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Quantification of small enthalpic differences in anaerobic microbial metabolism—a calorimetry-supported approach

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Abstract

A calorimetry-supported approach to the measurement of small enthalpic differences in microbial metabolism is presented. Anaerobic fermentations with the bacterium *Zymomonas mobilis* and the yeast *Saccharomyces cerevisiae* were carried out in a fermenter–calorimeter at a constant dilution rate ($D=0.15\ h^{-1}$). The glucose concentration of the feed was increased stepwise from 10 to 80 $g\,l^{-1}$. Salts, vitamins and trace elements were present in excess. Different steady state conditions were analysed.

For *Z. mobilis*, specific production rates for ethanol, carbon dioxide and heat were three times higher than those measured for *S. cerevisiae*. If the glucose feed concentration exceeded 20 $g\,l^{-1}$ during the *S. cerevisiae* fermentations, glucose could be measured in the fermenter. Further increasing glucose concentrations of the feed caused higher specific ethanol, carbon dioxide and heat production rates, whereas the biomass yield $Y_{X/S}$ decreased remarkably. This result can be explained as the Crabtree effect under anaerobic conditions and interpreted as metabolic uncoupling of anabolic biomass formation and catabolic energy substrate consumption. A futile cycle contributing to the heat production is considered to provide *S. cerevisiae* with a regulatory mechanism in the control of the flux of metabolites through the glycolytic pathway.

Keywords: Calorimetry; Futile cycle; Growth efficiency; Heat production; Metabolic control; *S. cerevisiae*; *Z. mobilis*

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1. Introduction

Calorimetry-supported research is focusing increasingly on the thermodynamic aspects of microbial growth and production processes [1–5]. Research mainly involves aerobic processes causing reasonable enthalpy changes. Despite their potential in biotechnology, calorimetric measurements of anaerobic processes have attracted minor interest. This is due to the mostly small enthalpy changes during these processes and the technical limitations of the systems available [6,7]. In this contribution, a calorimetry-supported approach is presented to measure small enthalpic differences under anaerobic growth conditions. Physiologically well-characterized microorganisms were selected in order to compare their metabolic properties.

The bacterium *Zymomonas mobilis* and the yeast *Saccharomyces cerevisiae* are known to gain the energy required for growth and maintenance under anaerobic conditions by substrate phosphorylation processes along the Entner–Doudoroff pathway and glycolysis, respectively (Eq. (1))

$$1\ glucose \Rightarrow 2\ ethanol + 2CO_2\ \Delta H_R = -84\ \text{kJ mol}^{-1} \quad (1)$$

The conversion of glucose yields the same stoichiometric amounts of ethanol and carbon dioxide. The net yield of energy available for growth and maintenance is 1 ATP for *Z. mobilis* and 2 ATP for *S. cerevisiae* [8]. Due to the different amounts of ATP available for metabolic activity, growth yields and heat production are expected to be different: the biomass yield of *S. cerevisiae* should be higher compared to *Z. mobilis*, whereas the latter should exhibit a considerably higher specific heat, carbon dioxide and ethanol production. As the enthalpy of the anaerobic reaction is low ($\Delta H_R = -84\ \text{kJ mol}^{-1}$) compared to the aerobic reaction ($\Delta H_R = -2.816\ \text{kJ mol}^{-1}$), the total enthalpic differences for heat production and biomass formation are expected to be very small. In addition, the influence of the medium composition on the biomass production has to be considered (Eq. (2)) including the rates of glucose (ΔS_1), ammonium (ΔS_2), and yeast extract (ΔS_3) consumption, and ethanol (ΔP_1), carbon dioxide (ΔP_2), biomass (Δ_X) and heat (ΔQ) production. Therefore, reasonable deviations from the different theoretical yields are to be expected for real fermentations, depending on the specific conditions

$$\Delta S_1 + \Delta S_2 + \Delta S_3 \Rightarrow \Delta P_1 + \Delta P_2 + \Delta X + \Delta Q \quad (2)$$

The experiments were carried out in order to determine the advantages and limitations of a calorimetry-supported approach to the measurement of small enthalpic differences in anaerobic microbial metabolism. The aims were: (i) to specify conditions which allow the overall metabolic comparison of different species; (ii) to quantify the heat and biomass production rates for the specific experimental conditions applied; and (iii) to consider the theoretical aspects of the results. The experiments were performed in continuous culture. Under these conditions, the substrate from the feed is rapidly consumed by the cells. The growth rate depends on the dilution rate, if the maximum growth rate is not exceeded. Steady state conditions were used to monitor the effects of stepwise-increased glucose feed concentrations. Substrate consumption,

product and biomass formation and the enthalpy changes of the reaction were measured by means of fermentation calorimetry.

2. Experimental

2.1. The fermentation calorimeter

An isothermal Berghof fermentation calorimeter BFK with a working volume of 2 l was used (Fig. 1). The calorimetric system is an improved version of a former prototype described elsewhere [6, 7]. It offers all the functions of a standard laboratory fermenter. The experiments were carried out at 30.0°C, pH 5.0, an agitation rate of 500 rpm and at a dilution rate of $D = 0.15\ h^{-1}$. To achieve anaerobic conditions and to avoid undesired heat effects [7], the medium storage bottle (10 l) was exposed to a constant carbon dioxide flow (1 ml s^{-1}). Exhaust gas was vented from the fermenter through a pressure control valve (1000 hPa) and its volume was measured. The pH was kept constant by alkaline feeding (1 N NaOH diluted in the feed before it entered the fermenter.) The temperature of the feed was adjusted exactly to the fermenter temperature.

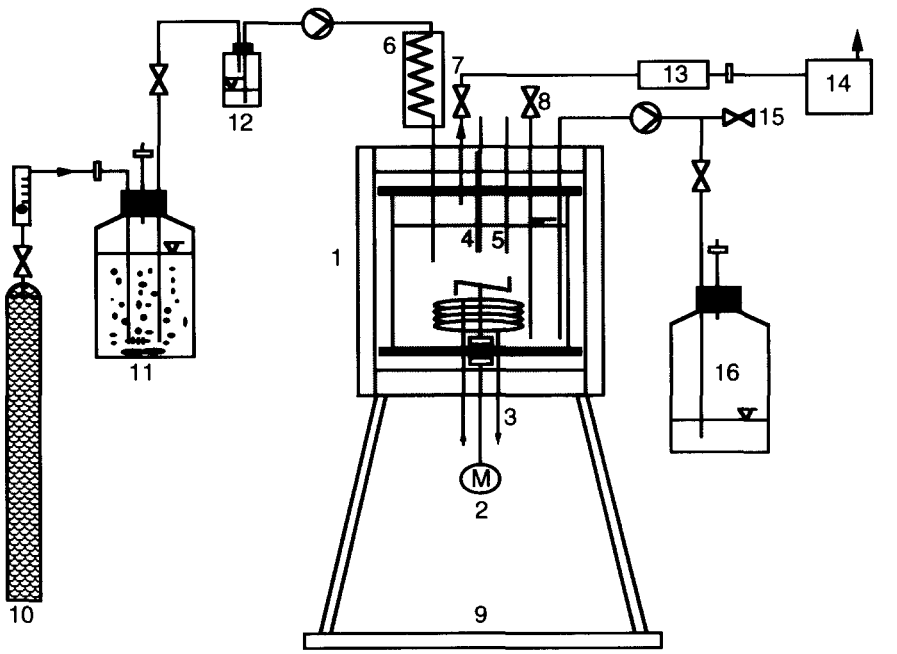

Fig. 1. Experimental set-up: 1, fermentation calorimeter consisting of the fermenter surrounded by an adiabatic jacket; 2, magnetic stirring system; 3, heating/cooling unit; 4, thermopile; 5, thermistor; 6, feed heat-exchanger; 7, pressure control valve for exhaust gas; 8 and 15, sampling probes; 9, balance; 10, CO_2 storage; 11, feed saturation with CO_2; 12, flow control; 13, exhaust gas dehumidification; 14, exhaust gas analysis; 16, fermenter effluent storage.

After in situ sterilization of the calorimeter, 2 l of medium were pumped into the vessel and the calorimeter control system was started. When constant conditions of the preset parameters were achieved, a zero offset calibration was performed and the base-line monitored. If no significant changes occurred, the fermenter was inoculated. After a batch phase, continuous culture was started and the experiments were carried out.

2.2. Organisms and culture conditions

Zymomonas mobilis ATCC 29191 and *Saccharomyces cerevisiae* CBS 426 were selected as test organisms. The media consisted of glucose, vitamins, salts and trace elements described elsewhere [9, 10]. In addition, 25 mg l^{-1} of yeast extract per g glucose consumed was added. The media were mixed from stock solutions which were sterilized separately.

The growth rate was maintained constant by a constant dilution rate throughout the experiments ($D = 0.15\ h^{-1}$), while the glucose concentration of the feed was increased stepwise from 10 to 80 g l^{-1}. After a transition from one glucose feeding rate to a higher rate, steady state conditions were usually achieved again after 3 retention times. The difference to the former situation was a higher cell density while the specific growth rate was the same for all steady state conditions. Vitamins, trace elements and salts were added to be always in excess.

2.3. Analyses

All measurements used for the calculation of consumption and production rates were carried out at dynamic equilibrium conditions. Therefore, samples were withdrawn from the feed, the fermenter and the liquid efflux. The optical density (OD) of the suspension was determined at 550 nm by a photometer. The dry weight was measured gravimetrically and related to the OD. Glucose, ethanol, glycerol, and lactic and acetic acids were analysed simultaneously by HPLC using a refractive index detector [11]. The heat of reaction was measured by the fermentation calorimeter. In addition, the exhaust gas volume was measured.

3. Results and discussion

3.1. Carbon balance, energy balance and the ratio of products

The experiments carried out were to measure small enthalpic differences during anaerobic microbial conversion of glucose to ethanol and carbon dioxide. Therefore, conditions were specified which allowed the overall metabolic comparison of different species. For *Z. mobilis* and *S. cerevisiae*, seven and eleven respectively, steady state conditions were analysed. With respect to the consistency of the data, first the carbon balance was checked at steady state conditions according to Eq. (3). The rates of glucose (S_1) and yeast extract consumption (S_3), and ethanol (P_1), carbon dioxide (P_2)

and biomass (P_X) production (carbon content of yeast extract and biomass assumed to be 44%) were considered. S and P give the consumption or production rates in g l^{-1}, whereas C with the different subscripts give the carbon contents of the compounds. C_{rc} represents the carbon recovery in g l^{-1} h^{-1}, which was converted into percent recovery with respect to the comparison of the different fermentations carried out.

$$C_{rc} = \sum_S \Delta C_S \Delta S - \sum_P \Delta C_P \Delta P$$

$$= (\Delta C_{S1} \Delta S_1 + \Delta C_{S3} \Delta S_3)$$

$$- (\Delta C_{P1} \Delta P_1 + \Delta C_{P2} \Delta P_2 + \Delta C_{Px} \Delta P_x) \quad (\mathrm{g\,l^{-1}\,h^{-1}}) \tag{3}$$

The loss of ethanol by evaporation through external gassing was reported to reach up to 2.5% for a gassing rate of 0.6 vvm [12, 13]. During the experiments carried out here, temperature and pressure conditions were similar but not the external gassing. The maximum exhaust gas production was much lower here (0.07 vvm) which means that ethanol stripping could be expected to be negligible. Although the influx medium was pre-saturated with carbon dioxide, the supersaturation of the efflux had to be considered. The CO_2 concentration of the efflux was approximated to be 0.46 mol l^{-1} or 0.31 l h^{-1} ($D = 0.15\ h^{-1}$, carbon dioxide pressure 1000 hPa). The neutralization heat from the alkaline reaction was also considered [7, 14].

If the yeast extract added to the media (25 mg per g glucose consumed) was completely incorporated into the biomass, the carbon balance based on glucose consumption would be altered by a maximum of 2.8%. Its contribution was neglected. For *Z. mobilis*, the average carbon recovery calculated was 92.3% ($S = 7.4$) and for *S. cerevisiae* 95.9% ($S = 3.5$). Thus, the carbon balance proved to be consistent with reasonable accuracy.

The energy balance of the fermentation was calculated (Eq. (4)). The balance was based on standard state reaction enthalpy measurements for glucose (S_1) and ethanol (P_1) of $\Delta H_R = -2.816$ and -1.366 kJ mol^{-1} [15]. For biomass (P_X), a standard state reaction enthalpy of $\Delta H_R = -23.2$ kJ per g dry weight was averaged [15]. The reaction enthalpy of ammonium (S_2) was calculated to be $\Delta H_R = -246$ kJ mol^{-1}. The amount of ammonium consumed was recalculated from the nitrogen content (15%) of the biomass formed [1, 16]. The reaction enthalpy for yeast extract (S_3) was $\Delta H_R = -16.67$ kJ g^{-1} [16]. The enthalpy of the reaction ($H_{P_{cal}}$) was recorded by the fermentation calorimeter. S and P give the consumption or production rates, whereas H with the different subscripts give the reaction enthalpies of the compounds and P_{cal} the heat production measured. H_{rc} is the enthalpy recovery which was converted into percent.

$$H_{rc} = \sum_S \Delta H_S \Delta S - \sum_P \Delta H_P \Delta P = (\Delta H_{S1} \Delta S_1 + \Delta H_{S2} \Delta S_2 + \Delta H_{S3} \Delta S_3)$$

$$- (\Delta H_{P1} \Delta P_1 + \Delta H_X \Delta X + \Delta H_{P_{cal}} \quad (\mathrm{kJ\,l^{-1}\,h^{-1}}) \tag{4}$$

The enthalpy content of the glucose consumed was chosen as the basis of the balance. This balance is clearly dominated by glucose consumption and ethanol formation and, to a minor extent, by (i) the biomass formed, (ii) the heat released, (iii) the nitrogen source, and (iv) the yeast extract consumed. According to Eq. (1), the energy recovery by ethanol formation is theoretically 97%. However, the values of the experiments varied between 85 and 90% for *S. cerevisiae* and *Z. mobilis* respectively. The minor remainder was expected to be energy fixed as biomass or dissipated as heat. Based on the enthalpy content of glucose, the energy converted to biomass varied between 11 and 4% and the heat production varied between 8 and 12% (*S. cerevisiae* and *Z. mobilis* respectively). If the yeast extract added could be completely incorporated into the biomass during the consumption of 1 mole of glucose, it would contribute 2.6% of the energy balance. The influence of ammonium on the energy balance is in a similar range: for *Z. mobilis* the maximum contribution calculated was 0.5% of the energy available and for *S. cerevisiae* 2.3% at most. This means that all the variables mentioned had to be carefully considered. The energy balances calculated for the different steady states proved to be slightly better for the *S. cerevisiae* fermentations compared to the *Z. mobilis* experiments (energy recovery 96–103% and 92–110% respectively). Thus, the consistency of the energy balance was in a similar range as the carbon balance. Comprehensive statistical data processing is not yet possible, because the number of different measurements was too low.

For each steady state measured, *Z. mobilis* and *S. cerevisiae* had different biomass yields. However, the biomass yields changed throughout the different steady states, which also affected the ethanol, carbon dioxide and heat yields (see Sections 3.2 and 3.3). Therefore, the stoichiometry of the conversion of glucose to ethanol cannot be averaged for the different steady state conditions. But the relation between carbon dioxide and ethanol production and the relation between carbon dioxide and heat production proved to be constant for all glucose consumption rates and for all steady states, as expected (Fig. 2). The theoretical values are 2.05 g l^{-1} ethanol per 1 CO_2 and 1.86 kJ per 1 CO_2. The average difference from theory was −0.5% (ethanol/carbon dioxide) and 4% (heat/carbon dioxide) for *Z. mobilis* and −8.5% (ethanol/carbon dioxide) and −2.0% (heat/carbon dioxide) for *S. cerevisiae*. The differences are presumably due to the contributions from yeast extract which were neglected.

Summing up the results of this section, the carbon and energy balance as well as the ratio of the products of the anaerobic conversion of glucose to ethanol proved to be consistent with reasonable accuracy. This is very important with respect to the reliability of different metabolic effects considered in the following sections.

3.2. Specific glucose consumption, and ethanol, carbon dioxide and heat production rates

In order to identify conditions suitable for the metabolic comparison of the microorganisms studied, the dilution rate was maintained constant but the substrate feed rate was increased stepwise during the experiments. Steady state conditions were quantified.

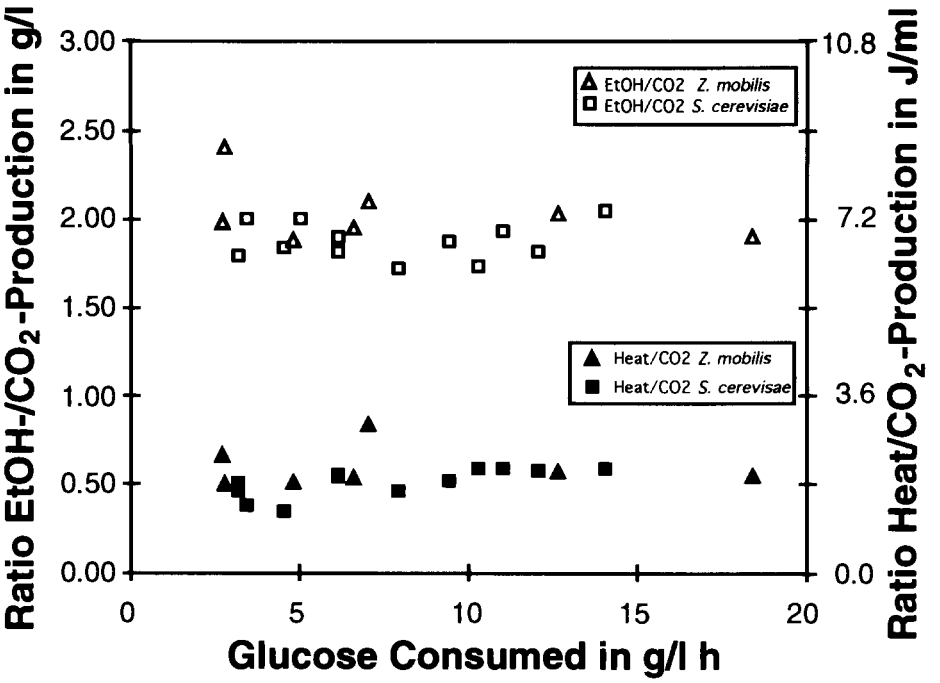

Fig. 2. Ratio of carbon dioxide to ethanol production and ratio of heat to carbon dioxide production for steady state conditions of anaerobic growth of *Z. mobilis* and *S. cerevisiae* on glucose (30°C; agitation, 500 rpm; $D = 0.15\ h^{-1}$; pH 5.0).

Specific substrate consumption always increased with higher feed concentrations, causing higher specific ethanol and carbon dioxide formation and heat production rates for both microorganisms (shown for *S. cerevisiae* steady state conditions in Fig. 3). This behaviour was expected from the kinetics of reaction involved. However, the specific glucose consumption and the specific production rates mentioned were approximately three times higher for *Z. mobilis* than for *S. cerevisiae* at $D = 0.15\ h^{-1}$ (shown for specific heat production in relation to the biomass concentration in Fig. 6, below). This is very important with respect to the comparison of their metabolic properties, because high glucose consumption, product formation and heat evolution rates were expected to be linked to a low biomass production rate and vice versa. These results are described in Sections 3.3.1 and 3.3.2.

3.3. Yields

3.3.1. Biomass yield $Y_{X/S}$, ATP yield $Y_{X/ATP}$ and heat yield $Y_{Q/S}$

For both microorganisms, the biomass yield $Y_{X/S}$ (that is the amount of biomass formed per g glucose consumed) decreased with increased substrate conversion rates

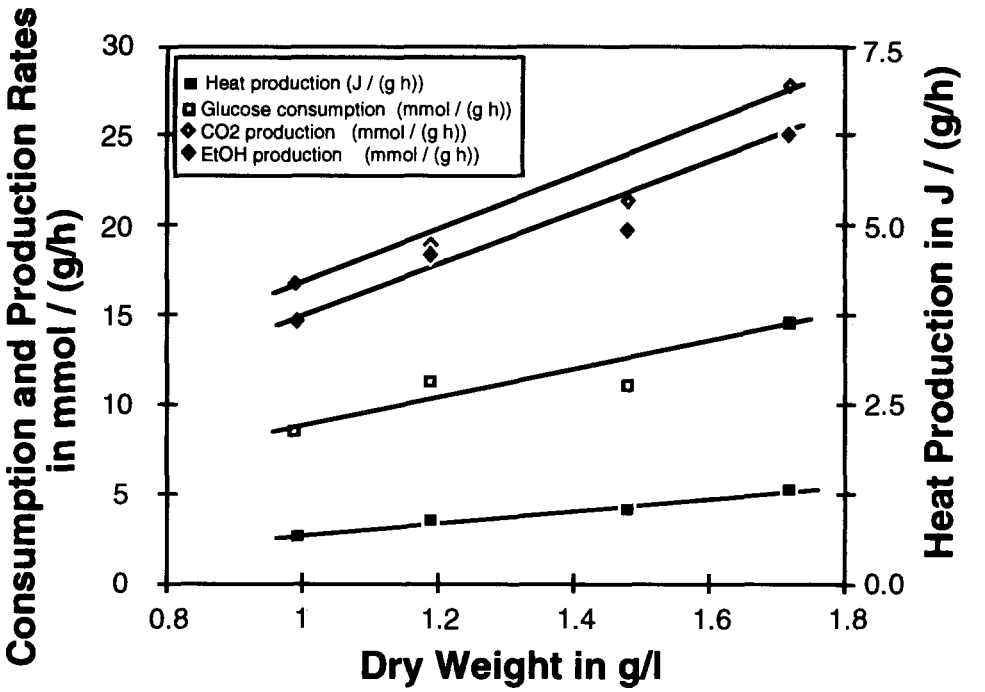

Fig. 3. Specific rates of substrate consumption, and ethanol, carbon dioxide and heat production during steady states of an anaerobic *S. cerevisiae* fermentation in relation to biomass (30°C; agitation, 500 rpm; $D = 0.15\ h^{-1}$; pH 5.0).

(Fig. 4). In other words, high glucose consumption rates diminished the biomass yield. For *Z. mobilis*, the already low biomass yield decreased from $Y_{X/S} = 31$ mg biomass g^{-1} glucose consumed (glucose consumption rate, 2.8 g l^{-1} h^{-1}) to 19 mg biomass g^{-1} glucose (glucose consumption rate, 18.0 g l^{-1} h^{-1}), i.e. by −38%. For *S. cerevisiae*, the initially high yield, $Y_{X/S} = 135$ mg biomass g^{-1} for the low glucose consumption rate of 3.0 g l^{-1} h^{-1} decreased to 51 mg biomass g^{-1} for a glucose consumption rate of 13 g l^{-1} h^{-1} (−63%). Thus, significant differences between the microorganisms concerning their biomass production could be quantified.

For decreasing biomass production, enhanced heat production rates were to be expected. The plot of the heat yield $Y_{Q/S}$ (that is the amount of heat evolved per g glucose consumed) against the glucose consumption rates showed that this expectation was only valid for *S. cerevisiae* (increase from $Y_{Q/S} = -52$ kJ mol^{-1} glucose for a glucose consumption rate of 3.0 g l^{-1} h^{-1}, to −85 kJ mol^{-1} glucose for a glucose consumption rate of 13 g l^{-1} h^{-1}). The heat yield for *Z. mobilis*, however, decreased from an initially very high −108 kJ mol^{-1} for a glucose consumption rate of 2.8 g l^{-1} h^{-1}, to −83 kJ mol^{-1} for a glucose consumption rate of 18.0 g l^{-1} h^{-1} (data not shown). If glucose is completely converted to ethanol, a theoretical yield of $Y_{Q/S} = -84$ kJ mol^{-1} glucose is expected (Eq. (1)). The higher initial heat yields for *Z. mobilis* were the results of correct

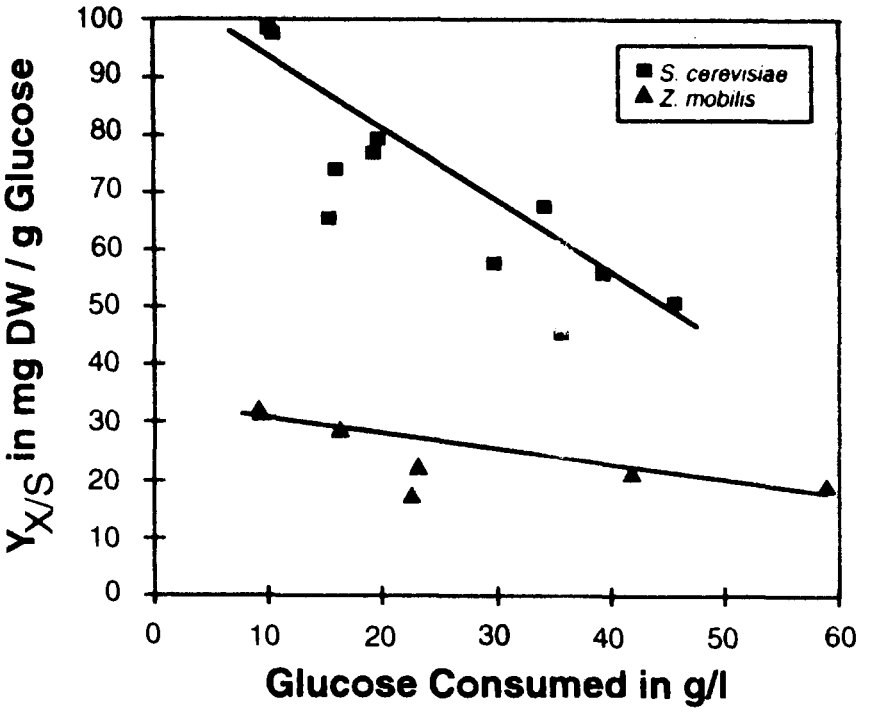

Fig. 4. Changing biomass yields $Y_{X/S}$ of *Z. mobilis* and *S. cerevisiae* are caused by increased glucose consumption (30°C; agitation, 500 rpm; $D = 0.15\ h^{-1}$; pH 5.0).

calorimetric measurements. They are presumably due to limitations in the accuracy of glucose analysis, which is explained in detail in Section 3.4. However, the expectation that *Z. mobilis* should deliver higher amounts of heat than the yeast but that the latter should have the higher biomass yield was confirmed.

If the ratio of dry weight to heat production is plotted against glucose consumption (Fig. 5), the ratio was constant for *Z. mobilis* which means that yield effects were counterbalanced. It was mentioned above that increased substrate consumption diminished the biomass yield. The explanation is that glucose may be partly converted to by-products [17]. By-product formation may have no effect on ATP generation and biomass production. By-product formation may be interpreted in terms of metabolic control. However, this was not studied in detail. For *S. cerevisiae* the decreasing dry weight produced per amount of glucose consumed was initially counterbalanced by increased heat production (first straight line). When glucose consumption exceeded 20 g l^{-1}, the ratio of dry weight to heat production remained constant indicating by-product formation (second straight line), but glycerol, the most likely by-product, was not observed. However, the negative influence of accelerated substrate availability on biomass production already described for aerobic metabolism [12, 16] was confirmed and quantified for the specific anaerobic conditions applied here.

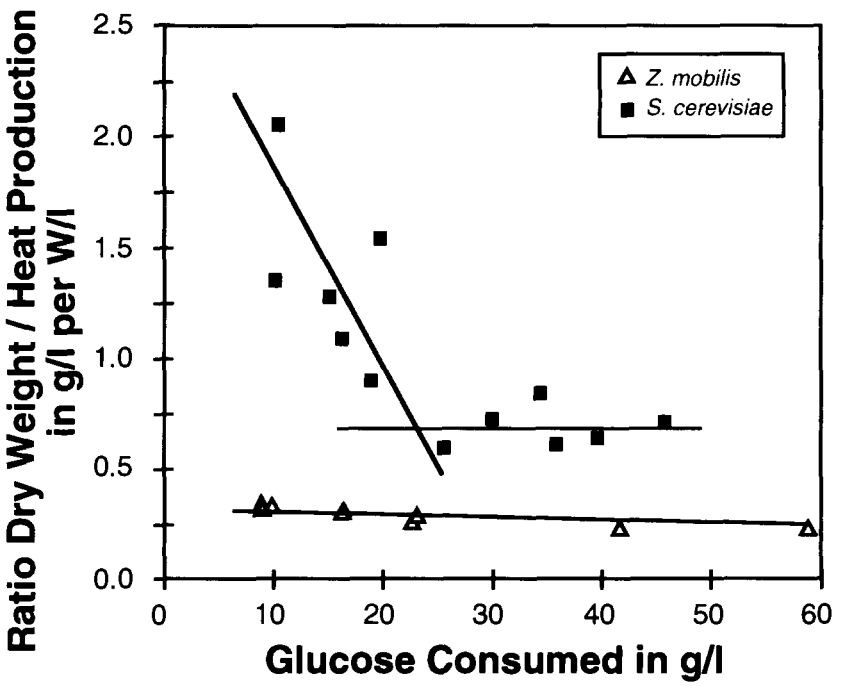

Fig. 5. Ratio of dry weight to heat production in relation to increased glucose consumption for anaerobic *Z. mobilis* and *S. cerevisiae* fermentations (30°C; agitation, 500 rpm; $D = 0.15\ h^{-1}$; pH 5.0).

The ATP yields $Y_{X/ATP}$ (that is the amount of biomass formed per mole ATP consumed) were calculated according to Ref. [8] from the ethanol production and the biomass production rates measured. As for the biomass yield, the ATP yield decreased with increasing substrate consumption especially for *S. cerevisiae* (data not shown). Therefore, the average value of 8.14 g dry weight per mol ATP consumed has a significant standard deviation of 22%. The average ATP yield for *Z. mobilis* was 4.59 g dry weight per mol ATP. Both values differ significantly from the average reported, namely 10.5 (*S. cerevisiae*) and 8.3 (*Z. mobilis*) g biomass per mol ATP [18].

3.3.2 Comparison of metabolic properties of Z. mobilis *and* S. cerevisiae

The plot of specific heat production rates for *Z. mobilis* and *S. cerevisiae* against the dry weight formed gives straight lines (Fig. 6). The specific heat production rate of *Z. mobilis* was three times higher than for *S. cerevisiae* (average production 2.97 kJ g^{-1} dry weight h^{-1} and 0.97 kJ g^{-1} dry weight h^{-1}, i.e. 0.83 W g^{-1} and 0.27 W g^{-1} dry weight respectively). This result is in good accordance with the different specific glucose conversion and ethanol and carbon dioxide production rates mentioned above (see Section 3.2). The biomass production rates were inversely related by the same factor.

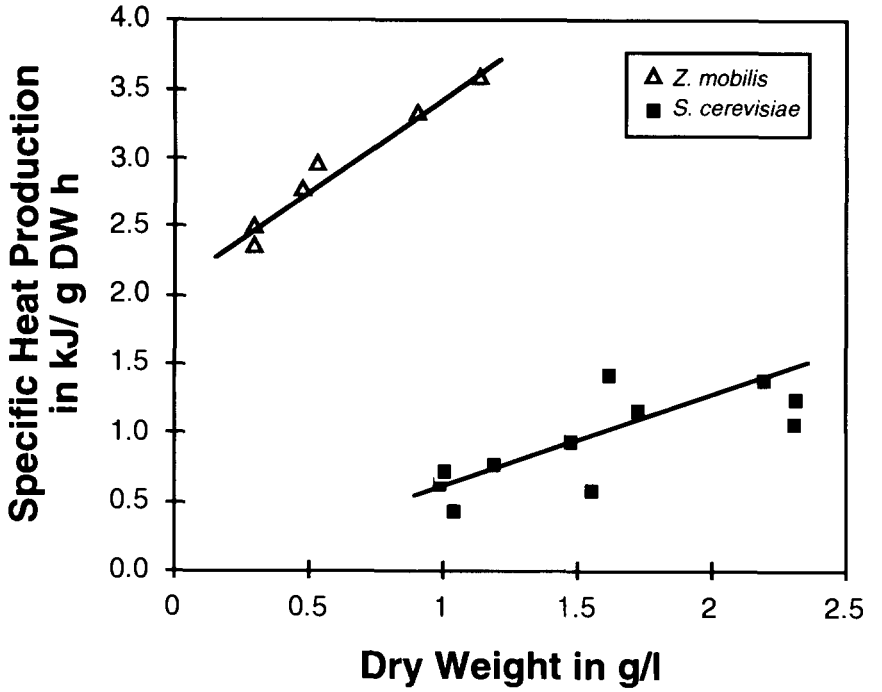

Fig. 6. Specific heat production rates for *Z. mobilis* and *S. cerevisiae* in relation to the dry weight formed.

Note that the medium used for the experiments contained, as well as glucose as a source of carbon and energy, equimolar amounts of yeast extract. *Z. mobilis* is thought to utilize yeast extract mainly as a source of vitamins, whereas *S. cerevisiae* can produce additional biomass from the amino acids available. Thus, yeast extract may influence the specific metabolic rates as well as the reaction enthalpy of the conversion itself (see below).

According to the law of conservation of mass and energy, the reaction enthalpy of the anaerobic conversion of glucose to ethanol for the specific fermentation conditions applied must be equal to the sum of energy either dissipated as heat (specific heat production rate) or fixed in the biomass formed (biomass production rate). These sums calculated for each steady state measured can be used to average the sums of reaction enthalpy. For defined media, the enthalpy recovery ($\Delta Q_{cal} + \Delta Q_X$) should be equal for both organisms, i.e. the quotient of enthalpy recovery should be 1. The sums of the average reaction enthalpies ($\Delta Q_{cal} + \Delta Q_X$) were calculated to be $\Delta H_R = -615$ kJ (standard deviation S = −74 kJ) and −477 kJ (standard deviation S = −86 kJ) per mol glucose consumed (for *S. cerevisiae* and *Z. mobilis*, respectively). The quotient calculated here was 1.29. This difference may be explained by the biomass production of *S. cerevisiae*, which was approximately 30% higher than that reported for defined media [18, 19]. However, the sums of the enthalpies were very different from the theoretical

expectation of $\Delta H_{R^\circ} = -84\ \mathrm{kJ\ mol^{-1}}$; the latter value is in the range of the deviation of the practical values. The discrepancy between the reaction enthalpy of the biochemical conversion of glucose to ethanol derived from fermentation practice and theoretical expectation can be explained by the multiplication of analytical errors, which is discussed in detail in Section 3.4.

3.3.3. *Different energy wasting of* Z. mobilis *and* S. cerevisiae—*life strategies versus metabolic control strategies*

Z. mobilis exhibits a clearly higher specific heat production rate than *S. cerevisiae*, whereas the latter had the higher biomass yield. With respect to growth efficiency (that is the percentage of substrate converted into biomass), *Z. mobilis* wastes energy compared to the yeast. According to the literature, it is a common feature that the growth rate of an organism may be high at the expense of growth efficiency, and vice versa [5]. A high growth rate causes high ethanol, carbon dioxide and heat production rates. Therefore, a high growth rate should be associated with an enhanced entropy production, which diminishes growth efficiency. The entropy change ΔS of a chemical reaction can be calculated (i) from the entropies of formation of products and reactants, or (ii) from the calculated ΔG values and the measured ΔH values. For growth processes it is still difficult to obtain reliable values for the entropy ΔS and free enthalpy ΔG changes of biomass formation [1]. Moreover, the biomass formation changed during the experiments described here. Therefore, a thermodynamic interpretation of the data collected is not yet possible from our point of view.

The maximum growth rate under anaerobic conditions is reported to be $\mu_{max} = 0.397\ \mathrm{h^{-1}}$ for *Z. mobilis* [20] and $\mu_{max} = 0.100\ \mathrm{h^{-1}}$ for *S. cerevisiae* CBS 426 [21]. The behaviour of the cells in either wasting or not wasting energy is probably related to different survival strategies in their natural environments. Throughout the experiments reported here, a steady state growth rate of $\mu = 0.15\ \mathrm{h^{-1}}$ was established for both microorganisms. This seems to be unreasonably high for the yeast compared to the maximum growth rate cited above. The reproducibly higher growth rate was presumably caused by the specific fermentation conditions. Stepwise increased feed concentrations were expected to cause transiently higher growth rates until the transient glucose excess in the fermentation calorimeter was consumed. The former growth rate should then be reestablished, because μ depends on the dilution rate. As a consequence of the experimental procedure, the biomass concentration in the fermentation calorimeter should simply increase. These expectations were satisfied by the growth behaviour of *Z. mobilis* measured up to feed concentrations of 59 g glucose $\mathrm{l^{-1}}$. The response of *S. cerevisiae* was different: when the glucose feed concentration exceeded 20 g $\mathrm{l^{-1}}$, which corresponds to a feed rate of 3.0 g $\mathrm{l^{-1}\ h^{-1}}$, accumulation of the cells occurred but traces of glucose became measurable in the fermenter. These effects increased at higher glucose feed concentrations until a maximum of 34 g $\mathrm{l^{-1}}$ glucose in the fermenter at the maximum feed concentration of 80 g $\mathrm{l^{-1}}$ glucose was reached. This phenomenon was accompanied by flocculation of *S. cerevisiae*, which was reversible, and the more intensive the flocculation, the higher the glucose concentration in the fermenter. A nutrient limitation contributing to flocculation of the cells was excluded, because

salts and trace elements in the medium were calculated to be in excess. Under the experimental conditions applied, the growth rate determined by the dilution rate of $D = 0.15\ h^{-1}$ was already in the vicinity of μ_{max}. During the transition phase from one glucose shift to another, the μ_{max} of *S. cerevisiae* was readily exceeded, indicating that the growth rate was not sufficient to consume the substrate available. Thus, the stepwise increase of the feed concentration turned out to be different extensions of the transition, which were then analysed under steady state conditions.

For the strain of *S. cerevisiae* used for this study, the aerobic oxidation of high glucose concentrations leads to a well-known transient excretion of ethanol until the glucose is nearly exhausted. Finally, ethanol is aerobically oxidized. This behaviour is called the Crabtree effect [2, 11, 16, 22]. This phenomenon is thought to be linked to a limited transfer of cytosolic pyruvate into the mitochondria of the cells. The dynamics of metabolic response to glucose shifts has been studied intensively in aerobic continuous culture [23]. The phenomenon described in this study differs from the reports mentioned, because (i) increased substrate availability caused enhanced ethanol production under anaerobic conditions (complementary to the aerobic Crabtree effect), and (ii) there is no other known study that focuses on different phases of the anaerobic transition under steady state conditions.

Throughout these phases, a characteristic profile of the specific rates of heat, ethanol and carbon dioxide production in relation to the glucose accumulation in the fermentation calorimeter was observed (shown for heat and carbon dioxide production in Fig. 7). Already a slight increase in the initially low glucose feed concentrations caused

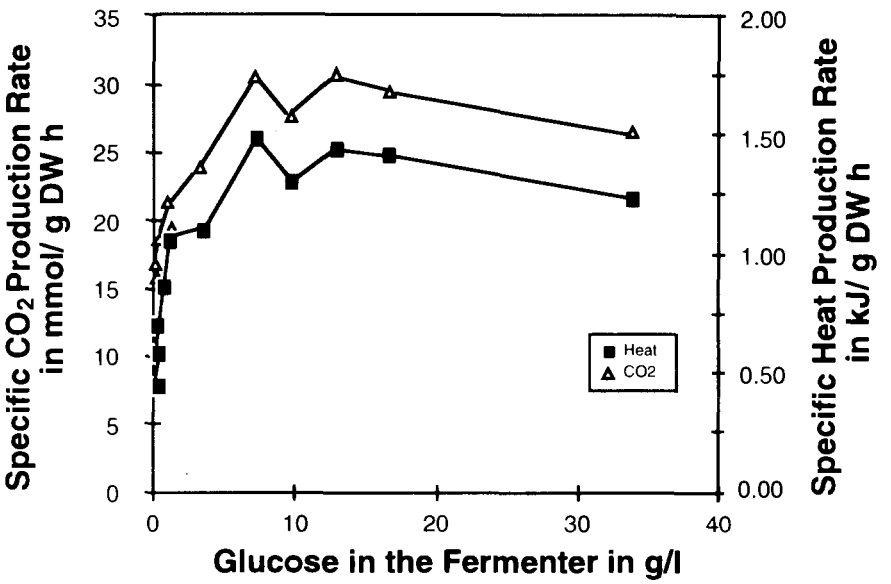

Fig. 7. Specific rates of heat and carbon dioxide production in relation to the glucose accumulation in the fermenter during the anaerobic fermentation with *S. cerevisiae*.

an accumulation of glucose in the fermenter which was linked to a rapid increase of the specific production rates mentioned. The highest slope of this energy wasting occurred in the early phase of glucose accumulation in the fermenter. These rates turned out to be more or less constant at glucose concentrations in the fermenter higher than 10 $g\,l^{-1}$. In other words, the enzymes and coenzymes involved in glycolysis and TCA-cycle-dependent anaplerotic reactions were presumably saturated (zero-order reaction). Thus, growth under anaerobic conditions is obviously limited by the same rate-determining reaction of pyruvate transfer leading to the low growth rates mentioned above.

Fig. 7 provides some information concerning the metabolic regulation of the yeast cells. The profile of the specific rates indicates that the transfer sites in the mitochondrial membranes were rapidly saturated with metabolites, but that the substrate uptake still proceeded. Because additional biomass production was impossible, heat, ethanol and carbon dioxide production increased, indicating the metabolic uncoupling of anabolic biomass formation and catabolic energy substrate consumption. In other words, the energetic efficiency is markedly lower during this overflow metabolism. Is there any advantage in this for the yeast?

The so-called futile cycles are considered to provide the cell with a regulatory mechanism to control the flux through the metabolic pathway. This is particularly prominent under conditions with great variation in the flux, which is valid for the experiments carried out here. A futile cycle is defined as a cyclic metabolic pathway, which consumes net ATP accompanied by heat production [16, 18, 24, 25]. For the glycolytic pathway involved here, the futile combined action of phosphofructokinase and fructose-1,6-bisphosphatase is thought to be relevant. The energetic efficiency is dramatically decreased for each turn of the cycle per unit glucose going through glycolysis (decrease in the net ATP yield by 50%). Thus, the results obtained may be explained by differential futile action (maximum biomass decrease by 63%, differential increase of heat production by 39%).

In addition, metabolic regulation seems to be directly linked to cellular heat production by other mechanisms. As reported for *E. coli*, the implementation of non-expressed plasmid material may already cause enhanced heat production [26]. It was also shown that the profiles of heat production rates are different for wild-type strains compared to mutants [16, 27, 28]. In the attempts of genetic pathway engineering to improve the production properties of strains [29], it may be possible to apply calorimetry to analyse the growth and conversion properties of relevant constructs.

3.4. Facilities and limitations of calorimetry-supported analysis of anaerobic fermentations

The yield calculations were based on the analysis of glucose, ethanol, biomass carbon dioxide and heat. The error levels of the measurements vary between 1 and 3%. For most purposes, this accuracy is sufficient except for heat yield calculations, remembering that the reaction enthalpy of the anaerobic conversion of 1 glucose ($\Delta H_R = -2.816$ kJ mol^{-1}) to 2 ethanol ($\Delta H_R = -1.366$ kJ mol^{-1}) is calculated to be -84 kJ mol^{-1} (standard state conditions, see Sections 1 and 3.1). A deviation of $\pm 2\%$, e.g. for the

glucose initially available, already influences the reaction enthalpy calculated as -140 or -28 kJ mol^{-1}. Obviously, the analytical errors of the different measurements may multiply leading to marked deviations in the yield calculations (see Figs. 2, 4–6). Note that the glucose in the feed was added from stock solutions. Deviations derived from the addition may multiply with analytical errors. Thus, it is neither possible to calculate the heat production nor the biomass production of anaerobic processes with reasonable accuracy, as suggested by Dekkers et al. [21]. As shown in this contribution, the enthalpic yield, i.e. the amount of energy dissipated as heat or fixed as biomass, derived from a real anaerobic fermentation can differ significantly from theoretical expectations. In other words, the heat production of an anaerobic process can be estimated only roughly from theory. Calorimetry, however, is the only tool which supports direct and therefore reliable information about the energy dissipation of anaerobic metabolic reactions. Thus, heat and biomass measurements can be used to calculate the real enthalpy change for the fermentation conditions applied. This allows some thermodynamic considerations of the growth properties of the strains tested to be made and also contributes to a better understanding of cellular regulation as described in Section 3.3.3.

4. Conclusions

The experiments presented here were carried out in order to determine the advantages and limitations of a calorimetry-supported approach to the measurement of small enthalpic differences in anaerobic microbial metabolism. It was shown that the enthalpy change of the anaerobic reactions cannot be estimated from substrate consumption and product formation rates, because of the influence of the normal analytical errors. It was also shown that the energy biochemically available from the substrates could be analysed quantitatively by means of calorimetry, even for small enthalpic differences. The interpretation of the data in terms of growth efficiency, growth rate and production rates is possible, giving valuable insight into the metabolic processes. However, it turned out to be very difficult to specify the experimental conditions under which the metabolic action of different species might be compared, because their metabolic properties changed. Small changes in the reaction enthalpy are predicted to be relevant both for aerobic and anaerobic processes. Thus, the calorimetry-supported analysis of fermentations is proposed as a powerful tool for applied aspects of the understanding and improvement of a variety of growth and production processes, e.g. genetically modified microorganisms, as well as for basic research, e.g. concerning the question of the existence of endothermic growth [30].

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