Monitoring of Yeast Metabolism with Calorimetry

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A system for calorimetric measurements is established in a 1500 L pilot bioreactor. *Saccharomyces cerevisiae,* baker's yeast has three metabolic pathways: 1) purely oxidative, glucose consuming; 2) purely reductive, glucose consuming and ethanol producing and 3) purely oxidative, ethanol consuming. Designing experiments to lead the yeast to a single pathway, the accuracy of the calorimetric measurements are verified. Using on-line data of microbial heat production and substrate consumption, the combustion enthalpies of glucose and ethanol in purely oxidative (aerobic) fed batch fermentations are determined as 15900 and 29000 kJ/kg respectively, Combustion enthalpy of glucose in purely reductive (anaerobic) environment is determined as 511 kJ/kg. These values are in good corresponding with literature data. It is now possible to determine the fraction of substrate uptake utilized for energy (catabolism, k) and biosynthesis (anabolism, a) metabolisms on-line. In oxidative (aerobic) fermentations we determined k_{Sox}=0.45 for glucose, k_{Eox}=0.63 for ethanol and in reductive (anaerobic) fermentations k_{Sred}=0.96 for glucose; the anabolism factors are a_{Sox}=0.55, a_{Eox}=0.37 and a_{Sred}=0.04 respectively.

The single pathways can occur together so that an experiment is designed with changing environmental conditions to prove the overall calorimetric model. The result is that measured and the calculated microbial heat energy rates are in good accordance. Calorimetric measurements can be used to monitor yeast metabolism on-line, for advanced control strategies or, to predict fermentations or for designing heat exchanger or bioreactor systems.

Key words:
Continuous calorimetry, baker's yeast metabolism, anabolism, catabolism

Introduction

In the production of baker's yeast, *Saccharomyces cerevisiae* converts the substrate under aerobic conditions into energy and utilizes it for growth and maintenance. Being these biochemical reactions exothermic, the produced energy rises the temperature in the reactor. Since, the temperature is required to be held at a constant value of 30 °C, excess energy must be removed from the system. The raise of temperature, i.e. the production of heat, is related with the feedings, mainly the carbon source, during the fermentation.

The measurement of the heat produced during microbial reactions is an excellent tool for determining the metabolism of the yeast.[1,2,3] Several authors described methods for calorimetric monitoring in bioreactors.[2,4] Although the temperature can be determined very exactly with the help of common sensors, continuous calorimetry in laboratory scale fermentations still remains a problem. The reason of this fact is that the ratio of surface to volume is too great. Heat losses and side effects disturb the measurement severely. In the extensive review of Stockar and Marison[2] is claimed that continuous calorimetry could be used at production scale as an on-line sensor for indirect determination of biomass concentration, product formation and state of the culture.

Voisard et al.[36] used a 300 L pilot scale bioreactor as a calorimeter and demonstrated that quantitative calorimetry is not only possible at pilot scale but could be applied at larger scales. Truly, scaling-up results in a higher ratio of heat producing volume to heat exchanging surface and thus increases the sensitivity. This is demonstrated by Türker[38] for baker's yeast production in a bioreactor of 100 m^3 volume. The, so-called, mega calorimetry is a robust and cost effective technique for process development, monitoring and control. Schubert et al.[44] achieved to turn a bench-scale bioreactor of 2 L volume into a useful calorimeter using two sensitive control circuits. The first circuit kept the bioreactor temperature constant and the second controlled the temperature difference between water jacket and the reactor.

Maskow et al.[40] emphasizes the advantages of calorimetry to give insights into dynamic biological processes and to use this information to control technical and natural systems. To be able to use calorimetry in bench-scale, immediately responding small detectors are needed. Reduction in calorimeter size diminishes the amount of energy needed for heating up the calorimetric device, while speeding up the measurement. The recent developments are resulting in integrated circuit (IC) calorimetry (also called thin film calorimetry, chip or nanocalorimetry) and enthalpy arrays.[40,42,43]

Calorimetry is a promising monitoring tool as it provides real-time information about growth rates and stoichiometry.[40,41] Voisard et al.[37] accomplished to control the nutrient feed in a fed-batch cultivation of *Bacillus sphaericus*, using measurement of the metabolic heat release. *Bacillus sphaericus,* strictly aerobic, changes the morphology due to sporulation and the excretion of proteases and crystal proteins, which are toxic to mosquito larvae. The calorimetry driven feed control enabled cells to grow at the maximum rate due to non-limiting and non-inhibiting substrate concentrations. More examples are given in the review of Maskow et al.[40]

Types and problems of calorimeters[16,40] are:

1. Microcalorimetry: problems are aeration and stirring

2. Flow calorimetry: fermentation broth is pumped from the fermenter into a microcalorimeter. Problems: time lag; oxygen and nutrients are consumed that their concentrations change during the measurement.

3. Dynamic calorimetry: an energy balance is established around the reactor. Problem: the temperature increase of the insulated reactor is measured after switching off the cooling.

4. Continuous calorimetry (mega calorimetry): a heat balance is established around the insulated reactor. Problem: good results in pilot and production scale but special precautions are needed for bench scale bioreactors.

5. Integrated circuit (IC) calorimetry: miniaturized calorimeters are in development since last ten years.

Our experiments are carried out in a pilot scale stirred tank reactor of 1500 l volume. Knowing all the sources and sinks of heat, it is possible to establish a heat balance around the reactor and than to determine the heat flow produced by the yeast. An important factor at the heat balance is the heat capacity of the liquid in the reactor.[4] If the temperature in the reactor could be kept at a constant value, this term could be ignored during the continuous calorimetry. In our reactor, the fermentation temperature is controlled by regulating the flow of cooling water by a control valve. The heat exchanger is the double jacket of the reactor. Cooling water is supplied from the groundwater wells. Climatic conditions and the consumed amount of water may change the temperature of cooling water between 15 and 23 °C. On the other hand, due to our fed batch process, the microbial heat production is about three times higher at the end of the fermentation than at the beginning. A precise control of the temperature during the fermentation by using a common single loop PID-controller (SLC) is difficult because of these two facts: changing heat production rate and cooling water temperature. So we can control the fermentation temperature, Tf, only within an accuracy of 1 °C.

The accuracy of 1 °C is not enough to ignore the effect of the heat capacity during the calorimetric measurements. A solution for controlling the temperature by changing system parameters, cooling water temperature and heat production rate, is to use another control strategy: the model predictive control (MPC).

The essence of MPC is to optimize and to forecast the process behaviour. The forecasting is accomplished with a process model, and therefore, the model is the essential element of an MPC controller. The model of the process may be not good enough to forecast perfectly. So a feedback can overcome some effects of a poor model. The difficulty that MPC introduces into the robustness question is the open loop nature of the optimal control problem and the implicit feedback produced by the receding horizon implementation.[5,6] So using MPC, an optimizer computes future control actions that minimize the difference between a model of the process and desired performance over a time horizon.[7]

MPC has been used in over 2000 industrial applications, in the refining, petrochemical, chemical, pulp and paper and food processing industries.[5,7] In biotechnology there are several attempts to use MPC and model based control systems. Ramaswamy et al.[39] for example, describes an MPC system to control the optimal biomass growth based on substrate concentration in a non-linear bioreactor (some more examples).[8-11]

We have already worked out dynamic models of the yeast growth and the reactor system[12,13] and applied in control algorithms. Having jumped the main hurdle of the MPC, description of the process model, MPC could be used for a precise temperature control.[14,15] The intention of this work is to establish a pilot reactor system for continuous calorimetric measurements to monitor the metabolism of *Saccharomyces cerevisiae*.

Material and methods

Cultures and Fermentation

A production strain (PAKMAYA) of *Saccharomyces cerevisiae* is used in the experiments.

The fermentation is a combined batch and fed-batch process. After the batch phase, glucose is fed as the carbon source until the end of the fermentation. The separate sterilization of the glucose solution prevents the Maillard reactions and the substrate remains microbial available.

The medium used is synthetic, containing per 100 kg of glucose:

3.64 kg H_3PO_4 (85%), 1.5 kg $MgSO_4 \cdot 7H_2O$, 1.4 kg $CaCl_2 \cdot 2H_2O$, 3 kg KCl, 30 g $ZnSO_4 \cdot 7H_2O$, 48 g $FeCl_3 \cdot 6H_2O$, 7.8 g $CuSO_4 \cdot 5H_2O$, 35 g $MnSO_4 \cdot 2H_2O$, 0.2 g $Na_2MoO_4 \cdot 3H_2O$ and 200 g m-inositol. These compounds are sterilized in the reactor before the inoculum. The thermo-sensitive compounds of the medium: 100 g Ca-pantothenate, 20 g Vitamin B1 (Thiamin-HCl), 5 g Vitamin B6 (Pyridoxin-HCl), 0.5 g Biotin and 20 g Nicotinic acid are sterile filtrated and added separately into the reactor.

The pre-culture is prepared in an inoculation flask. The flask containing 145 g glucose, 32 g $(NH_4)_2SO_4$, 10.24 g $(NH_4)_2HPO_4$ and the other substances calculated according to glucose amount as mentioned above is inoculated from an agar tube. The vitamins are sterilized by filtration. The flask, with the total volume of 8 L, is aerated through a sterile filter and incubated at 30 °C for 72 h. The pre-culture is inoculated into the reactor through a sterile port.

During the fermentation the ammonia, used as the N-source, is fed as a base by the pH control. The experiments are done without N-limitation. The temperature and pH are controlled at 30 °C and 5.0, respectively.

Reactors, instrumentation and control systems

The fermentations were carried out in a pilot scale stirred tank reactor of 1500 L volume 955 mm inner-diameter, 2.1 m height and 3x6-blade Rushton turbines.[12,13] The reactor is fully equipped with instrumentation and control systems for the fed-batch operation. The measured values are the fermentation, air, cooling water and environment temperatures, pH, pressure in the reactor, stirrer's sped, dissolved oxygen in the liquid, oxygen, carbon dioxide and ethanol in the exhaust gas, substrate feed, air and cooling water flow rates, air humidity and liquid volume.[12,13]

The reactor is connected to a SCADA system; the SCADA software running on a PC can manage all the monitoring, control, historical trends, graphics and documentation as an interface between the operator and sensors and controllers. The changing set points of the feedings, like substrates and air, are sent to the controllers from the profile files established at the beginning of the fermentation. Complicated control algorithms can be programmed and driven from the PC. Different calculations, like the oxygen transfer rate (OTR), carbon dioxide production rate (CPR), respiration quotient (RQ), volumetric mass transfer coefficient (k_La) and microbial heat evolution (Qf) can be calculated from the on-line measurements and used for the control algorithms. Samples taken during the fermentations were analysed off-line.

Off-line analysis

The samples taken are cooled on ice and centrifuged. The pellets are washed with isotonic water twice and centrifuged. The dry matter of the pellets is determined gravimetrically after drying at 105 °C until they reach a constant weight.

The total N-content of the cells is determined according to Kjeldahl.[17] In this method, all substances containing nitrogen will be transformed to $(NH_4)_2SO_4$ by H_2SO_4, heat and catalyst. The liberated NH_4 is distilled by steam, captured in boric acid and the N-content is determined by titration.

The protein content of the cells is calculated by multiplication of Kjeldahl-N with a factor of 6.25.[18]

Alcohol in the supernatant is determined by distillation into acidified potassium dichromate solution of known volume and concentration. Ethyl alcohol is oxidized to aldehyde and chromium gives a colour indication which is determined colourometrically.[19] The glucose is determined with a YSI-analyser using an enzymatic assay. The ammonium in the fermentation broth is analysed by buffering the supernatant of the samples with a borate buffer and distilling into a solution of boric acid and determined by titration with H_2SO_4.[20]

There are several carbohydrate compounds in the yeast cell: trehalose, glycogen, manna and glucan. They are extracted using different chemicals and purification steps. The trehalose is extracted with trichloroacetic acid according to Steward.[21] The extraction methods of glycogen, manna and glucan are described in Herbert et al.[22] The extracts containing the solute carbohydrates are treated with the anthrone reactive[23] at a defined temperature and the absorbance of the formed glucose is determined colourometrically at a wavelength of 630 nm.

RNA in the yeast cells is extracted with perchloric acid and the formed colour after treating

the extract with orcinol reactive is determined colourometrically at a wavelength of 670 nm.[21]

Ash content of yeast is determined by burning at 600 °C for 20 h per gravimetry.

Glycerol is determined by the enzymatic test kit of Boehringer Mannheim (Cat.no. 148270).

Total organic carbon, TOC and inorganic carbon IC analysis are performed by an Ionics I 555B analyser.

Calorimetry in yeast fermentations

In the production of baker's yeast, *Saccharomyces cerevisiae* converts the substrate into energy and utilizes it for growth and maintenance. These biochemical reactions are exothermic and the produced energy, Qf, rises the temperature in the reactor. Therefore, the temperature is required to be held at a constant value of 30 ^{0}C; excess energy must be removed from the system. The rising of temperature, i.e. the production of heat, is related with the feedings, mainly the carbon source, during the fermentation.

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We carried out our experiments in a pilot-scale stirred tank reactor of 1500 L volume. Knowing all the sources and sinks of heat, it is possible to establish a heat balance around the reactor and than to determine the heat flow produced by the yeast. An important factor at the heat balance is the heat capacity of the liquid in the reactor.[1] If the temperature in the reactor could be kept at a constant value, this term could be ignored during the continuous calorimetry. In our reactor, the fermentation temperature is controlled by regulating the flow of cooling water by a control valve. The heat exchanger is the double jacket of the reactor. Cooling water is supplied from the groundwater wells. Climatic conditions and the consumed amount of water may change the temperature of cooling water between 15 and 23 °C. On the other hand, due to our fed-batch process, the microbial heat production is about three times higher at the end of the fermentation than at the beginning. A precise control of the temperature during the fermentation by using a common single loop PID-controller (SLC) is difficult because of the following two facts: the changing heat production rate and the cooling water temperature. So we can control the fermentation temperature, Tf, only within an accuracy of 1°C.

Model predictive control, MPC

The accuracy of 1°C is not enough to ignore the effect of the heat capacity during the calorimetric measurements. A solution for controlling the temperature by variable system parameters, cooling water temperature and heat production rate, is to use another control strategy: the model predictive control (MPC).

The essence of MPC is to optimize and to predict the process behaviour. The prediction is accomplished with a process model, and therefore, the model is the essential element of an MPC controller. We have already worked out dynamic models of the yeast growth and the reactor system[12,13] and applied them in control algorithms. Having jumped the main hurdle of the MPC, which is the description of the process model, we used MPC for precise temperature control.[14] We established a system predicting the flow rate of cooling water in order to control the temperature by using fermentation data collected from the beginning of the process until the present time. The model and control algorithms are established in the SCADA program running on a standard PC.

Comparing the data of two identical fed-batch experiments we showed that the accuracy of Tf with MPC (0.1 °C) is more precisely than with SLC (1 °C) over the whole fermentation.[14]

To prove that this accuracy is enough to make calorimetric measurements we made experiments with strictly oxidative fermentations and verified our calorimetry.[15]

Process models

Microbial heat production model

With a heat balance around the reactor we calculate the volumetric heat production in the reactor, Qt [kW/m^3] (1), as microbial heat production, Qf, plus friction energy, Qr, minus heat losses by evaporation, Qv, radiation and convection, Qk, and cooling by aeration, Qa (Fig. 1).[22,23] In order to keep the temperature Tf constant, the heat flow has to be removed from the system by cooling. The cooling energy, Qs, via the double jacket, is determined by the available heat exchange area, the cooling water flow rate Fs [m^3/h], and cooling water temperature Ti (Fig. 1).

$$Qt = Qf + Qr - Qv - Qk - Qa - Qs \qquad (1)$$

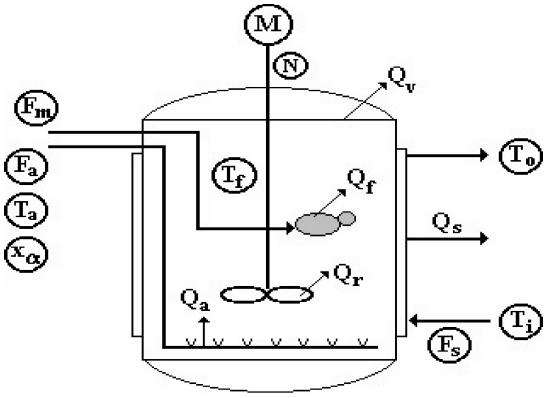

Fig. 1 – *Heat balance around a bioreactor*

The model equations of the single terms are described previously in.[15]

Calorimetric model

In the fermentation, the baker's yeast can ran through three different metabolic pathways:[19]

1. Purely oxidative growth on glucose (2)
2. Purely reductive growth on glucose (3)
3. Purely oxidative growth on ethanol (4)

These states are dependent on the specific substrate consumption rate, r_S, and on the mass transfer capacity of the reactor (OTR).

In a purely oxidative (aerobic) cultivation, where $r_S < r_{Scrit}$, with excess oxygen and without alcohol production the substrate glucose is utilized according Eq. (2):

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow \rightarrow 6\ CO_2 + 6\ H_2O + 38\ ATP + 2874\ kJ \quad (2)$$

The anaerobic process, the purely reductive pathway, is according to Eq. (3)

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 + + 2\ ATP + 92\ kJ \quad (3)$$

The produced alcohol is consumed in the presence of O_2 according to Eq. (4)

[ATP gain according to our own calculations]

$$C_2H_5OH + 3\ O_2 \rightarrow \rightarrow 3\ H_2O + 2\ CO_2 + 12\ ATP + 1310\ kJ \quad (4)$$

The value of the energy lost by heat for the case of substrate glucose, i.e. the combustion enthalpy of glucose in the oxidative reaction (2), h_{Sox} [kJ/kg$_S$], is found to have a range of values in the literature:

15 400 kJ/kg$_{glucose}$,[27] 15 667 kJ/kg$_{glucose}$,[29] and 15 967 kJ/kg$_{glucose}$.[26]

In a previous work we could verify our calorimetric measurements experimentally and proof that we can obtain the value h_{Sox} = 15900 kJ/kg$_S$.[15]

According to literature the combustion enthalpy of glucose in the reductive reaction (3) is h_{Sred} = 511 kJ/kg$_S$[29] and the combustion enthalpy of ethanol in the oxidative reaction (4) is h_{Eox} = 28 460 kJ/kg$_E$,[19] 29 130 kJ/kg$_E$.[29]

These values are to be verified in this paper.

The ATP gain in the both pathways of yeast sums to

Oxidative pathway:

211.11 Mol$_{ATP}$/kg$_S$ for glucose

260.89 Mol$_{ATP}$/kg$_E$ for ethanol [according to our own calculations]

Reductive pathway:

12.42 Mol$_{ATP}$/kg$_S$ for glucose

According to the stoichiometry in Eq. (2), the oxygen quantity required for the complete combustion of glucose, h_O [kJ/kg$_{O2}$], is 1.07-times higher than for h_{Sox}:

$$OTR = 1.067\ SCR$$

With the oxygen transfer rate, OTR [kg/m^{3}h], and the substrate (here glucose) consumption rate, SCR [kg/m^{3}h]. SCR can be calculated using on-line fermentation data: substrate feed rate Fm [m^3/h] and glucose concentration in the substrate feed, c_s [kg/m^3].

$$SCR = Fm\ c_s / V_L \quad (5)$$

Further stoichiometric relations between OTR, ethanol production EPR and consumption ECR rates [kg/m^{3}h], are as follows:

$$OTR = 2.09\ ECR$$

$$SCRred = EPR / 0.51.$$

$$SCRox = SCR - SCRred$$

If all the substrate consumed would be used in the cell for the energy metabolism, the catabolism (k), the given heat-flow in for example Eq. (2) would be released and could be measured. But we know that the cells use the carbon source also in the biosynthesis, the anabolism (a). So the consumed glucose follows two different paths in the cell:

$$r_S = k\ r_S + a\ r_S \quad (6)$$

With r_S [1/h] as the specific substrate uptake rate and the factors k and a, are

$$k + a = 1 \quad (7)$$

The main idea for our calorimetric measurements is to determine the value of k, and naturally a, for different states of baker's yeast fermentation on-line. Here we can use the following model equation:

$$Qf,mod = h_{Sox}\, k_{Sox}\, SCRox + \\ + h_{Sred}\, k_{Sred}\, SCRred + h_{Eox}\, k_{Eox}\, ECR \qquad (8)$$

The values of Qf, SCR and ECR are determined on-line with the help of signals from the sensors, the combustion enthalpies are known. So single terms of Eq. (8) can be solved for k for defined environmental conditions and calculated in the SCADA system.

The oxidative yeast cultivation is determined by the critical specific substrate uptake rate, $r_{S,crit.}$.[30] Exceeding this value, the culture produces ethanol according to the bottleneck model of Sonnleitner and Käppeli.[31] The critical substrate uptake rate is a strain specific parameter. We determined $r_{S,crit}$ for our strain using experimental data and a growth model established with MATLAB-SIMULINK software: $r_{S,crit} = 0.47$.[14] This value for our strain is in good agreement with the literature.[30]

In our experiments we detect all the above mentioned states of yeast fermentation. Fig. 2 symbolizes our simplified model of yeast metabolism.

Having shown, that the fermentation temperature can be controlled at a constant set point with high accuracy, the model equation (1) can be used for calorimetry (Fig. 2).[15] The on-line signals of cooling water flow and the temperatures Ti and To are calculated to the cooling energy Qs. This value equals to the total heat energy Qt (1), where Qr, Qa and Qv can be determined using on-line signals coming from the field. So the microbial heat production, Qf, can be calculated on-line in the SCADA system.

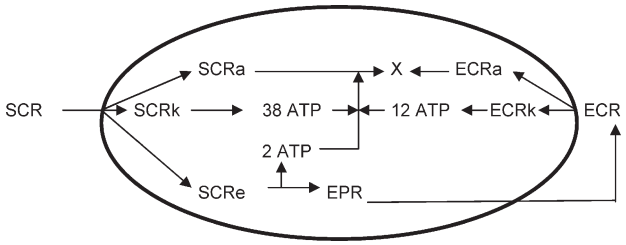

Fig. 2 – *Simplified model of yeast metabolism*

Results and discussion

We made three experiments to obtain the environmental conditions leading the yeast to the metabolic pathways described in the Eqs (2, 3, 4). All the experiments are in the fed-batch period of the fermentation in order to have a sufficient stock of biomass. Table 1 shows the fermentation parameters: biomass, X, Ethanol, E, substrate (glucose) consumption rate, SCR, substrate (glucose), S, air flow rate, Fa, carbon dioxide in the exhaust gas, CO_2 and the dissolved oxygen in the broth, pO_2, in the selected time intervals.

In experiment I, glucose feed and aeration rates enable the yeast to maintain the purely oxidative pathway. In experiment II, the anaerobic environment is obtained by setting the air flow rate to zero and continueing with the glucose feeding. The carbon source in experiment III is ethanol and the aerobic environment is maintained by aeration. The ethanol is produced by the yeast itself, following an impulse of glucose feed of high concentration, before the time interval given in Table 1.

Factors of catabolism and anabolism are determined with the help of these experiments of defined metabolic pathways. The microbial heat production, Qf, is calculated using Eq. (1) and field measurements on-line. On the other hand we used the carbon balances in the time intervals to determine the corresponding average biomass/substrate-yields on carbon base, i.e. factor a. So we could determine with the help of Eq. (7) the average factor k for the given time interval. Using one term of Eq. (8),

Table 1 – *Fermentation parameters*

Exp. (Eq.)	t [min]	X [kg]	E [kg]	SCR[kg/m^{3}h]	S [kg]	Fa [m^3/h]	CO_2 [kg]	pO_2 [%]
I	1800	39.7	0	8.7	73.3	100	39.7	30
(2)	2100	60	0	7.7	112.7	100	59.1	25
II	3000	62.8	0	27	177.7	0	n.a.	0
(3)	3275	66	37	22	261.7	0	n.a.	0
III	2100	40.2	15.5	0	0	100	61.2	0
(4)	2400	45.5	2.6	0	0	100	76.3	0

which represents the metabolic pathway present, we can now determine the combustion enthalpy. The calculated value of the combustion enthalpy should be in the range of the values given in the literature. If this is true, the measurements are verified and the determined values for the factors k in varying metabolic pathways are reliable.

Experiment I: aerobic growth on glucose

As seen in Fig. 3 the conditions for a purely oxidative metabolism are given in experiment I: the specific substrate uptake rate $r_S < r_{Scrit}$, (r_{Scrit} = 0.47) and the dissolved oxygen is non-limited, neither ethanol production is detected.

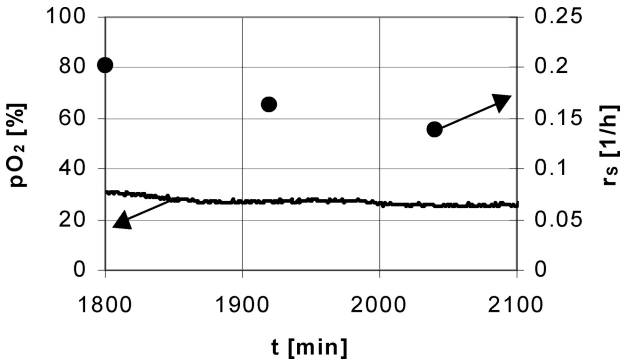

Fig. 3 – *Dissolved oxygen tension pO_2 [%] and specific substrate uptake rate r_S [1/h] in experiment I*

According to Eq. (12), a part of the substrate, S, is converted to CO_2 in the energy metabolism and the remaining part to biomass, X. The mass balance over the period investigated (1800^{th} – 2100^{th} min.) with the values given in Table 1 is as follows:

$$39.4\ kg_S = 20.3\ kg_X + 23.6\ kg_{CO_2}$$

According to this equation the yield is $Y_{X/S}$ = 0.51. For transforming the mass balance to a carbon balance we need to know the carbon content of the biomass.

Cell composition

As known in the literature, the composition of a yeast cell can be broken down to four main polymers: protein, carbohydrate, nucleic acid and lipid. In addition, part of the biomass consists of inorganic metals (ash).[32] We evaluated protein, carbohydrate, RNA, lipid and ash contents at the end of the fermentation with a regaining rate of 96.8 % (Table 2).

The results are in good corresponding with reported data by several authors (s.[32]).

Table 2 – *Cell composition of baker's yeast at the end of the experiment I given in [%] of cell dry mass*

Cell components	[%]	C-content [%]
Trehalose	16.8	6.7
Mannan	5.4	2.2
Glycogen	8.7	3.5
Glucan	5.7	2.3
Sum of carbohydrates	36.6	14.7
Glycerol	0.6	0.2
Protein	41.6	22.2
RNA	8	2.8
Lipid	4	2.6
Ash	6	–
Sum	96.8	42.5

The carbon balance over the investigated period in experiment I is as follows:

$$15.76\ kg_{S\text{-}C} = 8.63\ kg_{X\text{-}C} + 6.43\ kg_{CO_2\text{-}C}$$

$$15.76\ kg_C = 15.06\ kg_C$$

Accuracy –4.4%

The yield for carbon is $Y_{X\text{-}C/S\text{-}C}$ = 0.55, i.e. 55 % of the substrate-carbon is utilized for the anabolism and 45 % remains for the catabolism (a = 0.55; k = 0.45).

We have determined the factor k = 0.45 for the energy metabolism. This value is to be regarded as an average for the period between 1800^{th} and 2100^{th} min. This value is used to verify the accuracy of our calorimetric measurements. Relating the Qf values (Fig. 4) to (k SCR) we can calculate the combustion enthalpy of glucose, h_{Sox}. Fig. 5 shows h_{Sox}., calculated with the constant and average k-value of 0.45,

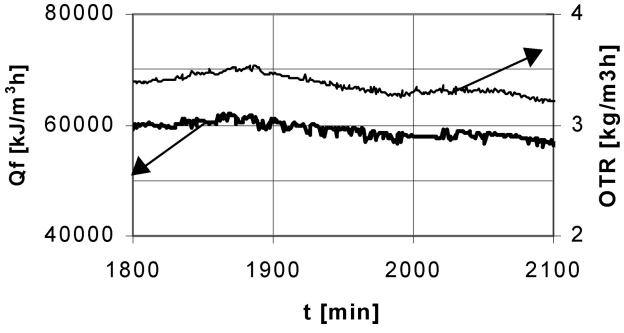

Fig. 4 – *Microbial heat production Qf [kJ/m³h] and oxygen transfer rate OTR [kg/m³h] during experiment I*

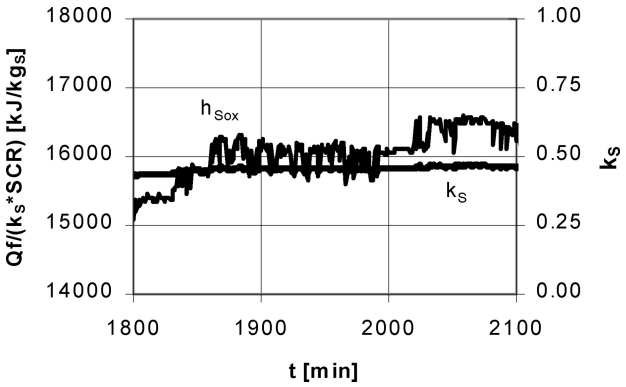

Fig. 5 – *Combustion enthalpy of glucose $h_{So}x$ (Qf / k_S SCR) and catabolism factor k_S during experiment I*

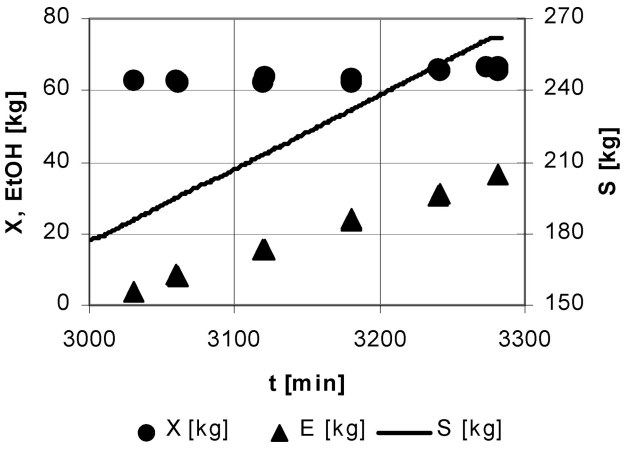

Fig. 6 – *Biomass X, Ethanol EtOh production and substrate S consumption during experiment II*

varying between 15300 and 16550 kJ/kg. In the literature we find values between 15400 and 15967 kJ/kg as mentioned before. For further calculations we chose for the combustion enthalpy of glucose in aerobic environment the value of 15900 kJ/kg.

As given in Table 1, the feed rate decreases in the period of the fermentation we analyse, constantly in order to keep the culture non oxygen limited. The result is decreasing growth rates (Fig. 3). The protein content of yeast biomass is strongly influenced by the growth rate. With decreasing growth rates the protein content decreases. The carbohydrate is inversely related to the protein content, i.e. shows an increase with decreasing growth rates.[32] In our experiment we determined the protein contents of yeast at the beginning and end of the fermentation. According to the literature we find 52.02 % protein for t = 1st min and 41.60 % for t = 2336th min. This fact means that the carbon content of the cells may change along the fermentation. Another point is, that different ATP-demands are needed for the synthesis of different biomass polymers. The ATP-demand for the synthesis of protein, grown on glucose, is about 3 fold more than for carbohydrates.[32] These facts indicate that the factor k could change in a fermentation with changing growth rates.

The k-value calculated using the measured Qf and chosen h_{Sox} varies between 0.44 and 0.47 with an increasing tendency (Fig. 5). With the decreasing SCR (Table 1) also the metabolic activity of the culture decreases as seen by the values of Qf and OTR in Fig. 4. The answer of the yeast to this is a slightly increasing k.

Experiment II: anaerobic growth on glucose

At the 3000th min. of experiment II the aeration was stopped and an anaerobic environment was achieved (pO_2 = 0 %, Table 1). The feeding was kept at a constant rate and the substrate was converted to ethanol, glycerol, biomass and CO_2 (Fig. 6, Fig. 7 and Table 1). The measured values of produced acetic acid were neglected (not shown here). Having no appropriate device, we could not measure the CO_2 rate driven from the exhaust line. CO_2 is calculated according to stoichiometry in Eq. (3). Only the soluble CO_2 content could be determined (inorganic carbon dioxide). The mass balance over the anaerobic period between 3000th and 3275th minutes is as follows:

$$84.04\ kg_S = 3.2\ kg_X + 37\ kg_E + 35.46\ kg_{CO2} + \\ + 1.07\ kg_{ICO2} + 0.35\ kg_G + 0.14\ kg_S$$

We calculate an average yield factor of $Y_{X/S}$ = 0.04.

Cell composition

The yeast cell composition changes with varying environmental conditions. Among the cell components given in Table 2, protein and trehalose show a fast and drastic response to the changes.The carbohydrate is inversely related to the protein con-

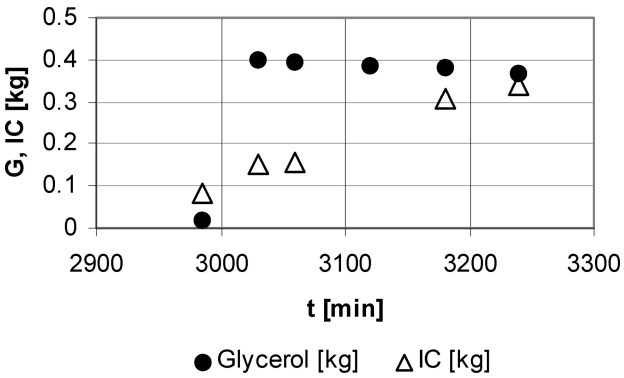

Fig. 7 – *Glycerol and inorganic carbon IC production during experiment II*

tent.[32] In this experiment we followed protein and trehalose contents of the cell mass during the anaerobic period (Table 3). The sum of the carbon content of these both substances remains almost constant. We assumed that the remaining cell components have the same values like given in Table 2 and added the carbon of protein and trehalose to the sum of the rest. The regaining rate of the sum of the cell components is between 94.7 and 99.8 %. So we gained an average carbon content of the yeast in the period between 3000th and 3275th min. as 44 %.

Table 3 – *Cell composition of baker's yeast, anaerobic growth on glucose, experiment II*

t [min]	Protein [%]	Trehalose [%]	Protein + Trehalose [%]	Sum C [%]
3000	51.53	6.57	58.1	30.1
3030	56.47	4.94	61.4	32.1
3060	52.94	3.95	56.9	29.8
3120	52.94	3.41	56.4	29.6
3180	52.94	3.34	56.3	29.6
3240	54.34	4.04	58.4	30.6
3275	55.06	4.11	59.2	31.0
average			58.1	30.4

The carbon balance gives now

$$33.62\ kg_{S\text{-}C} = 1.4\ kg_{X\text{-}C} + 18.87\ kg_{E\text{-}C} +$$

$$+ 9.65\ kg_{CO2\text{-}C} + 0.34\ kg_{ICO2} + 0.14\ kg_{G\text{-}C} + .06\ kg_{S\text{-}C}$$

$$33.62\ kg_{C} = 30.47\ kg_{C}$$

The accuracy of –9.4 % can be accepted as far as the produced CO_2 is not measured but estimated according to the stoichiometry.

With the carbon yield of $Y_{X\text{-}C/S\text{-}C} = 0.04$ we can calculate the average factors for anabolism, a = 0.04 and catabolism, k = 0.96 in the anaerobic environment.

ATP gain in the reductive metabolism is far below the ATP gain in the oxidative metabolism for the same amount of substrate. So in anaerobic environment almost all of the substrate is used for energy metabolism but barely for biosynthesis. Yeast uses the energy for maintenance.

The combustion enthalpy of glucose in anaerobic conditions can be calculated with this average k-value. As seen in Fig. 8 $h_{S,red}$ is between 450 and 550 kJ/kg, a value in good accordance with the literature, i.e. 511 kJ/kg. 'The fermentation heat' of an anaerobic alcohol fermentation was firstly determined in 1856 by Dubrunfaut.[45] 2559 kg sugar in oak vessels of 21400 L volume (3 m in height and diameter) was converted to alcohol. The temperature increased from 23.7 to 33.7 °C during four days of fermentation at ambient temperatures between 12-16 °C. Taking heat losses by radiation, convection, evaporation and heat storage by the wood into account, a vaule of 527 kJ/kg glucose equivalent was calculated; suprisingly close to our results.

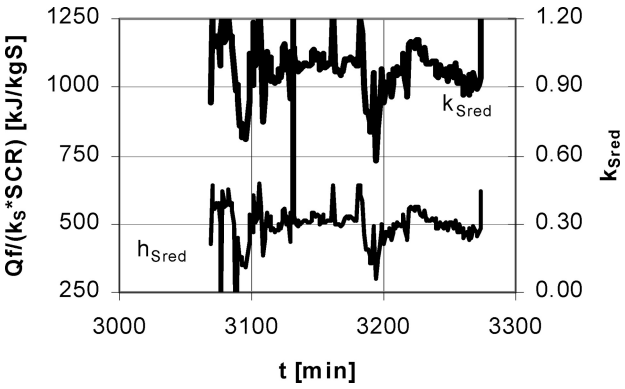

Fig. 8 – *Combustion enthalpy of glucose h_{Sred} (Qf / k_S SCR) and catabolism factor k_{Sred} during experiment II*

The on-line calculated value for $k_{S,red}$ is between 0.9 and 1.1. In the following we use the average value 0.96 for the anaerobic conditions.

In Fig. 9 we see the microbial produced heat energy and the substrate consumption rate. Qf has an instability at the 3181st min. Substrate feeding is not the reason as seen in the SCR-progress in Fig. 9. In the 3181st min we changed the stirrer's speed from 200 rpm to 150 rpm. The instability is the response of the control system to this disturbance of changing energy input via stirrer.

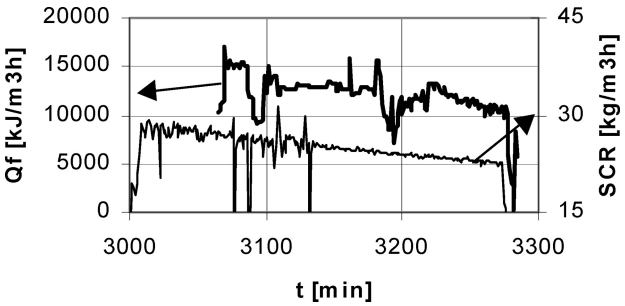

Fig. 9 – *Microbial heat production Qf [kJ/m^{3}h] and substrate consumption rate SCR [kg/m^{3}h] during experiment II*

Experiment III: aerobic growth on ethanol

In experiment III we had at the 2040th min. 39.5 kg biomass, no ethanol and a volume of 485 L. We stopped the continuous feeding in order to give 80 L glucose solution into the reactor as an impulse

to enforce the yeast to produce ethanol with a high ethanol yield. The ethanol production reached its maximum at the 2080th min. with the declining of the glucose concentration. Our experiment begins at the 2100th min., with the consumption of ethanol as the single carbon source. At the beginning of the investigated period we had 40.2 kg biomass, 15.5 kg ethanol and 565 L liquid volume. Although the aeration rate was 100 m^3/h the dissolved oxygen tension show 0%, i.e. all the oxygen transferred to the liquid phase is consumed (Table 1).

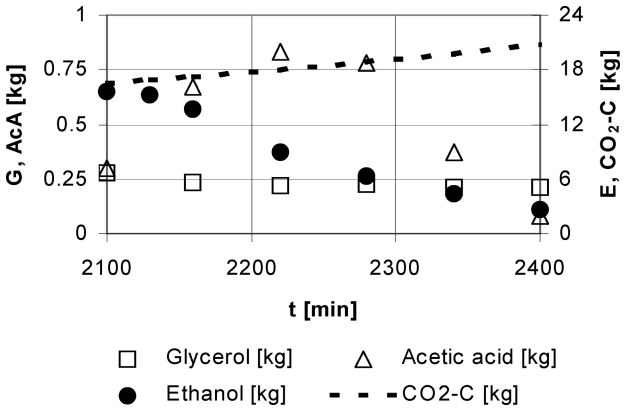

Fig. 10 – *Glycerol, ethanol, acetic acid and carbon-dioxide during experiment III*

The substrate ethanol is converted to biomass and carbon-dioxide according to Eq. (4) in the presence of oxygen. Besides this main metabolic reaction there are side reactions like production and consumption of glycerol and acetic acid under these environmental conditions. Fig. 10 shows the mass of glycerol, ethanol, acetic acid and carbon-dioxide in the period between 2100th and 2400th minutes. The mass balance in this period is as follows:

$$12.94\ kg_E + 0.22\ kg_{AcA} + 0.065\ kg_G = 5.3\ kg_X + 15.1\ kg_{CO_2}$$

We calculate an average yield factor of $Y_{X/E} = 0.41$

Cell composition

Also in experiment III we followed the protein and trehalose contents of the biomass during the investigated period (Table 4). The sum of the carbon content of these both substances remains almost constant (32 – 35 %). We again assumed that the remaining cell components have the same values like given in Table 2 and added the carbon of protein and trehalose to the sum of the rest. The regaining rate of the sum of the components is between 99.7 and 105.3 %. The average carbon content of the yeast in the period between 2100th and 2400th min. is 47 %.

Table 4 – *Cell composition of baker's yeast, aerobic growth on ethanol, experiment III*

t [min]	Protein [w%]	Trehalose [w%]	Protein + Trehalose [w%]	Sum C [w%]
2045	50.1	15.9	66	33.1
2100	62.5	2.4	64.9	34.3
2160	58.6	2.7	61.3	32.4
2400	60.4	6.5	66.9	34.9
2640	48.4	15.6	64	32.1
Average			64.4	33.9

The carbon balance gives now

$$6.6\ kg_{E\text{-}C} + 0.09\ kg_{AcA\text{-}C} + 0.025\ kg_{G\text{-}C} = 2.49\ kg_{X\text{-}C} + 4.12\ kg_{CO2\text{-}C}$$

$$6.71\ kg_C = 6.61\ kg_C$$

Accuracy –1.5 %

With the carbon yield of $Y_{X\text{-}C/E\text{-}C} = 0.37$ we can calculate the average factors for anabolism, a = 0.37, and catabolism, k = 0.63, in the aerobic environment for the substrate ethanol.

The combustion enthalpy of ethanol in aerobic conditions can be calculated with this average k-value. As seen in Fig. 11, $h_{E,ox}$ is between 31300 and 27400 kJ/kg, a value in good accordance with the literature (we chose for recent calculations 29000 kJ/kg). The on-line calculated value for $k_{E,ox}$ is between 0.7 and 0.6. In the following we shall use the average value 0.63.

Growth on substrate ethanol have a lower substrate yield compared to glucose. Meyenburg[34] already suggested that this is due to the necessity of

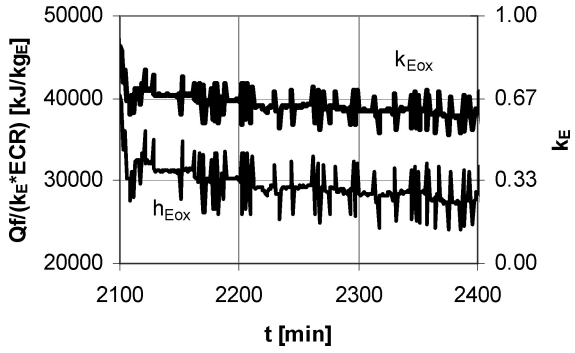

Fig. 11 – *Combustion enthalpy of ethanol h_{Eox} (Qf / k_E ECR) and katabolism factor k_{Eox} during experiment III*

gluconeogenesis and the need of ATP activation before ethanol could enter the TCA cycle. Entian[32] also remarks that yeast cells synthetise hexoses for the cell wall biosynthesis. Ethanol, consisting C_2., trigger the necessity of gluconeogenesis so the catabolism factor for oxidative growth on ethanol is higher than on glucose.

The yield for substrate ethanol is given as $Y_{X/E} = 0.4$.[33,35] The yield is determined using a flow-through microcalorimeter,[33] fed-batch process, in a 4 L fermenter with a working volume of 2.5 L at the end of the experiment which is fed with glucose solution. The yield determined with microcalorimetry in a batch experiment was $Y_{X/E} = 0.38$. The culture was in the early respiratory phase which is defined as, the produced ethanol is being consumed as the single c-substrate and oxygen was not limited.[33] These values correspond with our factor for anabolism a = 0.37.

In Fig. 12 we see the microbial produced heat energy and the oxygen transfer rate. Qf differs between 80000 and 70000 kJ/m^{3}h with a decreasing tendency. Also the OTR declines from 5.2 to 3.6 kg/m^{3}h. Both signals indicate a decreasing of the metabolic activity rate. During this period of the experiment the volume of the reactor does not change due to the lack of feeding. Also the aeration rate has the constant value of 100 m^3/h. So the reason should be metabolic and not due to mass transfer effects. The declining of k signals a lessening need to energy and the tendency to use the substrate as cell substance in the anabolism.

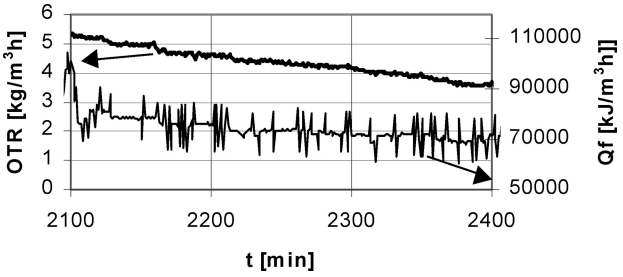

Fig. 12 – *Microbial heat production Qf [kJ/m^{3}h] and oxygen transfer rate OTR [kg/m^{3}h] during experiment III*

Experiment IV: combination of metabolic pathways in one fermentation

The results of three experiments are given in Table 5. The combustion enthalpies for the chosen environmental conditions and substrates are in good accordance with the values in the literature. This is the proof that our calorimetric measurement system is reliable. With the help of the combustion enthalpies the factors for catabolism are determined. Until yet we have shown that we are able to calculate calorimetric values for single metabolic reactions described by the Eqs (2, 3, 4). These reactions can occur together so that we designed an experiment to prove our overall calorimetric model equation (8).

Table 5 – *Combustion enthalpies h and average catabolism factors k*

Experiment/(Eq.)	Environment and carbon source	k	h [kJ/kg]
I (2)	Aerobic, glucose	0.45	15900
II (3)	Anaerobic, glucose	0.96	511
III (4)	Aerobic, ethanol	0.63	29000

In experiment IV we constructed environmental conditions in order to trigger the ethanol production. Also simultaneous consumption of ethanol and glucose was enforced by decreasing specific substrate uptake rate, r_S. (Table 6).

At the beginning of the experimental period, the 1320th min., there is no oxygen limitation (s. Fig. 13) and the specific substrate uptake rate r_S = 0.59 (Fig. 14, Table 6). This is greater than the critical value, $r_{S,crit} = 0.47$ (s. 3.2). The response of the culture is ethanol production as seen in Fig. 14. At the 1360th min the feed rate is set to a constant value, so that r_S decreases with increasing biomass concentration. The culture stops to produce ethanol. At the 1400th min. the cells begin to consume ethanol.

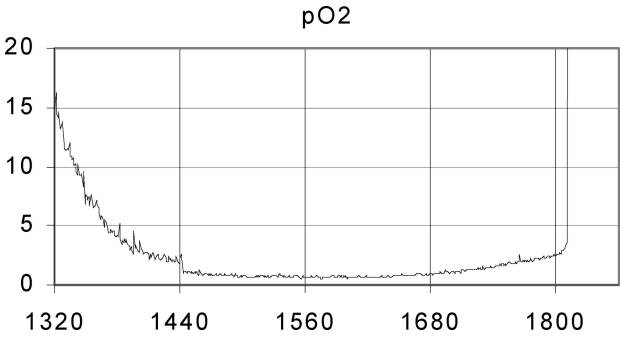

Fig. 13 – *Dissolved oxygen concentration pO2 [%] during experiment IV*

At the1440th min. we decreased the aeration rate of 100 m^3/h to 30 m^3/h in order to create an environment with oxygen limitation. The glucose feed rate is still kept constant that results in a decreasing substrate consumption rate, SCR (Table 6). The response of the reactor system is oxygen limitation (Fig. 13: pO_2 = 0 %). The culture reacts with production of ethanol up to the 1590th min. With increased biomass concentration the specific substrate

Table 6 – *Data of Experiment IV*

time [min]	Fa [m^3/h]	V [L]	SCR [kg/m^{3}h]	X [kg]	r_S [1/h]	E [kg]
1320	100	761	9.85	13.11	0.59	0.13
1440	100 → 30	799	9.61	20.32	0.38	0.05
1560	30	837	9.17	26.91	0.29	0.86
1680	30	875	8.77	34.88	0.22	0.98
1800	30 → 100	914	8.4	42.8	0.18	0.38
1820	100	924	8.3	–	–	0

uptake rate is low enough (s. Table 6) so that the ethanol production stops and the consumption begins at the 1680th min.

At the 1800th min we again increased the aeration up to 100 m^3/h. Within 20 min. the residual ethanol is consumed with increasing OTR (Fig. 14 and 15).

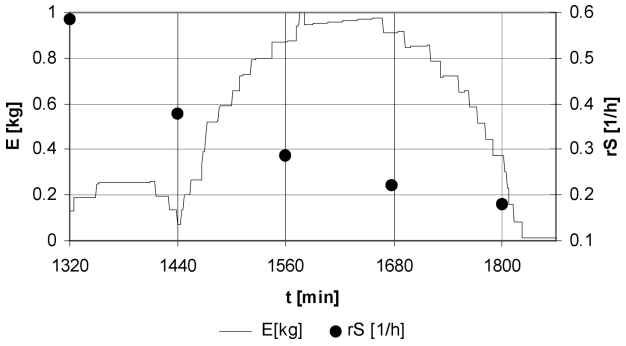

Fig. 14 – *Ethanol production and consumption and substrate consumption rate r_S during experiment IV*

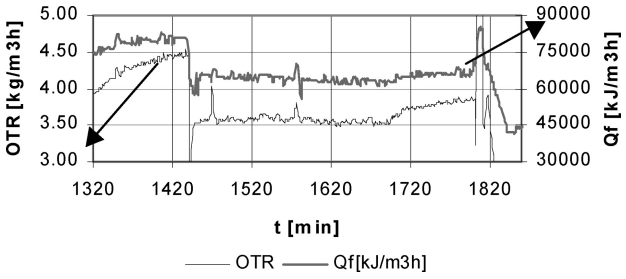

Fig. 15 – *Microbial heat production Qf [kJ/m^{3}h] and oxygen transfer rate OTR [kg/m^{3}h] during experiment IV*

Fig. 15 shows the course of the microbial heat energy. Qf has a strong dependency to OTR. Our aim with this experiment was to proof the model equation for the heat production (8). Eq. (8) has three terms according to the Eqs. (2-4). Depending on the environment, i.e. metabolism, one or more of the terms should be used to describe Exp. IV. k and h values in Eq. (8) are taken from Table 5. SCR is a process variable determined by the set value of the substrate feed rate (Eq. (5)). The ethanol production and consumption rates are calculated using he on-line ethanol signal (Fig. 14). The substrate consumed for ethanol production according to Eq. (3), SCRred is calculated using the stoichiometric relationship: SCRred = EPR / 0.51. The remaining substrate is than consumed according to Eq.: (SCRox = SCR – SCRred).

In Fig. 16 we see the parity plot of the measured, Qf, and the calculated, Qf,mod according to Eq. (8), microbial heat energy rates in the period between 1320th and 1860th min. The model equation represents the reality in good accordance.

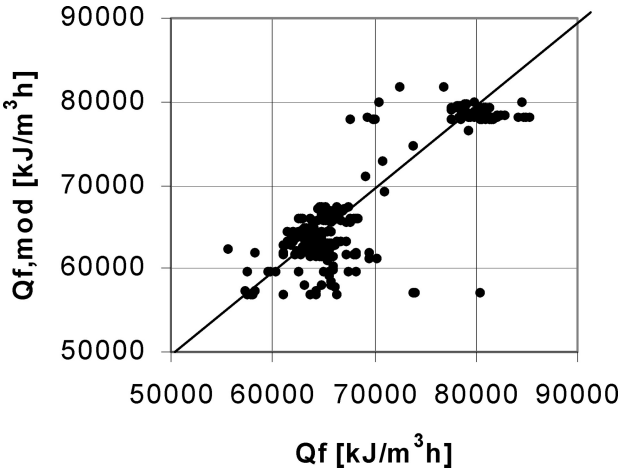

Fig. 16 – *Parity plot of the measured, Qf, and the calculated, Qf,mod according to Eq. (8) in experiment IV*

Conclusions

In a pilot scale bioreactor of 1500 L volume we established a system to make calorific measurements. The microbial heat production is determined using a heat balance around the reactor. In order to overcome the disturbing effects of the latent heat, the temperature in the reactor has to be controlled in a very narrow band. We obtained this using a model predictive temperature control.

Saccharomyces cerevisiae, baker's yeast, is used to verify the calorimetry. Yeast has three metabolic pathways: 1) purely oxidative, glucose consuming; 2) purely reductive, glucose consuming and ethanol producing and 3) purely oxidative, ethanol consuming. Three experiments are designed to obtain the environmental conditions leading the yeast to the three metabolic pathways described above. Calorific measurements obtained by these experiments are verified with literature data for

combustion enthalpies of the substrates in different pathways of the yeast.

With the help of the combustion enthalpies, the factors for catabolism and anabolism are determined for the three pathways of yeast metabolism. The single pathways can occur together so that an experiment is designed with changing environmental conditions to prove the overall calorimetric model. The result is that measured and the calculated microbial heat energy rates are in good accordance.

Calorimetric measurements can be used to monitor yeast metabolism on-line for advanced control strategies. Predicting the coming metabolic shifts, both free parameters substrate feeding and aeration can be driven in a way that the objectives biomass or product formation can be maximized. The results of calorimetry can also be used for designing heat exchangers or bioreactors.

List of symbols

A_{wt} – area of the heat exchanger, m^2
a – factor for anabolism, –
c_S – substrate concentration in feed, g/l
CPR – carbon dioxide production rate, kg/m^3h
D – reactor diameter, m
D_R – stirrer's diameter, m
ECR – ethanol consumption rate, kg/m^3h
ECRa – ethanol consumption rate used for anabolism – energy metabolism, kg/m^3h
ECRk – ethanol consumption rate used for catabolism – biosynthesis, kg/m^3h
EPR – ethanol production rate, kg/m^3h
Fa – air flow rate, m^3/s
Fm – substrate feed rate, l/h
Fs – cooling water flow rate, m^3/h
h_{Eox} – combustion enthalpy of ethanol in oxidative (purely aerobic) environment, $kJ/kg_{ethanol}$
h_{Sox} – combustion enthalpy of glucose in oxidative (purely aerobic) environment, $kJ/kg_{glucose}$
h_{Sred} – combustion enthalpy of glucose in reductive (purely anaerobic) environment, $kJ/kg_{glucose}$
h_O – oxygen quantity required for complete combustion of glucose, kJ/kg_{oxygen}
k – factor for catabolism, –
k_{Sred} – factor for catabolism for substrate in reductive (purely anaerobic) environment, –
k_{Eox} – factor for catabolism for ethanol in oxidative (purely aerobic) environment, –
k_La – volumetric mass transfer coefficient, 1/h
μ – specific growth rate, 1/h
μ_m – specific growth rate for maintenance, 1/h
OTR – oxygen transfer rate, kg/m^3h
pO_2 – partial pressure of oxygen (dissolved oxygen) in the liquid, %
Qa – heat loss by evaporation, kW/m^3
Qf – cooling by aeration, kW/m^3
Qr – microbial heat production, kW/m^3
Qs – cooling energy, kW/m^3
Qt – heat generated through agitation, kW/m^3
Qv – total heat energy in the system, kW/m^3
RQ – respiration quotient, –
r_S – specific substrate uptake rate (r_S = SCR/X), l/h
S – substrate concentration in the reactor, g/l
SCR – substrate consumption rate, kg/m^3h
t – fermentation time, h, min
Ta – air temperature, °C
Tf – fermentation temperature, K
Ti – cooling water temperature, at entrance of heat exchanger, K
To – cooling water temperature, at exit of heat exchanger, K
OTR – oxygen transfer rate, kg/m^3h
V_L – liquid volume, l
X – biomass concentration (dry mass), g/l
$Y_{X,S}$ – yield, biomass/substrate, –

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