewater treatment line is possible and advantageous (Can-Dogan et al., 2010).

4.2.2. Biological oxidation of sulfides with different oxidized forms of nitrogen

By comparison of nitrates and nitrites as electron acceptors in the oxidation of sulfides by means of an anaerobic culture adapted to sulfides, it was ascertained that this mixed culture is resistant to high concentrations of the substrate, mainly nitrites, which are toxic at higher concentrations to other autotrophic denitrifiers and prefers nitrites as electron acceptors, i.e. that nitrites utilize the electrons released during the oxidation of sulfides much more efficiently than nitrates. The reactor that uses nitrites tolerated a very high substrate load, in the case of sulfides up to 1920 mg/L and 2265 mg/L of N-NO_2^-. An important parameter is also an appropriately set hydraulic retention time, which greatly affects the reaction rate (Mahmood et al., 2007b).

Similar study monitored the effect of various nitrogen electron acceptors on the oxidation of sulfides. If nitrates were used for desulfurization their influent concentration was lower than for nitrites, but in nitrate presence the process coped with a higher concentration of sulfides. The process occurred in a bioreactor with suspended biomass and recycling of fluid. The culture was tolerant to higher concentrations of sulfides, but more sensitive to the presence of nitrites, so nitrates are recommended as a more suitable electron acceptor (Jing et al., 2010).

It is therefore clear that a very important parameter in the process of biological oxidation of sulfides with oxidized forms of nitrogen is the mixed culture of denitrifying microorganisms used and the representation of their various species in it.

4.2.3. Combination of autotrophic and heterotrophic denitrification

Heterotrophic denitrifiers use organic substances as electron donor for reduction of sulfides; autotrophic denitrifiers use reduced sulfur compounds. If the system contains both types of denitrifiers, it is possible to simultaneously remove sulfides, nitrates and organic compounds with high efficiency. However, excessive sulfides inhibit the activity of denitrifiers and it is therefore necessary to control their concentration efficiently. In the case of the system under micro-aerobic conditions, the inhibitory effect of sulfides is limiting for denitrifiers. Chen set the inhibition limit of sulfides at 200 mg/L under anaerobic conditions for granulated biomass of heterotrophic and autotrophic denitrifiers (Chen et al., 2008b). However, they succeeded in isolation of a new consortium H7 comprising *Penibacillus sp.* and micro-aerophilic *A. aneurinilyticus*, which was in micro-aerobic conditions (dissolved oxygen concentration 0.4 – 0.8 mg/L) capable to remove of sulfides at concentration of 280 mg/L in the presence of nitrates and acetic acid effectively. Nitrates were completely reduced, but nitrites were a significant product of this reduction. However, their concentration decreased with increasing sulfide doses (Chen et al., 2010).

Removal of sulfides associated with denitrification represents a complex of interactions between autotrophic and heterotrophic denitrification bacteria and it is very difficult to describe this system and define a proper approach to management and control of this process. Moreover, autotrophic denitrification bacteria are not flock forming; they grow mainly dispersed and thus subject to washout from the system. On the contrary, heterotrophic denitrifying bacteria form micro flocks enable immobilization of autotrophic bacteria.

Activated sludge enriched with a pure culture of *T. denitrificans* in a semi continuous reactor resulted in suspension culture of sulfur denitrifiers with good sedimentation properties, because non-flocculate bacteria such as *T. denitrificans* have a chance of capture and immobilization with flocculate heterotrophic bacteria. Heterotrophs present subsequently used waste products from the microbial metabolism and dead cell content as sources of organic carbon and energy. However, given the fact that the concentration of sulfides may be a limiting factor for the growth of autotrophic denitrifiers and heterotrophic denitrifiers are rapid growing, heterotrophic denitrification in the presence of organic substances usually prevailed (Ongcharit et al., 1989).

Comparison of heterotrophic and autotrophic denitrification with different concentrations of organic substrate was accomplished by Pokorna et al. in batch experiments with activated sludge from a large wastewater treatment plant. In experiments without organic substrate, it was demonstrated that the addition of sulfides has a positive effect on the removal rate of oxidized forms of nitrogen only up to a sulfide concentration of 200 mg/L. Upon exceeding this concentration limit, significant accumulation of nitrites occurs and no nitrogen was practically removed from the system. In the case of heterotrophic denitrification with abundant organic substrate, any applied concentration of sulfides caused inhibition. Significant slowing of denitrification was observed already from sulfide concentration about 50 mg/L. The same as for autotrophic denitrification, there is substantial accumulation of nitrates upon exceeding the concentration of 200 mg/L. In case of combination of organic substrate and sulfide, organic substrate was preferentially used, and after depletion autotrophic denitrification with sulfides occurred. It confirmed that in the case of low concentration of organic substrate, sulfides are capable of replacing it as an electron donor (Pokorna et al., 2013). The presence of organic substances, e.g. acetic acid serving as a carbon source, may increase total denitrification rate (Cardoso et al., 2006). However An using a mixed culture enriched with *Thiomicrospira* CVO ascertained that in the presence of acetic acid, first chemolithotrophic denitrification took place and it was followed by heterotrophic nitrate reduction only after depletion of the sulfides (An et al., 2010). Due to the addition of sulfide, the operating costs of denitrification in wastewater treatment plants could be decreased by saving external sources of organic carbon with subsequent removal of undesirable pollution of sulfides.

The study on the combined two-stage heterotrophic denitrification process in a fluidized bed reactor, and autotrophic denitrification with sulfur was presented by Liu. Given the influent concentration of sulfides 200 mg/L, organic carbon 33.6 mg/L and the hydraulic retention time of 2.67 h, they attained removal efficiency of 99.9% for sulfide and 91.8% for organic carbon. Volumetric loading rate of sulfides was 800 mg/(L d), and corresponding loading rate of oxidized forms of nitrogen was 472.5 mg/(L d) of NO_3^- and 180 mg/(L d) of NO_2^-. This simultaneous autotrophic and heterotrophic denitrification and desulfurization process showed the feasibility of removing sulfides with nitrites and nitrates also in the presence of organic substances (Liu et al., 2009).

4.2.4. Combination of biogas desulfurization and denitrification

The impact of the oxidizing agent used, pure oxygen, air and nitrates was studied on biogas from the anaerobic reactor with a high concentration of sulfide. Addition of oxygen to the reactor at the rate of 0.25 Nm^3/m^3 of the input substrate reduced the concentration of hydrogen sulfide from 15 811 mg/Nm^3 to 400 mg/Nm^3 of biogas. Supply of air at the rate of 1.27 Nm^3/m^3 removed more than 99% of the sulfide with a final concentration of 50 mg/Nm^3. The addition of nitrate to the reactor did not have the desired effect; it is thus not appropriate to associate denitrification with desulfurization directly in the anaerobic fermenter, but an external bioreactor is necessary (Díaz et al., 2010).

The combined process of washing hydrogen sulfide from biogas and denitrification on the WWTP line was also tested on a pilot scale. The biogas scrubber volume was 5 m^3 and activated sludge and a nitrified wastewater flow were fed to it from the WWTP operations. The biogas flow rate was from 5 to 25 m^3/h and the wastewater flow rate was from 2.5 to 15 m^3/h. The sulfide volume load was in the range of 2 to 22 $kg/(m^3\ d)$. The optimal ratio of the flow of wastewater with sludge and biogas seemed to be 2 – 3 m^3/m^3; the minimum retention time was 10 min. The removal of nitrogen in the form of nitrates and nitrites was proportional to the removal of sulfides. At a sulfide volume load of 2 – 4 $kg/(m^3\ d)$, the system had a hydrogen sulfide removal efficiency of 95%. The results of this study show that biogas desulfurization may be successfully integrated into wastewater treatment technology (Baspinar et al., 2011).

5. Conclusions

Considering sulfur pollution, serious problems are caused mainly by sulfur in reduced form as sulfides due to their odor and toxic characteristics. Recently a growing interest in the application of biological methods of sulfide removal has led to increase in research of sulfur oxidizing bacteria and their technological application. This review has collected and evaluated the most important data about microbiology and ecology of sulfur oxidizing bacteria and possibility of technological utilization of their promising properties. The perspectives of successful exploitation of biotechnology with sulfur oxidizing bacteria are especially in environment protection.

Acknowledgments

This research was supported by the Technological Agency of the Czech Republic—project TA01020798.

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