ELSEVIER

Thermochimica Acta 394 (2002) 185–190

thermochimica acta

www.elsevier.com/locate/tca

Continuous estimation of product concentration with calorimetry and gas analysis during anaerobic fermentations of *Saccharomyces cerevisiae*

Eva Albers [a,*], Christer Larsson [a], Gunnar Lidén [b], Claes Niklasson [c], Lena Gustafsson [a]

[a] *Department of Molecular Biotechnology, Chalmers University of Technology, Box 462, SE-405 30 Göteborg, Sweden*
[b] *Department of Chemical Engineering II, Lund Institute of Technology, Box 124, SE-221 00 Lund, Sweden*
[c] *Department of Chemical Reaction Engineering, Chalmers University of Technology, SE-412 96 Göteborg, Sweden*

Received 10 September 2001; received in revised form 7 February 2002; accepted 13 March 2002

Dedicated to Prof. Richard Kemp on the occasion of his 60th birthday

Abstract

Product concentrations may be estimated from gas analysis or calorimetric measurements. These techniques of analysis are fast, simple, and provide a continuous output. To check the accuracy of these estimations, the anaerobic ethanol formation of an industrial strain of the yeast *Saccharomyces cerevisiae* was selected as a test system. Cultivations were carried out as batch cultures with different nitrogen sources (ammonium, glutamate and a mixture of amino acids) and 20 g/l of glucose as the carbon and energy source. The results showed that there was a good agreement between measured and calculated ethanol values. However, especially when using heat data the results are very sensitive to small changes in the stoichiometry of the catabolic reaction, i.e. ethanol is not the only product but there are also minor amounts of glycerol and acetate. The problems generated by these products when using heat data to calculate ethanol concentrations are discussed in detail.

Keywords: *Saccharomyces cerevisiae*; Anaerobic growth; Ethanol estimation; Calorimetry; Gas analysis

1. Introduction

Measurements of metabolites are important to survey the condition of a culture and to have possibilities for process control. However, most analyses are time-consuming and in an industrial process the scanning has to be simple and fast. Any parameter, which is possible to analyse fast, may then be used to estimate the desired concentrations. Two suitable techniques are gas analysis and heat measurements.

All reactions of "life" involve alteration in heat. This heat is the change in enthalpy of all metabolic reactions in the cell and can be measured by calorimetry [1–4]. The heat evolution characteristics in batch cultures of *Saccharomyces cerevisiae* have been investigated in some studies. Under aerobic conditions a diauxic growth pattern is obtained [5,6]. Initially, glucose is consumed in a respiro-fermentative metabolism, producing, apart from biomass, also ethanol. The ethanol, subsequent to glucose depletion,

* Corresponding author. Fax: +46-31-773-2599.
E-mail address: eva.albers@molbiotech.chalmers.se (E. Albers).

0040-6031/02/$ – see front matter
PII: S0040-6031(02)00249-6

will be consumed via respiration. These different phases are clearly visible in the heat production rate curve obtained from a calorimeter [5,6]. Thus, the overall catabolic conversion of glucose is to form carbon dioxide and water, and the enthalpy change of this reaction is −2814 kJ/mol glucose [1]. Anaerobically, *Saccharomyces* has a mono-auxic growth. This is seen in both the heat and carbon dioxide curves. When glucose is depleted, the growth process stops and the heat and carbon dioxide evolution rates drop to zero [7]. The amount of heat produced is substantially lower during anaerobic conditions, since a large part of the energy available in glucose is maintained as ethanol.

Most of the evolved heat originates from catabolic processes [3]. Hence, the heat measured with a calorimeter may be used to estimate the catabolic product concentrations on-line. If, beside biomass, there is only one product or if there is a fixed relation between the products, measurements of heat production will be sufficient to calculate the product concentration. Advantages of the calorimeter are the very fast measurement and in a flow microcalorimeter the cells can be recirculated back to the fermentor from the instrument, eliminating loss of the culture. Gas analysis has similar advantages, but can of course only be used if the reactions involved are connected to gas evolution.

The aim of this work was to investigate if calorimetry and/or carbon dioxide measurements are suitable methods to use for estimation of product concentrations. As a test system the anaerobic ethanol formation of the yeast *S. cerevisiae* was selected. The ethanol concentration was estimated from the measurements of the heat production rate and carbon dioxide evolution rate. The accuracy and generality of such estimations are checked on cultivations with different nitrogen sources. The different nitrogen sources were ammonium, glutamic acid and a mixture of amino acids representing an industrial medium. The amino acids decrease the amount of glycerol formed via reducing the surplus of NADH formed and hence give different yields of ethanol production [7,8].

2. Theory and calculations

An overall macroscopic reaction of anaerobic fermentation from glucose may be written as

Table 1
Molar enthalpies of combustion at 25 °C [1]

Substance	Molar enthalpy of combustion (kJ/mol)
Glucose (aq)	−2813.6
Ethanol (aq)	−1356.8
Carbon dioxide (g)	0
Carbon dioxide (aq)	+19.8
Glycerol (aq)	−1648.5
Acetic acid (aq)	−873.4

$$\text{Glucose (aq)} + Y_{\text{N/S}}\,\text{nitrogen source (aq)} \rightarrow Y_{\text{X/S}}\,\text{biomass} + Y_{\text{E/S}}\,\text{ethanol (aq)} + Y_{\text{P/S}}\,\text{products (aq)} + Y_{\text{C/S}}\,\text{carbon dioxide (g)} + Y_{\text{H/S}}\,\text{water (l)} + \Delta H_{\text{R1}}$$

where $Y_{i/\text{S}}$ are the yields for the different products and ΔH_{R1} is the heat of the reaction. Using experimentally determined yields, the ethanol may be predicted continuously from heat or carbon dioxide measurements, assuming all yields are constant during the whole cultivation. A value of ΔH can be achieved with a calorimeter or calculated from the molar enthalpies of combustion or formation, e.g. see Table 1. The yields give a relationship between the heat of reaction and the amount of ethanol, the ethanol-heat yield ($Y_{\text{E/H}}$) and thus provides an estimation of the ethanol concentration from the accumulated heat evolution rate. The same type of yield may also be determined from the measurements of carbon dioxide evolved. The disadvantage of using either of these yields is that they have to be determined experimentally.

However, it is suggested that heat of anabolism for heterotrophic growth on carbohydrates is close to zero [3,4] even though it is not always obvious how to distinguish between anabolic and catabolic reactions [3]. Hence, it is not necessary to determine the biomass yield in order to calculate catabolic product formation from heat measurements. Therefore, two simple approximations were made in order to estimate the ethanol formation during anaerobic batch cultures of *S. cerevisiae*.

Approximation 1 (Catabolic reaction).

$$\text{Glucose (aq)} \rightarrow 2\,\text{ethanol (aq)} + 2\,\text{carbon dioxide (aq)} + \text{heat}$$

In this approximation the only catabolic products considered are ethanol and CO_2. The ΔH for this reaction is −139.6 kJ/mol glucose or −69.8 kJ/mol ethanol and 1 mol CO_2 = 1 mol ethanol. The thermodynamic state of carbon dioxide was chosen as aqueous. Previously in aerobic systems, a gaseous form has been proposed as the correct thermodynamic state for this system [6]. However in the aerobic case, an air stream was introduced in the flow of culture entering the calorimeter while in the present anaerobic study, no gas stream was introduced (see Section 3). Therefore, it was considered that aqueous carbon dioxide was more appropriate in this case.

Approximation 2 (Catabolic reaction).

$$\begin{aligned}\text{Glucose (aq)} \rightarrow\ & 1.7\,\text{ethanol (aq)} + 0.2\,\text{glycerol (aq)} \\ & + 0.1\,\text{acetic acid(aq)} \\ & + 1.8\,\text{carbon dioxide (aq)} + \text{heat}\end{aligned}$$

In this approximation the formation of glycerol and acetate is also included. The amount of glycerol formed is obtained from Albers et al. [7] using ammonium as a nitrogen source. Acetate formation is simply chosen as to supply the necessary amounts of NADH and maintain redox neutrality. This is a simplification since it is known that the main part of the NADH originates from biomass formation [7]. The ΔH for this reaction is −125.7 kJ/mol glucose or −73.9 kJ/mol ethanol and 1 mol CO_2 = 0.94 mol ethanol. It should be emphasised that it is only the catabolic part of glucose consumed that is represented in the above approximations. Furthermore, it might seem strange to use the same amount of glycerol formed irrespective of the nitrogen source. However, ammonium as a nitrogen source is a worst case, since glutamate and especially a mixture of amino acids result in less glycerol [7], i.e. making Approximation 2 more similar to Approximation 1.

3. Material and methods

3.1. Strain, growth conditions and cultivations

In this study an industrial strain of the yeast *S. cerevisiae* (supplied by Swedish Baker's yeast manufacturer, Jästbolaget AB, Rotebro, Sweden) was used. The cells were grown in a minimally defined medium [7], with 20 g/l glucose as the carbon and energy source and three different nitrogen sources, ammonium, glutamate, and a mixture of 20 amino acids. The mixture of amino acids corresponds to the composition of an industrial media based on wheat hydrolysate, where the major part of the nitrogen content originates from only six of the acids, for complete composition see [7]. The yeast was grown anaerobically in pH (5.0), temperature (30 °C) and agitation (500 rpm) controlled batch cultures with a Belach bioreactor system (Belach Bioteknik AB, Stockholm, Sweden), see [7]. The working volume of the reactor was 3.0 or 2.0 l (no differences in the results due to different cultivation volume were observed). The fermentor was flushed with nitrogen (<1 ppm O_2, N48, Air Liquide, Malmö, Sweden) to maintain anaerobic condition at a controlled flow rate of 0.25 vvm (vol/vol/min, at NTP).

3.2. Analyses

Carbon dioxide was analysed continuously by an acoustic carbon dioxide and oxygen monitor, type 1308 (Brüel and Kjær, Nærum, Denmark) [9]. The heat evolution rate was measured with a flow microcalorimeter 2277 Thermal Activity Monitor (Thermo-Metric AB, Järfälla, Sweden) of the heat conduction type at 30 °C. This microcalorimetric set-up has been described previously [6], but in this study no air was added to the flow, resulting in an effective volume of the flow-through cell of 0.52 ml. The flow-through cell and the connected steel tubes were sterilised with 70% (w/v) ethanol and then rinsed with sterile water until baseline stability was reached. The culture was sucked from the bioreactor by a peristaltic pump (Alitea, Sweden). After the measurement, the flow was returned to the reactor. Base addition to keep the pH constant is done outside the calorimeter and, thus, does not influence the heat measurements. Furthermore, the enthalpy of neutralisation of the buffer (phosphate) was considered negligible at the pH used (5.0) [1]. Metabolites were measured with a HPLC system (Waters) with a polymeric ion exchange column as described previously [7].

4. Results and discussion

It was indeed possible to obtain a good estimation of the ethanol concentrations by using heat as well

as carbon dioxide measurements (Fig. 1). The deviation between the estimated and measured concentrations of ethanol at the end of fermentation was less than 1% for ammonium and glutamate as nitrogen sources using carbon dioxide measurements and only considering ethanol and carbon dioxide as products (Approximation 1). Including glycerol and acetate (Approximation 2) gave slightly less accurate results with a larger discrepancy between calculated and measured ethanol levels except when using amino acids as nitrogen source (Fig. 1). The calculated ethanol concentrations using carbon dioxide measurements were exaggerated compared to the measured values with amino acids as a nitrogen source.

However, it might be that the measured concentrations of ethanol are too low compared to what has been produced. First of all, because of ethanol evaporation which can be a serious problem [10]. Even if a condenser was fitted to the fermentor, it is close to impossible to prevent evaporation of ethanol completely. In an experimental set-up similar to the one used in this study, 1–5% of the ethanol was lost per hour depending on the actual ethanol concentration in the reactor [10].

Furthermore, there was also a slight dilution effect due to the addition of NaOH in order to maintain a constant pH. Hence, it might be that the calculated ethanol levels are a better representation of ethanol formation than the measured concentration values, i.e. the calculated values show what the ethanol concentration would have been in the absence of evaporation and dilution. This is of course true for all nitrogen sources and not only when using a mixture of amino acids. In other words, if ethanol evaporation did occur, the estimated ethanol concentrations should have been higher than the measured ones also with ammonium and glutamate as nitrogen sources.

Using heat measurements instead of carbon dioxide for calculation of ethanol showed a slightly larger deviation from the measured concentrations (Fig. 1). However, using heat measurements for estimation of ethanol concentrations during anaerobic conditions requires careful considerations. Ethanol (and the connected CO_2) is by far the product(s) formed in largest amounts and this is coupled to a relatively small heat production. This means that small errors in the heat measurements will lead to gross errors in esti-

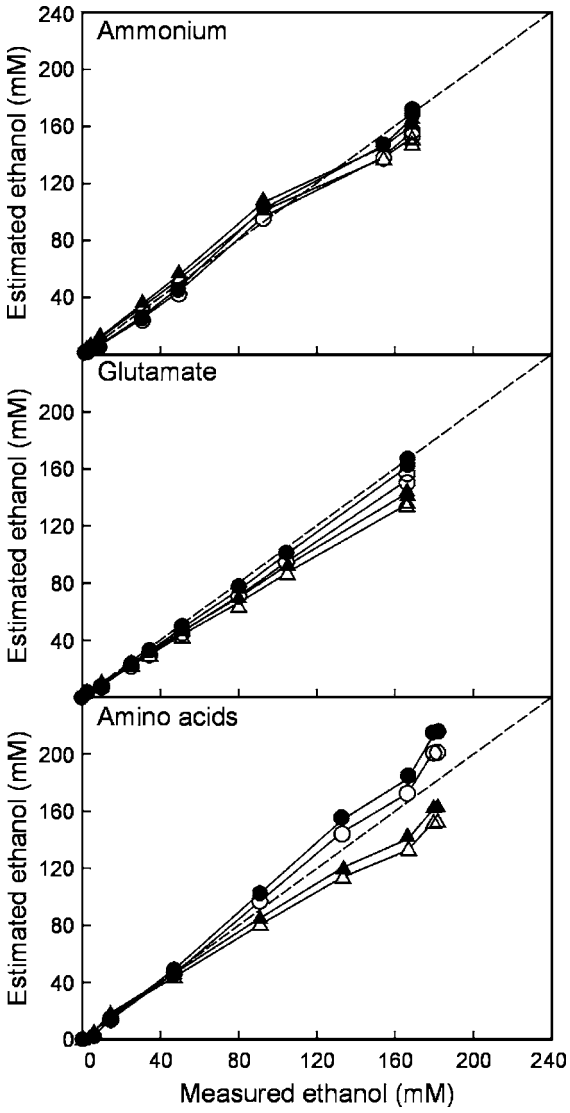

Fig. 1. A comparison between the measured and calculated ethanol concentrations during anaerobic batch cultures of *S. cerevisiae* using glucose as the carbon source and ammonium (top panel), glutamate (middle panel) or a mixture of amino acids (bottom panel) as nitrogen source. The dashed line indicates perfect agreement between the measured and calculated concentrations. The calculations were performed using measured heat (triangles) or carbon dioxide (circles) production rates. One calculation only considered ethanol and carbon dioxide as catabolic products (closed symbols) (Approximation 1, see Section 2), whereas glycerol and acetate were added in the subsequent estimation (open symbols) (Approximation 2).

mated ethanol concentrations. The relation between amount of heat produced and ethanol formation is also very sensitive to the assumptions made in the approximations. For instance, in Approximation 2 it was assumed that acetate formation was responsible for the NADH supply required for glycerol formation and as a result −73.9 kJ/mol ethanol was obtained. However, the major part of glycerol formed is known to be a consequence of a net NADH production in the biosynthetic reactions [7,8]. Therefore, if instead, it is assumed that anabolic reactions supply the NADH required for glycerol formation and no acetate is formed, Approximation 2 will change to

$$\text{Glucose (aq)} \rightarrow 1.8\,\text{ethanol (aq)} + 0.2\,\text{glycerol (aq)} + 1.8\,\text{carbon dioxide (aq)} + \text{heat}$$

The enthalpy change for this reaction would be −77.3 kJ/mol glucose or −42.9 kJ/mol ethanol compared to −73.9 kJ/mol ethanol when acetate was used. Using a value of −42.9 kJ/mol ethanol for the calculations would make the ethanol levels sky-rocket to absolutely unrealistic values. Most probably because if there is a net NADH production in biosynthesis, it is no longer correct to assume that heat of anabolism is zero [3]. A solution to this problem would be to add NADH as a substrate in the above reaction in order to restore redox balance. By doing this the enthalpy change would increase and more realistic values of estimated ethanol levels would follow.

Another complicating factor is the choice of thermodynamic state, e.g. aqueous, gaseous etc., for substrate and products when calculating the enthalpy change of a reaction. Especially, during anaerobic conditions, an incorrect thermodynamic state may result in large errors (for an extensive treatment of this see e.g. [1]). Usually, the aqueous state is appropriate in biological systems, but for gaseous compounds, gaseous as well as aqueous state may be correct. The correct choice of state depends on the system boundaries and these in turn are determined by the design of the calorimeter used for the heat measurements [1]. If, for instance, carbon dioxide is switched from aqueous to gaseous state in Approximation 1, the accompanying enthalpy change will decrease from −139.6 to −100 kJ/mol glucose. Adopting the latter value in the calculations would make the estimated ethanol levels to increase. This seems like a good idea since the calculated ethanol concentrations were in general lower than the measured ones (Fig. 1). However, using −50 kJ/mol ethanol in the calculations would lead to an estimated ethanol concentration at the end of fermentation that is 35% higher than the measured value with ammonium as the nitrogen source. An exaggeration of more than 20% of the ethanol levels would be obtained also with other nitrogen sources. It might very well be that the correct thermodynamic state of carbon dioxide in this system is a mixture of aqueous and gaseous.

To summarise, it is possible to estimate ethanol concentrations during anaerobic fermentations from measurements of heat as well as carbon dioxide production rates. However, especially calculations using heat data are very sensitive to, of course, errors in the heat measurements (due to a low heat production). In addition, it is also very important to make the correct assumptions when setting up the heat-producing pathway for calculation of the enthalpy change accompanying ethanol production. In that sense, calculations using carbon dioxide measurements is more straightforward with less complications. This is true at least under anaerobic conditions where there is a high rate of carbon dioxide production and, as stated before, a low rate of heat production. It should be remembered though that not all fermentations are coupled to carbon dioxide production. Furthermore, heat measurements could also provide additional information regarding, e.g., the thermodynamics of microbial growth.

Acknowledgements

This work was financially supported by the Swedish National Energy Administration (Energimyndigheten).

References

[1] U. von Stockar, L. Gustafsson, C. Larsson, I. Marison, P. Tissot, E. Gnaiger, Biochim. Biophys. Acta 1183 (1993) 221.
[2] L. Gustafsson, Thermochim. Acta 193 (1991) 145.
[3] C. Larsson, L. Gustafsson, in: R.B. Kemp (Ed.), Handbook of Thermal Analysis and Calorimetry, Vol. 4, 1st Edition, Elsevier, Amsterdam, The Netherlands, 1999, p. 367.
[4] E.H. Battley, Energetics of Microbial Growth, Wiley, USA, 1987.

[5] A. Blomberg, C. Larsson, L. Gustafsson, J. Bacteriol. 170 (1988) 4562.
[6] C. Larsson, A. Blomberg, L. Gustafsson, Biotechnol. Bioeng. 38 (1991) 447.
[7] E. Albers, C. Larsson, G. Lidén, C. Niklasson, L. Gustafsson, Appl. Environ. Microbiol. 62 (1996) 3187.
[8] E. Albers, G. Lidén, C. Larsson, L. Gustafsson, Recent Res. Dev. Microbiol. 2 (1998) 253.
[9] L.H. Christensen, U. Schulze, J. Nielsen, J. Villadsen, Chem. Eng. Sci. 50 (1995) 2601.
[10] U. Schulze, Ph.D. Thesis, Technical University of Denmark, Lyngby, Denmark, 1995.