Introduction

The sulfate-reducing bacteria (SRB – group 7) are capable not only of assimilative sulfate reduction but also of dissipatory reduction of sulfate or sulfur. The dissipatory pathway is the source of energy for SRB. The reductive character of metabolism, especially of the dissipatory pathway requires strictly anaerobic conditions for SRB growth. These pathways are coupled to the utilization of hydrocarbon derivatives; lactate is a very good substrate for most SRB. However, Widdel and Pfennig (1981) postulated that the Gram-positive strains of Desulfotomaculum acetoxidans never utilized lactate as an electron donor and sporulated only when acetate was the organic substrate. Consequently, Campbell and Singleton (in Bergey’s Manual of Systematic Bacteriology) described this species as growing on media with acetate, but not with lactate (Campbell and Singleton, 1986). In contrast, we found that Desulfotomaculum acetoxidans DSM 771 consumed lactate, too (Pado and Pawłowska-Ćwięk, 2004). Because the ability of this species to grow on medium with lactate remains controversial (Holt et al., 1994), we have attempted to thoroughly investigate the growth of Desulfotomaculum acetoxidans DSM 771 on acetate and on lactate. We also determined the effects of these carbon sources on the antioxidative activity of this bacterium.

Material and Methods

Material. Desulfotomaculum acetoxidans strain DSM 771 was grown at room temperature (19–23°C). The primary inoculum was 1 ml active culture from Deutsche Sammlung von Mikroorganismen. Half volume of the inoculum was used immediately
to inoculate 50 ml of medium with 42 mM acetate and the other half to inoculate 50 ml of medium with 42 mM lactate. After 3 weeks both cultures were supplemented with 50 ml of the respective fresh medium and further kept in the dark at room temperature. After the next 3 weeks these cultures broths were used as the inocula (45 ml) for the first 32-day culture (culture I). Each inoculum was supplemented with the respective fresh medium 3 weeks before the next culture (culture II – strictly on completion of the first cultures and culture III – six months after the first culture was completed). All the cultures were conducted in parallel for acetate or lactate-supplemented media in 500 ml Erlenmeyer flasks each containing 450 ml of the culture medium. The lactate concentration was determined on the basis of the results of earlier cultures (Pado and Pawłowska-Ćwiśk, 2004). In the first series of cultures, also the culture in lactate-supplemented medium but inoculated with the acetate-containing inoculum was executed. After fixing oxygen detectors and inoculation, the media were immediately covered with a liquid paraffin layer (about 5 mm thick), which was maintained throughout the culture. This paraffin layer made easier monitoring, particularly of oxygen level in the culture, without the risk of the culture being exposed to air.

Other medium components were as follows: 21.12 mM Na2SO4, 1.15 mM KH2PO4, 4.02 mM KCl, 5.61 mM NH4Cl, 1.13 mM CaCl2, 1.97 mM MgCl2, 85.55 mM NaCl and trace elements (according to DSM-bank instruction) (Pado and Pawłowska-Ćwiśk, 2004, Pawłowska-Ćwiśk and Pado, 2005).

Growth. The classic Monod’s method (Monod, 1949) of graphical representation of bacterial growth in continuous cultures (bacterial growth curve) consists in plotting the number of living cells in 1 ml of culture broth as a function of cultivation time but this assay is very time-consuming. Therefore it is frequently replaced by a simpler method, based on culture turbidity measurements (nephelometry) used for cell number evaluation (Gottschal, 1992). On the basis of the results of our earlier study the high correlation coefficient (0.6295) between the Monod’s and nephelometry methods was found. The culture turbidity measurements facilitated much faster determination of the cell division index (CDI). The turbidity of culture broth was measured throughout the cultures (as shown in Table I) at 580 nm using a Specol 11 colorimeter with a TK attachment (Carl Zeiss Jena). Each result in the table is an arithmetic mean from five measurements with standard deviation ranging from 4 to 11%. Prior to sampling the flasks were gently manually agitated for 10 min. On the basis of turbidity values the cell division index (CDI) was calculated:

\[ CDI = \frac{\tau_x}{\tau_0} \]

where: \( \tau_0 \) – turbidity at \( \tau_0 \) (CDI on the day of inoculation is 1.00), \( \tau_x \) – turbidity in successive days of the culture (\( \tau \)).

Chemical analysis. Proteins and reduced glutathione contents were assayed (without using any cell membrane disrupting agents) in culture broth supernatant after centrifugation at 6000×

The colorimetric analyses and spectrum scanning were performed using the CECIL 8020 spectrophotometer. The standard curves obtained for known concent-

### Table I

Average protein levels (mg/ml) in culture broth supernatant during successive cultures

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<tr>
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<th>0(^a)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>17</th>
<th>21</th>
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<td>13.3</td>
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<td>9.15</td>
<td>10.8</td>
<td>16.2</td>
<td>13.5</td>
</tr>
</tbody>
</table>

\( a \) – inoculation day; \( b \) – standard deviation [%]
Antioxidant activity of *D. acetooxidans* DSM7713
tations of respective standards. All the reagents (including standards: albumin for protein, Na₂S for hydrogen sulfide and GSH) were of analytical grade from Merck or Fluka. The results of triplicate assays are presented as arithmetic mean ± the standard deviation (the latter was similar for protein, GSH and H₂S) (Table I).

The oxygen level in culture broth was measured before mixing the cultures to avoid an error caused by air diffusion (the differences of oxygen concentration before and after mixing were 2.6–4.3 µM O₂). Because between the measurements, the cultures were kept without mixing, these measurements were made very gently for five different positions of the culture CTN-980 R oxygen detector (ELSENT Poland) coupled to a CX-315 microcomputer pH/oxygenmeter (ELMETRON Poland).

**Results and Discussion**

**Cell division index.** The obtained values of CDI revealed that the bacterium grew faster on lactate (about 2-fold higher turbidity) than on acetate within the first two weeks of the culture (Fig. 1). After this time, in the first and the third cultures, the CDI was slightly higher on acetate. After 24 days CDI was again higher (about 3-fold) for the lactate containing culture medium (in culture III). However, the culture on lactate medium but inoculated with acetate inoculum showed increase of neither cell division index nor hydrogen sulfide level. On the other hand, the lack of an increase in CDI values when the lactate medium was inoculated with acetate inoculum indicates that the adaptation process requires a relatively long time for the changeover of metabolic pathways, essential for switching on lactate catabolism. The necessity of a changeover of metabolic pathways was confirmed by an earlier observation regarding the synthesis of different redox proteins in cultures of three *Desulfovibrio* strains grown on hydrogen or lactate (Steger *et al.*, 2002).

The cell division is related to biosynthesis processes, particularly the production of proteins. According to some authors (Hancock and Poxton, 1988; Russel, 1988) “free” wall-associated proteins will continue to be synthesized and will be released directly into the culture supernatant. The obtained average content of determined protein in supernatant was at least 2-fold higher in lactate cultures and in culture I (when the cultures were inoculated with the youngest inoculum) were even 3-fold higher than in acetate cultures (Table I and II). Interestingly, as the inoculum grew older the average protein level was progressively reduced in both cultures, but more in the cultures on lactate. Thus, the above-mentioned decrease in the protein content in cultures inoculated with aged

![Cell division index during cultivation of cultures on acetate (thin lines) or lactate (thick lines): culture I – solid lines; culture III (six months after the first series was completed) – dashed lines; culture on a medium with lactate but inoculated with an acetate inoculum – pointed line. For more clear illustration culture II (after the first series was completed) is not presented, since it was similar to series I. Student’s t-test values for lactate to acetate culture in successive cultures: I – 1.967 (statistically insignificance where p>0.05); II – 3.969 (0.01 significance level); III – 2.588 (0.05 significance level).](image)
inoculum suggests that the age of the inoculum compromises the capability of cells to synthesis and secretion of proteins. The highest coefficient of correlation between CDI and protein level was found for culture I (Table III). However, this correlation coefficient in culture III (with 7-month-old inoculum) was much higher for the lactate than acetate culture.

Reports on *D. acetoxidans* are rather scarce. This species has not been grown earlier on lactate (Stackebrandt *et al*., 1997; Hristova *et al*., 2000; Scholten and Stams, 2000; Boscher *et al*., 2001; Londry and Des Marais, 2003; Londry *et al*., 2004), because it was commonly believed to be unable to grow on media containing lactate as a sole carbon source (Widdel and Pfennig, 1981; Campbell and Singleton, 1986; Holt *et al*., 1994). Our experience showed that *D. acetoxidans* DSM 771 was also capable of catabolic utilization of lactate (Pado and Pawłowska-Ćwięk, 2004; Pado and Pawłowska-Ćwięk, 2005; Pawłowska-Ćwięk and Pado, 2005). However, in agreement with earlier findings (Widdel and Pfennig, 1981; Campbell and Singleton, 1986), we did not observe sporulation, even after 80 days of culturing in the presence of lactate (Pado and Pawłowska-Ćwięk, 2004).

The obtained relationship between the secreted protein level and CDI is reflected in the correlation coefficient but only in the first series, especially in the acetate culture (Table III). These results are in accordance with the data of Londry and Des Marais (2003) who used $^{13}$C acetate. Those authors proved that *D. acetoxidans* (unlike three other species of SRB) effectively incorporated acetate into biomass via acetyl-CoA. Moreover, they observed that this species was capable of lithotrophic growth using carbonate and gaseous CO$_2$. This lithotrophic growth capability could explain the better growth of *D. acetoxidans* in acetate culture but only after two weeks of the cultivation (in the first and second cultures), when carbonate (including dissolved CO$_2$) accumulated as a consequence of acetate catabolism (Fig. 1).

**Reduced glutathione.** Also the GSH level was higher in cultures with lactate than in those with acetate (Fig. 2–4). As the cultures with lactate produced slightly higher levels of both GSH and H$_2$S it suggests that lactate is more advantageous for the assimilatory and dissimilatory sulfate reduction pathways too. It is known that lactate contains more hydrogen atoms than acetate and this is very important in sulfate reduction processes. Since cell membranes were not disrupted prior to GSH determination, the measured GSH was extracellular. The results obtained in all three cultures (designated I, II and III) indicate that GSH biosynthesis and secretion began immediately after inoculation and in early cultures the GSH level increased more rapidly than that of H$_2$S (Fig. 2–4). The early GSH domination over H$_2$S suggests priority of the assimilatory over the dissimilatory pathway.

The initial sulfate concentration in the media was 21 mM. On the basis of the highest GSH levels (always during the first four days of the cultures), sulfur incorporation from sulfate into GSH was counted: it ranged from 0.35 to 2.55‰ in acetate cultures and from 0.68 to 3.74‰ in lactate cultures. Contrary to

![Fig. 2. Hydrogen sulfide (solid lines) and glutathione (dotted lines) levels within culture I: acetate cultures (thin lines) or lactate cultures (thick lines).](image-url)
Antioxidant activity of *D. acetooxidans* DSM7713

In our expectation we did not find any correlation between GSH and H$_2$S levels (Table III). The absence of such correlation can explain different metabolic requirements of bacterial cells during the cultivation. The observed regular, significant and negative values of correlation coefficients between GSH and oxygen (the more GSH the less oxygen) prove that the reduced glutathione performs the role of an antioxidant, as expected (Table III, compare Fig. 2–4 and 5). The antioxidant role of GSH was especially clear at the beginning of cultivation (the first 4 days), because within this period the greatest decreased in oxygen level was observed (by about 100 µM).

As it is well known, GSH is the major antioxidant agent (both extra- and intracellular) in all live organisms (Poot *et al.*, 1995; Deneke 2000; Hand and Honek, 2005). The obtained results (the ratio of GSH and protein concentration) showed that lactate stimulated the production of GSH, so thus increasing the antioxidant activity of the examined strain. These levels of GSH were lower (Table II) as compared to Fareleira *et al.* (2003) (1.8±0.6 nmol GSH per mg protein of *Desulfovibrio gigas* cells). However, we determined the extracellular GSH, while those authors determined the total GSH. In this work, a rapid increase in GSH level was observed at the beginning of cell growth, which was in agreement with the stimulatory effect of lactate on GSH production. This indicates that the release of intracellular GSH into the external medium is a regular metabolic event during bacterial cultivation, as proven by the observed regular, significant and positive values of correlation coefficients between GSH and lactate (the more GSH the more lactate).

**Fig. 3.** Hydrogen sulfide (solid lines) and glutathione (dotted lines) levels within culture II: acetate cultures (thin lines) or lactate cultures (thick lines).

**Fig. 4.** Hydrogen sulfide (solid lines) and glutathione (dotted lines) levels within culture III: acetate cultures (thin lines) or lactate cultures (thick lines).
the culture (Fig. 2–4). However, Fareleira et al. (2003) did not observe any significant differences when oxygen concentration in the medium or the duration of the oxic period in D. gigas cells were increased. This phenomenon may be explained by GSH secretion initiated by O2 and/or reactive oxygen species present in the fresh medium and taxonomic differences between these strains. Extracellular oxygen-utilizing processes are well known in eucaryotic Deuteromycotina (Odier and Artaud, 1992; Leonowicz et al., 2001). This strategy should also apply to anaerobic bacteria because it protects the cells against the penetration of toxic radicals into the cytosol.

**Hydrogen sulfide.** The amounts of H2S (or sulfide) were much greater in cultures grown on lactate than on acetate (Table II): in the first culture about 10-fold higher (during the first week of cultivation – Fig. 2); in the second culture about 30-fold higher (during the first week – Fig. 3); and in the third culture over 45-fold higher (during the first week of cultivation – Fig. 4).

The absorption spectra of 2-days samples (in the first culture) after addition of methylene blue method’s reagents showed the presence of peaks at 411.6 and 665 nm (which is characteristic for product formed in the assay) only for the lactate culture (Fig 5A). Our earlier research showed that the complex of ferrous ions and 4-hydroxy-3-sulfobenzoate and this ligand was product of 4-hydroxybenzoate sulfonation. The 4-hydroxy-3-sulfobenzoate as extracellular metabolite was requisite for sulfate transport processes in this strain (Pawłowska-Ćwik and Pado, 2005). The obtained results suggest that lactate was more efficiently for sulfate transport processes than acetate in D. acetoxidans. The more efficiency of lactate requires further research. The incorporated sulfur index was counted: in lactate cultures it ranged from 4.66 to 6.07‰, while in acetate ones from 0.83 to 1.45‰ (Table II).

### Table II

Average levels of GSH (µM) and protein (mg/ml) in culture broth supernatant and H2S levels in culture broth and Student’s t-test values in individual series

<table>
<thead>
<tr>
<th>Series</th>
<th>Protein Acetate</th>
<th>Protein Lactate</th>
<th>A</th>
<th>H2S Acetate</th>
<th>H2S Lactate</th>
<th>A</th>
<th>GSH Acetate</th>
<th>GSH Lactate</th>
<th>A</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>30.78</td>
<td>94.73</td>
<td>3.08</td>
<td>4.480</td>
<td>40.923</td>
<td>9.13</td>
<td>27.738</td>
<td>53.126</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>5.186</td>
<td>0.146b</td>
<td></td>
<td>0.432b</td>
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<td>0.901a</td>
<td>0.561b</td>
<td>3.731</td>
</tr>
<tr>
<td>II</td>
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<td>58.97</td>
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<td>9.579</td>
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<td>3.61</td>
<td>7.719</td>
<td>13.249</td>
<td>1.72</td>
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<td></td>
<td>15.40b</td>
<td>37.75/a</td>
<td>11.123</td>
<td>0.368b</td>
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<td>0.296/a</td>
<td>0.225/a</td>
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</tr>
<tr>
<td></td>
<td>(0.001)</td>
<td>1.45/c</td>
<td></td>
<td>4.66/b</td>
<td>(0.01)</td>
<td></td>
<td>0.35/c</td>
<td>0.68/c</td>
<td>(0.02)</td>
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<td>21.62/a</td>
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<td>0.837/b</td>
<td>2.050</td>
<td>0.492/a</td>
<td>0.586/a</td>
<td>2.059</td>
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</table>

**A** – the ratio of protein or H2S or GSH average concentration in lactate culture to in acetate culture; bold values – Student’s t-test for lactate to acetate culture; ( ) – significance level; nss – statistically insignificance (p>0.05);

b – decrease relative to previous series (%); a – the ratio of H2S and protein concentration (nmol/mg protein);

b – sulfur incorporation from sulfate (initial concentration 21 mM) into hydrogen sulfide (on final cultivation day) (%); a – the ratio of GSH and protein concentration [nmol/mg protein];

### Notes

1. **SO4**– + 2 CH3CH(OH)COO– → → → 2 acetylCoA + + 2 HCO3– + HS–

2. **SO4**– + 2 acetylCoA → → → 4 CO2 + H2S
Antioxidant activity of *D. acetoxidans* DSM7713

Although the average H$_2$S level decreased in consecutive cultures, this decrease was smaller in the cultures with lactate than acetate (Table II). The calculated correlation coefficients show that H$_2$S amounts correspond to levels of determined proteins (Table III). Despite the levels of both proteins and hydrogen sulfide were reduced in the next culture, but their correlation coefficients increased in the successive cultures, both with acetate and lactate. In lactate culture, in the third culture, was found higher H$_2$S level in samples from immediately inoculated culture than on the next day (in contrast to other culture-compare Fig. 2, 3 and 4). The higher H$_2$S amount in samples from freshly inoculated lactate culture proves extracellular accumulation of hydrogen sulfide. Moreover, the average amount of H$_2$S per mg of protein increased in subsequent series, but only in lactate cultures (Table II). These results suggest that extracellular accumulation of hydrogen sulfide.

![Fig. 5. Absorption spectra of culture samples: 1 – acetate culture; 2 – lactate culture after 2 days of cultivation (A) and 17 days (lactate culture) or 32 days (acetate culture) of cultivation (B) with methylene blue method’s reagents.](image)

### Table III

<table>
<thead>
<tr>
<th>Series</th>
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<th>GSH vs. CDI</th>
<th>GSH vs. H$_2$S</th>
<th>GSH vs. O$_2$</th>
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<th>H$_2$S vs. O$_2$</th>
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<tr>
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<td>-0.5356$^{a}$</td>
<td>0.9357$^{a}$</td>
<td>0.2519$^{a}$</td>
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</table>

CDI – cell division index; $^{a}$ – whole period of cultivation; $^{b}$ – from the fourth day to the end; $^{c}$ – for the first 4 days of cultivation; $^{d}$ – from inoculation day to 21-th day; $^{e}$ – from the second day to the end of cultivation.
proteins bound dissimilated H₂S. Our earlier research showed H₂S accumulation by proteins linked to the cell wall this strain, because hydrogen sulfide levels were much higher in the lysozyme-treated samples than in the untreated samples (Pado and Pawlowska-Ćwik, 2004). Hydrogen sulfide can be bound by cysteiny1 residues of proteins forming disulfides, which release the so-called labile sulfur in acidic environment (Ogasawara et al., 1994). However, the results obtained in this work show a decrease of the amount of extracellular proteins with the age of used inoculum, but may be, the copies number of protein containing of Cys residues increased (e.g. proteins including in the transport processes). According to Russell (1988), these proteins could be associated with the cell wall, but not covalently linked.

Surprising was the fact that, contrary to expectation, O₂ did not decrease H₂S level; in acetate cultures, in the first and the second series oxygen presence was even advantageous, especially from the second day until the end of cultivation (compare Fig. 2–4 and 6). Also the high values of correlation coefficients for these cultures (Table III) show that oxygen could even be a positive factor for the dissipulatory sulfate reduction pathway (e.g. through the influence on sulfate transport processes) (Pawlowska-Ćwik and Pado, 2005). Also Johnson et al. (1997) found a positive influence of oxygen (48 µM) on the growth of Desulfovibrio vulgaris if 250 µM hydrogen sulfide was added to the medium.

Conclusion. Although growth of D. acetoxidans DSM 771 on lactate requires at least two successive passages on this medium the species grows better on lactate than on acetate, which is contrary to earlier observations of other researches. D. acetoxidans lactate cultures produced higher levels of both GSH and H₂S than cultures with acetate, so lactate is a better substrate for metabolic processes, especially the sulfate reduction pathways. The higher level of reduced glutathione in lactate cultures results in the increase of D. acetoxidans antioxidant activity, which could be very important for the survival in natural environment.

Literature


Chambers L.A., P.A. Trudinger, J.W. Smith and M.S. Burns. 1975. Fractionation of sulfur isotopes by continuous culture of...