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Characterization of sulfate-reducing bacteria in yeast industry waste by microcalorimetry and PCR amplification

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Abstract

Sulfate-reducing bacteria (SRB) were isolated from anaerobic sludge of yeast factory wastewater treatment plant by cultivation on Postgate C medium. Microcalorimetry was used to monitor the anaerobic digestion processes and to measure the growth rates of sulfate-reducing bacteria. The maximum growth rates determined by microcalorimetry and ATP analysis were different—$\mu_{max}(dQ/dt) = 0.165 \pm 0.008\,h^{-1}$ and $\mu_{max}(N_{ATP}) = 0.207 \pm 0.013\,h^{-1}$. Experiments on the cultivation of SRB from yeast industry wastewater treatment plant in batch culture showed that during the first 20 h the concentration of sulfate decreased from 78.3 mM down to 62.2 mM while the increase of sulfide production was negligible. Perceptible amount of sulfide (7.82 mM) appeared on the 33.5 h of fermentation together with a peak on the power–time curve and considerable increase in the cell count (1.26×10^9). First steps of sulfate metabolism (activation of sulfate by ATP sulfurylase, production of H_2) are accompanied by endothermic heat effects, therefore the values of thermal power remain moderate until the evolution of sulfide starts. The influence of green microalgae *Chlorococcum* sp. (preparation Biotreat 100) on the growth characteristics of microorganisms was also studied. Identification of one SRB strain was started by sequencing of PCR-amplified 16S rRNA gene. Two sets of primers were used for PCR amplification, both specific for domain Bacteria but giving different gene fragments. PCR-products were purified with JETQUICK kit according to the manufacturer instructions.
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Keywords: ATP; Bacterial growth; Green microalgae; Microcalorimetry; Sulfate-reducing bacteria

1. Introduction

Anaerobic treatment of sulfate-rich wastewaters, e.g. from food industry, fermentation industry, paper and pulp industry, etc. is inevitably accompanied by production of sulfides. Yeast industry wastewaters have high content of organic matter (COD 8900–27000 mg O_2 L^{-1}), betaine (up to 6% w/w) and sulfates (up to 5000 mg L^{-1}) but low content of easily degradable sugars and acids. Sulfate-reducing bacteria (SRB) interact competitively with other anaerobic bacteria involved in methanogenesis, resulting in formation of H_2S rather than methane. In addition to their toxicity and corrosive properties, sulfides have also inhibitory effect on methanogenesis. ATP bioluminescence method is a traditional method for determining growth characteristics of microorganisms, which has been also used for operational control of biological waste treatment [1–3]. We made an attempt to determine these characteristics by microcalorimetry, which is an appropriate method for studying anaerobic processes, however, not sufficiently exploited for this purpose during the past decade. The measurement of different growth characteristics simultaneously with the heat production rate has revealed that the thermal power–time curve is influenced by the metabolic activity and can be related to the different physiological states of bacteria [4,5]. Thus the aim of this study was to develop the microcalorimetric method further in order to adapt it for studying the growth characteristics of microorganisms.

2. Theory

2.1. Sulfate reduction by sulfate-reducing bacteria

The process of anaerobic digestion of sulfate-rich wastewaters consists of five sub-processes: hydrolysis,

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Table 1
The main reactions in anaerobic digester with sulfate-rich wastewaters, performed by SRB

No.	Reaction	$\Delta G'_0$ (kJ mol^{-1})	ΔH_{cat} (kJ mol^{-1})	Reference
Consumption of hydrogen				
1	$H_2 + 0.25SO_4^{2-} + 0.25H^+ \rightarrow 0.25HS^- + H_2O$			[6,7]
2	$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	−38.1		[8]
Production of hydrogen				
3	$Pyruvate^- + H_2O \rightarrow acetate^- + CO_2 + H_2$		−1.64	[6,9]
4	$Lactate^- + H_2O \rightarrow acetate^- + CO_2 + 2H_2$		+73.30	[7]
5	$Propionate^- + H^+ + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + 3H_2 + 2H^+$	+76.1		[10]
		+71.7		[11]
6	$Butyrate^- + H^+ + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2$	+48.1		[10]
		+48.3		[11]
Sulfate and proton as electron acceptors				
7	$Pyruvate^- + 0.2SO_4^{2-} + 0.15H_2O + 0.33H^+ \rightarrow CO_2 + 0.95\ acetate^- + 0.05\ ethanol + 0.087H_2S + 0.113HS^- + 0.1H_2$		−70.2	[6]
8	$Lactate^- + 0.37SO_4^{2-} + 0.56H^+ \rightarrow CO_2 + 0.98\ acetate^- + 0.02\ ethanol + 0.16H_2S + 0.215HS^- + 0.5H_2O + 0.48H_2$		−36.4	[6,12]
9	$2\ Lactate^- + SO_4^{2-} + 3H^+ \rightarrow 2\ acetate^- + 2CO_2 + 2H_2O + HS^-$	−74.5		[13]
10	$Propionate^- + 0.75SO_4^{2-} \rightarrow acetate^- + HCO_3^- + 0.75HS^- + 0.25H^+$	−37.7		[8]
11	$Propionate^- + 1.75SO_4^{2-} \rightarrow 3HCO_3^- + 1.75HS^- + 0.5H^+ + 0.25OH^-$	−88.9		[8]
12	$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−47.6		[8]
13	$Acetate^- + SO_4^{2-} + 3H^+ \rightarrow 2CO_2 + H_2S + 2H_2O$	−57.0		[13]

fermentation, acetogenesis, reduction of sulfates and methanogenesis. The reducing conditions prevailing in anaerobic processes of sulfate-rich wastewaters result in the increase of hydrogen sulfide content. Although part of the sulfides originates from the sulfur containing amino acids, most of them are formed during reduction of sulfates present in the raw sewage. Some of the reactions in anaerobic digester with sulfate-rich wastewaters performed by sulfate-reducing bacteria are presented in Table 1.Sulfate-reducing bacteria which contain hydrogenase (hydrogen: ferricytochrome c_3 oxidoreductase EC. 1.12.2.1) and cytochrome c_3 can either produce or consume molecular hydrogen. H_2 is consumed if they grow by anaerobic oxidation of hydrogen with concomitant reduction of sulfate (Table 1, reactions 1 and 2, [6–8]). H_2 is produced by some species of Desulfovibrio during growth on pyruvate [6,9] or any other volatile fatty acid medium lacking sulfate, using H^+ as electron acceptor (Table 1, reactions 3–6, [7]). In the absence of sulfate, SRB degrade very little volatile fatty acids to acetate, CO_2 and H_2, due to the relatively positive change in free energy, unless H_2 using bacteria (H_2-using methanogens) are present to maintain low concentration of H_2 (Table 1, reactions 3–6, [12]). H_2 is a stringent feedback inhibitor of hydrogenase and prevents the conversion of NADH to NAD^+ that is essential for growth.

Some species of Desulfovibrio utilize both sulfate and H^+ as electron acceptors and produce H_2 if grown in syntrophic association with an H_2 utilizing methanogen (Table 1, reactions 7–13, [6,12]). Already at low concentrations (1 mM) sulfate turned out to be a better electron acceptor than methanogen *Methanosarcina barkeri* or H^+ for SRB *Desulfovibrio vulgaris* Hildenborough (lactate and pyruvate as electron donors) [9]. At high sulfate concentrations (36 mM), molecular hydrogen was produced by the bacteria in a relatively big proportion (Table 1, reaction 8, [6]). This H_2 production was interpreted as a device to minimize H_2S production in the culture media. H_2 production seemed to precede sulfate reduction since a significant amount of H_2 was accumulated in the culture medium before H_2S could be detected [6]. The greater "lack" of energy observed during the growth on lactate ($\Delta H_{met} = -36.4$ kJ mol^{-1}, Table 1, reaction 8) as compared to that on pyruvate ($\Delta H_{met} = -70.2$ kJ mol^{-1}, Table 1, reaction 7) could be also attributed to the greater amount of H_2 produced by the former substrate. In natural conditions the loss of energy induced by H_2 production is counterbalanced by utilization of H_2 and CO_2 from SRB by MB. The main reactions of sulfate reduction in wastewater digesters performed by sulfate-reducing bacteria are the reactions 1–2 and 7–13 in Table 1 [8,13,17]. Sulfate metabolism by SRB comprises several individual stages, presented in Table 2.

Sulfate transport is accomplished by active symport with $3H^+$ or Na^+. Strong inhibitors of transport are molybdate, chromate and selenate. Activation of sulfate by ATP sulfurylase (Table 2, reaction 2) is the first reaction in the reductive assimilation of sulfate for biosynthesis as well as in dissimilative reduction (Table 1, reactions 1, 6–9). APS is referred to as adenosylsulfate or adenosyl phosphosulfonate or adenosine-5′-phosphosulfate. In the same manner molybdate and selenate can be converted by ATP sulfurylase into adenosine phosphomolybdate and adenosine phosphoselenate revealing thus the toxic effect. Formation of APS is thermodynamically unfavourable but the reaction is driven towards completion by coupling to pyrophosphate hydrolysis by pyrophosphatase (Table 2, reaction 3, [13]). After pyrophosphate hydrolysis, APS formation is still unfavourable

Table 2
The main stages of sulfate metabolism of sulfate-reducing bacteria [13,14]

No.	Stage/reaction	$\Delta G'_0$ (kJ mol^{-1})	Process
1	Sulfate transport		Active symport with $3H^+$ or Na^+. Strong inhibitors are molybdate, chromate, selenate
2	$SO_4^{2-} + 2H^+$ ATP $\rightarrow$ APS [AMP-SO_4] + PP_i^{3-}	+46	Activation of sulfate by ATP sulfurylase
3	$PP_i^{3-} + H_2O \rightarrow 2P_i^{2-} + H^+$	−33	Hydrolysis of pyrophosphate
4	APS reductase [4Fe4S], $FADH_2$	−69	
	APS [AMP-SO_4] $\rightarrow HSO_3^- +$ AMP $+ H^+$		Formation of bisulfite
5	$HSO_3^- \xrightarrow{3H_2(6e^-)} HS^- + 3H_2O$	−171	Reduction of bisulfite, formation of hydrogen sulfide

(net ΔG value + 13 kJ mol^{-1}). This reaction is driven by coupling to the exergonic reaction of reduction of APS by APS reductase and formation of bisulfite (Table 2, reaction 4). Bisulfite is further reduced to hydrogen sulfide in a six-electron reduction catalyzed by a soluble cytoplasmic reductase, accompanied again by a considerable exergonic effect (Table 2, reaction 5). From the point of view of energetics two high-energy bonds were used to activate sulfate (Table 2, reactions 2 and 3) and two ATPs per mole lactate oxidized to acetate are generated by substrate level phosphorylation [12].

2.2. Methods for avoiding the inhibitory effect of sulfides

Methods used for avoiding the inhibitory effect of sulfides on anaerobic bioprocesses include dilution of sulfate-rich wastewater, increasing the pH of the reactor, removal of sulfides from wastewater by chemical precipitation, oxidation or stripping [15]. Biological processes for sulfide removal consist in conversion of sulfide into elemental sulfur by colourless sulfur bacteria (Thiobacilli) [15,16,18,19]) according to the following reaction (1):

$$2HS^- + O_2 \rightarrow 2S^0 + 2OH^-, \quad \Delta G_0 = -169.35\,\text{kJ mol}^{-1} \tag{1}$$

or by genera of anaerobic photosynthetic bacteria from the families Chlorobiaceae and Chromaticeae that catalyze the photosynthetic van Niel reaction [20,21]:

$$2nH_2S + nCO_2 \xrightarrow{nv} 2nS^0 + (CH_2O)n + nH_2O \tag{2}$$

In the latter case light radiated to a photosynthetic reactor is coupled to the conversion of sulfide to elemental sulfur using the reverse citric acid cycle (Arnon cycle). According to experimental results, photoautotrophic bacteria produce a higher percentage conversion of S^{2-} to S^0 as compared to chemoautotrophic bacteria. There is also a thermodynamic advantage of using electrons from S^{2-} in comparison to those from S^0 or $S_2O_3^{2-}$: energy change per mole of electrons released from the oxidation of S^{2-} to S^0 is the highest (−26 kJ mol^{-1}) as compared to other sulfur species [21]. The greatest drawbacks of the system with the photosynthetic bacteria remain its low cost effectiveness due to the use of a light source and use of suspended-growth reactors to ensure the transparency of medium.

For the growth of colourless sulfur bacteria producing S^o (chemoautotrophs), it is important for microaerophilic conditions to prevail, e.g. in the presence of sulfide concentrations up to 80 mg L^{-1} only less than 10% sulfate was produced if O_2 concentration remained below 6 mg L^{-1}. Sulfide was concluded to be inhibitory to sulfate producing microorganisms or a more preferred electron donor than sulfur [15]. Later Janssen et al. [18] found the optimal concentration of oxygen to be 0.1 mg L^{-1}, guaranteeing the 92% conversion of sulfide into elemental sulfur. The optimal molar ratio of oxygen to sulfide consumption was 0.7 (autotrophic conditions) and 1.0–1.6 (heterotrophic conditions). Some colourless sulfur bacteria (*Thiobacillus denitrificans*) use sulfur compounds such as thiosulfate, elemental sulfur and sulfide as electron donors and in the absence of oxygen, they use nitrate as electron acceptor [22]. Environments such as sewage digester sludge contain large populations of denitrifiers that can use the sulfide produced by sulfate-reducing bacteria, or compete for electron donors required for sulfate reduction.

2.3. ATP bioluminescence method for determining the growth characteristics of microorganisms

Biological treatment of wastewater is based on the removal of organic matter by a combination of adsorption, oxidation, and assimilation by the microorganisms contained in the sludge biomass. The efficient operation of a wastewater treatment plant (WWTP) thus depends on the reliable monitoring and control of the viable organisms present in the mixed liquor [1]. Operational control of biological waste treatment has long depended on estimates of in situ biomass. A more appropriate and desirable parameter is evaluation of the metabolic activity of those organisms responsible for the treatment [2]. ATP is synthesized from intermediate and final products of substrate oxidation and is also utilized intracellularly for osmotic and mechanical work. In vitro light production by firefly lantern extract has been shown to depend upon the presence of luciferin, the enzyme luciferase, oxygen, magnesium ions, and ATP [2]. Thus, ATP assay technique is a feasible method for determining viable biomass and monitoring and controlling WWTP operation [1]. The occurrence of ATP in all living cells, its absence in nonviable matter, its direct proportionality to both viable biomass and metabolic activity, and the availability of accurate, rapid

and convenient assay have made this method attractive for many applications [1–3].

2.4. Identification of sulfate-reducing bacteria in anaerobic sludge from wastewater treatment plant

Several attempts have been made to identify sulfate-reducing bacteria in anaerobic sludge from wastewater treatment plants using different methods like 16S rRNA hybridization and sequencing. In past decades, various methodologies have been developed that allow rapid identification and quantification of bacteria in anaerobic bioreactors. Examples are detection methods based on direct visualization using transmission electron microscopy [23], use of specific antibodies against whole cells [24] or analysis of ether-lipids [25,26]. Other well-established techniques that specifically detect microbes are based on 16S rDNA or rRNA sequences and include the polymerase chain reaction (PCR) and 16S rRNA hybridisation approaches [27].

There are several advantages of using 16S rDNA or rRNA as target material to detect microorganisms. Currently, a huge database of sequence information is available [28]. This sequence information makes it possible to design primers and probes which allow for a species-specific or a more general identification (at genus or family level) of a bacterium. Furthermore, 16S rDNA primers and 16S rRNA probes can be designed and used for the detection of non-culturable bacteria [29].

3. Materials and methods

3.1. Instruments

Laboratory-scale anaerobic digester (W8, Armfield, UK) of 4.8 dm^{-3} working volume with biogas collection and measuring units was used at the temperature 35–37 °C. It consisted of two independently operating units—upflow anaerobic sludge blanket reactor (UASB) and upflow anaerobic fixed film reactor (UAFF). Sludge seed for the reactors originated from sugar industry waste anaerobic treatment plant, plastic “Bioball” filter elements were used as carrier for biomass in UAFF. The reactors were used as a two-stage setup [30].

For monitoring the process, samples were taken anaerobically from the reactors and placed immediately into the 2277 Thermal Activity Monitor (2277 TAM, Thermometric, Järfälla, Sweden)—an isothermal heat conduction microcalorimeter. Batch experiments were run in standard mode with 3 mL glass ampoules at 35 °C. To initiate experiment, substrate (yeast waste diluted with water to 36.6%), was added to the aliquot of sample taken from the reactor. Four parallel experiments, run simultaneously on the microcalorimeter were stopped at different time moments by removing the ampoule from the calorimeter and adding 2-propanol [30,31]. For analysing isolated cultures of SRB, samples were taken from cultivation test tubes and placed immediately into sterilized 3 mL ampoules. Sterilized growth media (Postgate C [32]) was used as the reference. Prior to the experiment the gas phase in the sample and in the reference ampoules was exchanged for argon gas. Power–time curves were dynamically corrected and registered using data acquisition program Digitam 3.0 (Thermometric, Järfälla, Sweden).

HPLC analyses of the samples from microcalorimeter were performed on Waters HPLC 600 with RI detector (512×), using column HPX 87H and 0.009n·H_2SO_4 as eluent [33]. Sulfate concentration was determined by turbidimetric method (Standard Method 4500-SO_4^{2-} for wastewater [34] and sulfide concentration by methylene blue method (Standard Method 4500-S^{2-} for wastewater [34]).

3.2. Materials and growth media

In order to remove dissolved oxygen, the flask with the growth medium (without reducing agent) was boiled for 2–5 min in the atmosphere of argon gas and cooled to the room temperature. The pH of the medium was adjusted to 6.8–7.2. The medium was dispensed into the 16 mL test tubes (5 mL per tube), adding the reducing agent (2% v/v of 0.1% $Na_2S·9H_2O$ in 0.1% Na_2CO_3) to each tube. Finally the tubes were flushed through with argon, sealed and autoclaved during 15 min at 120 °C.

The supernatant of yeast industry wastewater (pH ≅ 6.0, alkalinity 2.0–2.6 mequiv. L^{-1}), dry matter 152–408 g L^{-1}, COD 8900–27000 mg O_2 L^{-1}, BOD 7700–8700 mg O_2 L^{-1}, N_{total} = 798–1260 mg L^{-1}, P_{total} = 26–53 mg L^{-1}, SO_4 2640–5000 mg L^{-1}) was used in the experiments. Yeast industry wastewater share in the influent was increased step-by-step from 10% to 100%. Gas production was moderate in the start-up period, however COD removal efficiency in both reactors was about 70%. pH of the effluent from both reactors remained without adjustment at 8.0–8.1.

Bacterial growth regulator Biotreat 100 was kindly provided by BimKemi Eesti Ltd. It consists of dried biomass of green microalgae *Chlorococcum* sp.—a unicellular eucaryotic microalgae with spherical or ellipsoidal cells belonging to the phylum Chlorophyta (green algae). Biotreat is a mild natural preparation with non-toxic properties as it could be used to feed other organisms, e.g. minute freshwater crustaceans of the genus Daphnia (a common test organisms for measuring water toxicity).

3.3. Experimental method: isolation of sulfate-reducing bacteria

The cultivation technique used in this study for routine growth of strictly anaerobic bacteria in liquid media has been described by Balch and Wolfe [35], consisting of glass test tubes with thick rubber septa, a Freter-type anaerobic chamber, and a gassing manifold. Injection was carried out in the anaerobic chamber through the rubber septum with a

1 mL syringe to prevent introduction of air into the test tube. For isolation of the colonies of SRB, reduced sterile 2% agar medium (15 mL) was poured into plastic Petri plates maintained in the anaerobic chamber for at least 12 h to remove oxygen. After inoculation, the plates were transferred into the anaerobic cultivation box and the gas phase was exchanged for argon gas [36]. As the first inoculum, a sample (100 μL) from a pilot plant anaerobic reactor treating yeast industry wastewater was transferred into 5 mL liquid growth medium Postgate C [32], followed by inoculation to the agar medium of Postgate C. SRB were identified by production of sulfide and blackening the media containing ferrous ions [17]. The colonies surrounded by a characteristic black circle indicating formation of FeS were chosen and transferred further to the liquid medium of Postgate C. Culture purity was determined by microscopy and microcalorimetric analysis.

3.4. PCR conditions for 16S rRNA gene amplification

PCR conditions for 16S rRNA gene amplification consisted of 1 cycle of 95 °C for 1 min and 30 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 1 min. The reaction was completed by a final extension at 72 °C for 30 min. PCR amplifications were performed in a final volume of 25 μl, containing 2.5 μl of 10X PCR buffer with $(NH_4)_2SO_4$ (Fermentas, Lithuania), 0.2 mM of each deoxynucleoside triphosphates, 0.4 μM of each primer, 1.5 μl of 25 mM $MgCl_2$ solution, 1.0 μl of cell suspension and 1 u of *Taq* DNA-polymerase (Fermentas, Lithuania) [44]. PCR products were analysed by electrophoresis in agarose gels containing ethidium bromide (1 μg ml^{-1}) to check the purity of the bands.

4. Results and discussion

4.1. Identification of sub-processes in anaerobic digester

Microcalorimetry is a perspective and convenient method for monitoring complex anaerobic wastewater treatment processes [37]. Changes in the thermal power–time curves are reflecting changes in the state of bioprocesses in the anaerobic reactor during the experiment (Fig. 1). In the case of multi-stage processes, however, identification and study of the growth of individual bacteria is rather complicated. An attempt to identify and study the processes of sulfate reduction, acetogenesis and methanogenesis was undertaken in our experiments. It was observed that with the increase of sulfate removal in the reactor, the area under the power–time curve up to 10 h increased (Figs. 1 and 2). This allowed us to tentatively identify the process as reduction of sulfates, bacteria responsible for the process being most probably SRB. As seen in Figs. 1 and 2, the increase of acetate concentration during the 20–40 h of calorimetric experiments was

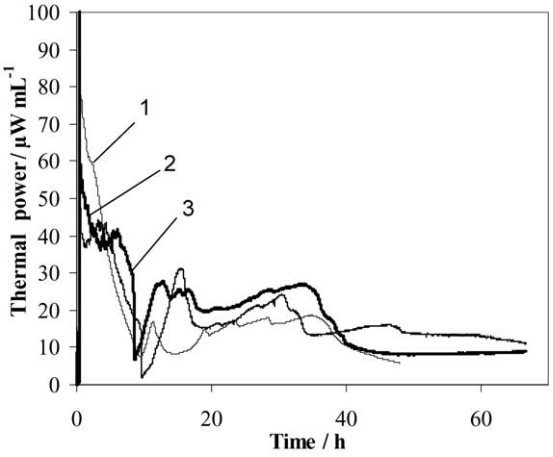

Fig. 1. Adaptation of acetogenic/methanogenic concortia to sulfate-rich yeast industry wastewater in anaerobic process (addition of 50 μl Biotreat from day 43). (1) Day 61, biogas 4.08 L day^{-1}; (2) day 72, biogas 5.51 L day^{-1}; (3) day 83, biogas 7.54 L day^{-1}.

accompanied with the simultaneous growth of several microorganisms observed from the thermal power–time curve (maximum at 25–30 h).

Taking into account the possible mechanism of acetate formation from betaine it could be speculated that these processes reflect among others the cleavage of betaine into trimethylamine and acetate, characteristic to some halophilic fermentative bacteria [38]. The strain *Haloanaerobacter salinarus* sp. nov. can grow by fermenting carbohydrates or by using the Stickland reaction with either serine or H_2 as electron donors and glycine-betaine as acceptor, which is reduced to trimethylamine:

$$2.5\,\text{Glycine-betaine} + 4.04H_2 \rightarrow \text{2-propanol} + 2.5\,\text{trimethylamine} + 0.95\,\text{acetate} + 0.1CO_2 + 1.9H_2O \quad (3)$$

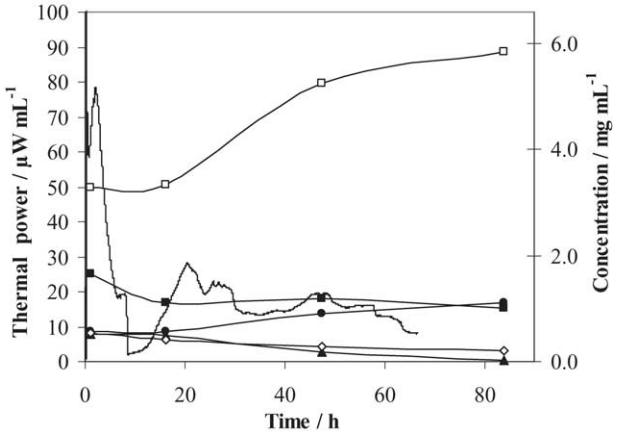

Fig. 2. Thermal power (—) and sugars and acids content in UASB reactor, day 86. Sulfate (■), lactate (▲), acetate (□), propionate (●) and butyrate (◇) concentrations were determined as described in methods.

$$\text{Glycine-betaine} + 1.32\,\text{serine} + H_2O \rightarrow \text{trimethylamine} + 2\,\text{acetate} + 1.32CO_2 + 1.32NH_3 \quad (4)$$

The similar cleavage mechanism for glycine-betaine under anaerobic conditions has been reported also for *Clostridium sporogenes* [39], while the fermentation products of *Eubacterium limosum* are *N*,*N*-dimethylglycine, acetic acid and butyric acid [40]. The produced acetate and trimethylamine can be readily used as carbon and energy sources by acetotrophic (e.g. *Methanobacterium soehngenii*) and methylotrophic methanogens (e.g. *Methanosarcina barkeri*), respectively [41]

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (5)$$

$$4(CH_3)_3N + H_2O \rightarrow 9CH_4 + 3CO_2 + 6H_2O + 4NH_3 \quad (6)$$

and the produced ammonia could participate in the reaction with sulfate [42].

4.2. Identification of sulfate-reducing bacteria from yeast factory wastewater treatment plant

Both fast growing (e.g. Desulfovibrio) and slow growing (e.g. Desulfococcus) sulfate-reducing bacteria are able to partially oxidize some organic compounds into acetate and CO_2 that in turn can be metabolized by methane producing bacteria or the SRB themselves [9,13]. Interactions between these two groups of microorganisms can be very diverse—trophic complementary, inhibition. Of particular importance is the competitive relationship towards the use of hydrogen since it can be the energy source for the both groups of bacteria.

In the case of multi-stage processes, identification and study of the growth of individual bacteria is complicated, therefore a sulfate-reducing bacterial strain was isolated from yeast industry wastewater using selective growth media for SRB (Postgate C [32]), with reduced Fe^{2+} concentration ($0.06\,g\,L^{-1}$) to avoid formation of the precipitate of FeS.

By light microscopy and staining by Gram the isolated sulfate-reducing bacteria (Gram negative *Bacillaceae*, dimensions 1.5-2.0 μm) could belong most probably to the genus Desulfovibrio. To specify the genus further, the 16 s rRNA analysis was started. DNA fragments encoding the 16 S rRNA gene of the domain Bacteria were amplified by PCR using two sets of primers as follows: 341F (5′-CCTACGGGAGGCAGCAG) plus 907r (5′-CCGTCAATTCCTTTRAGTTT) [43] and 16F (5′-AGAGTTTGATCATGGCTCAG) plus 1513r (5′-TACGGTTACCTTGTTACGACTT). 16 S rRNA gene is about 1.5 kb, so the first set of primers gives only a part of this gene as a PCR product while the second set of primers encodes almost whole gene. When gene fragments are purified and sequenced, it is easy to compare PCR-products got with different primers in order to define the position numbers of base pairs. Two samples of bacterial DNA were chosen for analysis, both from one SRB colony. Bacterial cells were re-suspended into sterile water with no extraction of nucleic acids.

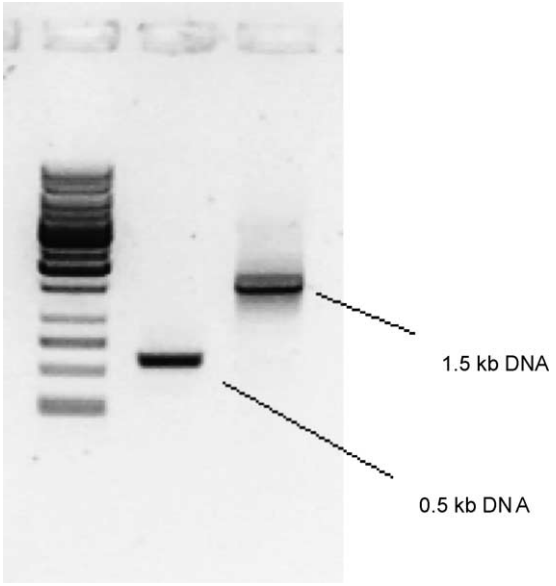

Fig. 3. The separated and purified PCR products (ca. 0.5 and 1.5 kb) extracted with JETQUICK Gel Extraction kit.

PCR products (ca. 0.5 and 1.5 kb) encoding the 16 S rRNA gene were excised from the gel and extracted with JETQUICK Gel Extraction kit. Agarose gel slice was excised and transferred into a tube. Therefore solution L1 was added (contains concentrated $NaClO_4$, sodium acetate and TBE-solubilizer) and the mixture was incubated at 50 °C for 15 min with vortexing. The mixture was loaded into the pre-prepared spin column and centrifuged at maximum speed for 1 min. After that the spin column was re-inserted into receiver tube and reconstituted solution L2 (contains ethanol, NaCl, EDTA and Tris–HCl) was added. After 5 min the mixture was centrifuged twice at maximum speed for 1 min. DNA was eluted by centrifuging at maximum speed for 2 min with 50 μl of sterile water [45]. The separated and purified PCR products (ca. 0.5 and 1.5 kb) are presented on Fig. 3.

4.3. Characterization of sulfate-reducing bacteria by microcalorimetry

By classification of SRB the genus Desufovibrio belongs to the fast growing sulfate reducers converting electron donors from substrate (volatile fatty acids) to acetate that accumulates [12]. Thus it can be concluded that the power–time curves characteristic to pure cultures of these bacteria up to 40 h describe essentially the phase of acetogenesis (Table 1, reactions 7–10) and to a lesser degree that of methanogesis originating from the reduction of a certain part of CO_2. Traore et al. noticed that due to the incomplete fermentation of lactate or pyruvate by the coculture of *D. vulgaris* and *M. barkeri*, the evolved heat quantities were only 57–58% of the theoretical value. Further analysis

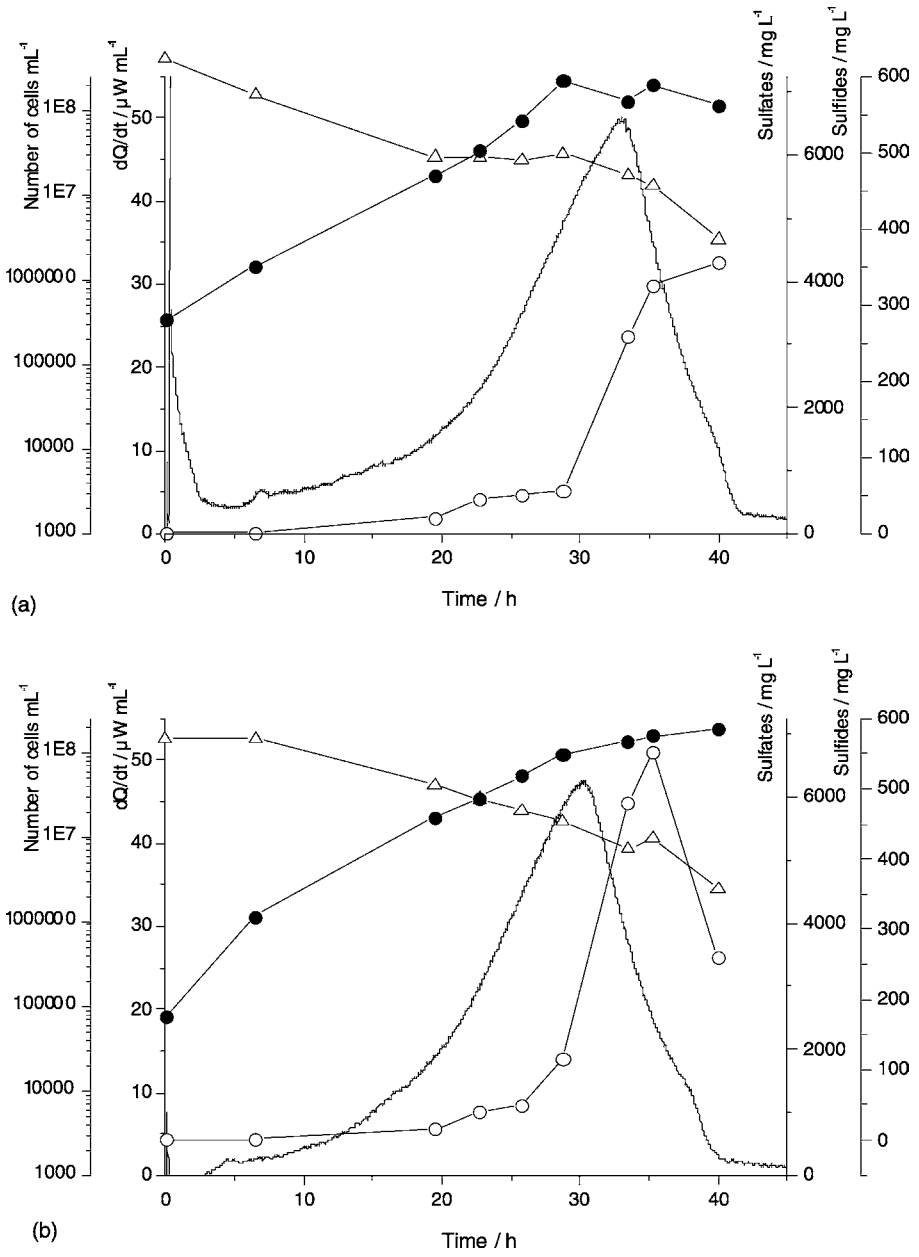

Fig. 4. Cultivation of sulfate-reducing bacteria in batch culture: (a) without preparation Biotreat 100; (b) with supplement of Biotreat 100. Thermal power (—); cell count (●); concentration of metabolites—sulfates (△); sulfides (○).

of fermentation products showed that lactate fermentation continued about 12 days, whereas methane production from acetate and CO_2 lasted for three more weeks [6,9]. This production could not be detected by microcalorimetry because of the extremely low level of heat flux evolved that is in part due to the low reaction rate (for methanogens $\mu_{max} \approx 0.1–0.2\,h^{-1}$) and low exothermicity. The production of about 0.5 mol H_2 per mol of lactate by *D. vulgaris* lowered the enthalpy of reaction from the theoretical value −74.5 (Table 1, reaction 8) to −36.36 kJ (Table 1, reaction 6, [6]).

Our experiments on the cultivation of sulfate-reducing bacteria from yeast industry treatment plant in batch culture in microcalorimeter 2277 TAM are shown in Fig. 4. During the first 20 h the concentration of sulfate decreased considerably (from 7520 down to 5967 ppm) while the increase of sulfide production was negligible. Perceptible amount of sulfide (258 ppm) appeared on the 33.5 h of fermentation together with a peak on the power–time curve and a considerable increase in the cell count (1.26×10^9) determined by ATP analysis. As shown above one of the first steps of sulfate metabolism (activation of sulfate by ATP sulfurylase) is accompanied by endothermic heat effect (Table 2, reaction 2,) therefore the values of thermal power remain moderate ($<10\,\mu W\,mL^{-1}$) until the evolution of sulfide starts. If the growth medium contains lactate as electron acceptor, H_2 production is also significant accompanied by endothermic

value of enthalpy (Table 1, reaction 4). This affirms the earlier presented statement that H_2 production precedes sulfate reduction since significant amount of H_2 is accumulated in the culture medium before significant amounts of hydrogen sulfide can be detected [6].

Algal preparation Biotreat is used in wastewater treatment it acts as an activator for various sulfur bacteria already present in the water. Interestingly, when adding Biotreat to the pure culture of SRB the concentration of sulfides increased (Fig. 4). Obviously this preparation is more effective to various jointly functioning bacterial consortia than to a single isolated pure culture. In sulfate-limited conditions, Biotreat could facilitate switching of some facultative sulfate degrading acetogens to use alternative electron acceptors (e.g. H^+ instead of $SO_4{}^{2-}$).

4.4. Comparison of growth characteristics determined by microcalorimetry and ATP bioluminescence

During the exponential phase of growth the heat evolved per unit mass of bacteria versus time is constant. A linear correlation between the rates of heat production and biomass production has been reported for several microorganisms [5] that makes microcalorimetry a prospective method for monitoring anaerobic bacterial growth processes [31].

Number of cells was counted both by ATP and by heat production taking the yield of biomass per evolved heat amount $Y_{Q/X} = 0.043\,\mathrm{g\,kJ^{-1}}$ for sulfate-reducing bacteria cultivated on lactate [46]. The curves obtained were slightly different, especially in the lag phase and stationary phase (Fig. 5). For metabolic processes for which small total enthalpy change can be assumed or where there is a large difference in the degree of reduction between the anabolic substrates and the biomass (autotrophic growth, reduction of sulfates) the contribution of the anabolic reaction can be significant that might be the reason for the differences between cell count and also the maximum growth rate μ_{max} determined by ATP and microcalorimetry.

Due to environmental factors the maintenance requirements may increase considerably. If this is the case, more energy will be transformed into heat, i.e. the heat yield dQ/dX increases [5] and a smaller part of the carbon and energy source will be conserved as biomass. At the same time determination of the number of cell by calorimetry is based namely on heat yield dQ/dX (or on its reciprocal, biomass yield per amount of evolved heat $Y_{Q/X}$) that is, depending on microorganism in the range of 1–25 $\mathrm{kJ\,g^{-1}}$ [46].

Determination of cell count by ATP is first of all connected with the determination of the amount of biomass. ATP is synthezised in intermediate steps and final reactions of substrate oxidation and it is used in cells also for performing osmotic and mechanical work. ATP is contained in all living cells and its content is directly proportional to the amount of viable biomass as well as to its metabolic activity [3]. Thus, these both express methods for determination of cell count complement each other. The advantage of calorimetric method is its prompt nature (i.e. possibility for on-line monitoring) and resulting from that, preciseness. ATP analysis needs pre-treatment of the sample, this sets limits to the possible number of samples and to the promptitude of analysis; on the other hand, the measurable value is directly the biomass. In the range of exponential growth the bacterial counts determined by both methods are almost identical (Fig. 5). Greater differences can be noticed at the beginning of the exponential phase (obviously due the increase of heat yield) and at the end of the exponential phase (due to the decrease of heat yield and greater conservation of energy into biomass). As by both methods the growth rates of bacteria are calculated from the relation of $\ln X = f(t)$ or $\ln Q = f(t)$ as tangent of slope angle, from Fig. 5 it is evident, why often $\mu_{max}(\mathrm{ATP}) > \mu_{max}(dQ)$. In Table 3 an example on the determination of maximum growth rates μ_{max} for SRB by both methods are presented. Moderate differences (about −20%) in the values of growth rates, determined by microcalorimetry and ATP bioluminescence assay can be observed. The maximum specific growth rate by microcalorimetric data was $\mu_{max}(dQ) = 0.165 \pm 0.008\,\mathrm{h^{-1}}$ and by the content of ATP $\mu_{max}(N_{ATP}) = 0.207 \pm 0.013\,\mathrm{h^{-1}}$.

Finally, plotting the heat production Q against the cell biomass, found from the cell count by ATP, the value of biomass per evolved heat amount for SRB isolated from yeast waste treatment plant was found to be $Y_{Q/X}$

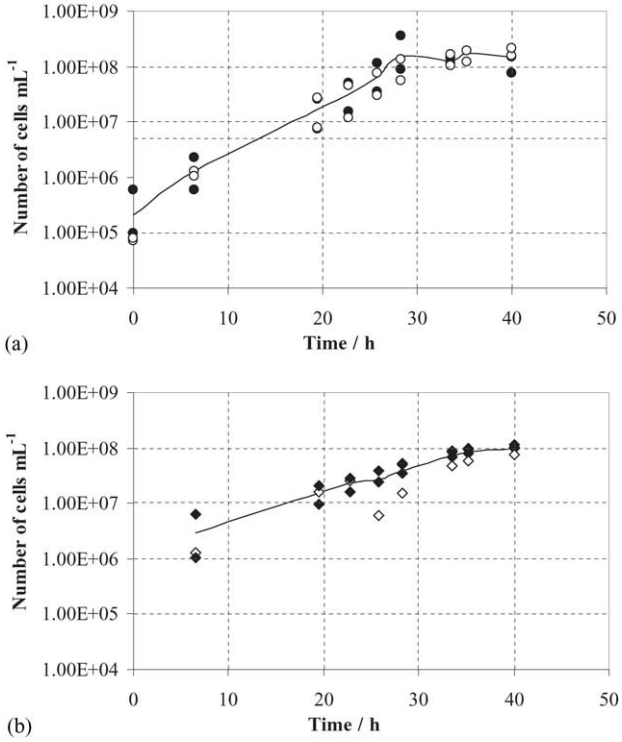

Fig. 5. Number of cells of sulfate-reducing bacteria isolated from yeast waste treatment plant (a) determined by ATP, without addition of Biotreat 100 (●), with 50 $\mathrm{mg\,L^{-1}}$ Biotreat 100 (○); (b) determined by microcalorimetry, without addition of Biotreat 100 (◆), with 50 $\mathrm{mg\,L^{-1}}$ Biotreat 100 (◇).

Table 3
Growth rates of sulfate-reducing bacteria, isolated from anaerobic reactor at various concentrations of preparation Biotreat 100, determined by ATP and thermal power

Concentration of Biotreat 100 (mg L^{-1})	μ_{max}(ATP) (h^{-1})	μ_{max}(dQ) (h^{-1})
0	0.220	0.150
50	0.195	0.153
500		0.171
5000		0.184
Average μ_{max}	0.207 ± 0.013	0.165 ± 0.008

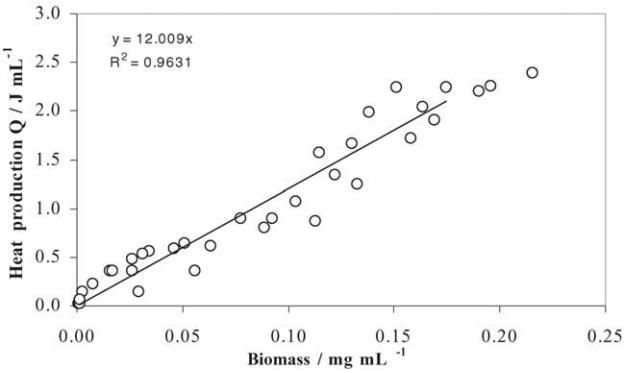

Fig. 6. Heat production as a function of biomass (on the example of SRB isolated from yeast waste treatment plant).

$= 0.083\,g\,kJ^{-1}$ (Fig. 6) that is similar to the analogous SRB grown on pyruvate ($Y_{Q/X} = 0.095\,g\,kJ^{-1}$) [29].

5. Conclusions

Microcalorimetry was used for determination of growth rates of sulfate-reducing bacteria and the value $\mu_{max} = 0.165 \pm 0.008\,h^{-1}$ was obtained. Addition of bacterial growth regulator Biotreat to the pure culture of SRB did not have any influence on the growth rate of these bacteria, but surprisingly the increase of sulfide production was noticed.

The maximum growth rates μ_{max} for sulfate-reducing bacteria determined by microcalorimetry and ATP analysis were different—$\mu_{max}(dQ/dt) = 0.165 \pm 0.008\,h^{-1}$ and $\mu_{max}(N_{ATP}) = 0.207 \pm 0.013\,h^{-1}$. The reason may lie in greater proportion of maintenance energy at low growth yields in the lag phase and in the early exponential phase. In calculation of growth rates from power–time curves the main prerequisite is the constancy of the stoichiometry of biomass during the exponential growth.

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