ELSEVIER

Thermochimica Acta 277 (1996) 7–16

thermochimica acta

Biocalorimetry and laser flow cytometry for characterization of the physiological state of microorganisms

Brigitte Zentgraf

Technical University Furtwangen, Department of Chemical Engineering and Biotechnology, Laboratory of Biochemistry, Jakob-Kienzle-Straße 17, D-78054 Villingen-Schwenningen, Germany

Received 11 September 1995; accepted 20 October 1995

Abstract

The physiological state of microorganisms should be considered in biotechnology since this influences the rate and efficiency of cellular processes. Biocalorimetry and laser flow cytometry are suitable techniques for the description of the physiological state of microorganisms.

Keywords: Biocalorimetry; Laser flow cytometry; Physiological state

List of symbols

$\dot{Q}$	heat flow rate
X	biomass concentration
μ	specific growth rate
r_x	rate of biomass production
S_o	initial carbon-substrate concentration in feed medium
BC	percentage of budding yeast
$Y_{S/X}$	specific carbon-substrate consumption
ρ	yield
$Y_{Q/X}$	specific heat production
ψ	dissipation function
ψ_u	internal energy dissipation
ψ_d	external energy dissipation
η_{enth}	enthalpic efficiency
η	thermodynamic efficiency

SSDI 0040-6031(95)02776-9

t_r residence time
t time

1. Introduction

The physico-chemical conditions in a bioreactor, such as the temperature, pH, and the composition of the reaction media, influence the physiological state of the cellular system, defined by Malek in 1958 [1] as a qualitative category as well as an auxiliary operational term.

In the last decade, a systematic compilation of the features of the physiological state has become available, considering the hierarchical structure of the cellular system in space and time [2–5]. Such features which could be applied are:

— microorganism shape and size, synchronization index of microorganism population,
— state distribution of microorganism population (DNA content, glycogen content or RNA content),
— microorganism age,
— enzyme activities,
— yield coefficients, e.g. biomass yield,
— composition of biomass,
— combustion enthalpy of biomass,
— respiratory quotient,
— oxy-caloric coefficient,
— specific heat flux and reaction heat,
— dissipation function (external and internal energy dissipation),
— enthalpic efficiency and thermodynamic efficiency,
— intracellular ATP/ADP concentrations,
— intracellular $NADH/NAD^+$ concentrations.

These approaches led to new knowledge and to application of structured mathematical models which significantly improved both the theory and practice of biotechnology [5–10].

In order to characterize the physiological state, physico-chemical methods, such as biocalorimetry and laser flow cytometry, are suitable [11, 12].

Whereas biocalorimetry looks at the physiological state of a microorganism population as a whole, laser flow cytometry measures the individual physiological state of a microorganism. When synchronous or partially synchronized microorganism populations are studied, biocalorimetry also delivers detailed data on the physiological state of a microorganism. The synchronization of microorganism functions can be carried out by the method of continuously synchronized cultures [5,13].

2. Biocalorimetry

The potential role of calorimetry in biotechnology research is based on heat production, especially that due to metabolism. This heat production depends on environmen-

tal conditions and the physiological state, respectively [3, 5, 10, 14]. Therefore, calorimetric measuring principles are suitable to indicate and characterize the physiological state.

Review articles on calorimeters and calorimetric measuring principles have been published by Brown [15], Sturtevant [16], Wadsö [17–19], Lamprecht and Schaarschmidt [20], Hemminger and Höhne [21], Lamprecht [22], von Stockar and Marison [23], Schügerl [11], and Gnaiger and coworkers [24, 25].

The present work was aimed at some aspects of calorimetric characterization of the physiological state in order to provide data on the use of calorimetric techniques.

Flow microcalorimeters belong to the class of twin-conduction calorimeters through which the culture sample flows. The instruments are composed of two calorimeter flow-through vessels surrounded by semi-conducting thermocouple plates which are placed in small aluminium blocks having thermal contact with the main calorimeter block [17–19]. The whole heat sink is surrounded by a thermostatted water bath. The samples are pumped to a central heat exchange unit before entering the measurement vessel. The heat flux is calculated from a differential voltage signal between the measurement and the reference vessel. Flow microcalorimeters are advantageous for research because of their small measuring volume (approx. 0.5 cm^3). No problems occur in taking samples during the calorimetric measurements compared to batch microcalorimeters. Typical experimental results of yeast growth and proliferation are shown in Fig. 1.

The heat flux rises during rapid microbial growth and proliferation as indicated by the increase in biomass dry weight. The growth limitation influences the heat flux.

The disadvantages of the flow microcalorimetric experiments are the possible change in the physiological state of the microorganism population during culture-medium transport to the measurement vessel and problems connected to aeration of the measurement vessel or microorganism adhesion to the walls of the measurement vessel. This could be partially avoided by a high flow rate of the culture medium via the measurement vessel.

Another possibility is the use of special principles for direct measurement of heat flux in the bioreactor. Such instruments include a modified standard bench-scale bioreactor in which the heat flux can be measured in situ [23], called "macro" or bench-scale calorimeters. The calorimetric measuring principles are dynamic and continuous calorimetry [23, 26–30]. Continuous calorimetry has several advantages compared to the dynamic technique. Automatic operation is possible and no temperature changes in the culture broth occur due to the actual calorimetric measurement.

According to the principle of continuous calorimetry, the heat flux resulting from cellular processes can be determined using an isothermal reaction calorimeter consisting of a bioreactor placed in a thermostatted chamber, Fig. 2 [27]. The temperature of the chamber was controlled and kept equal to the reaction temperature. The culture medium in the bioreactor was in contact with a water reservoir having a constant temperature below the reaction temperature. The temperature of the culture medium was adjusted to the reaction temperature using an immersion heater. To calculate the heat flux due to cellular processes, the voltage and the current of the electric circuit including the immersion heater, and the temperature of culture medium were measured

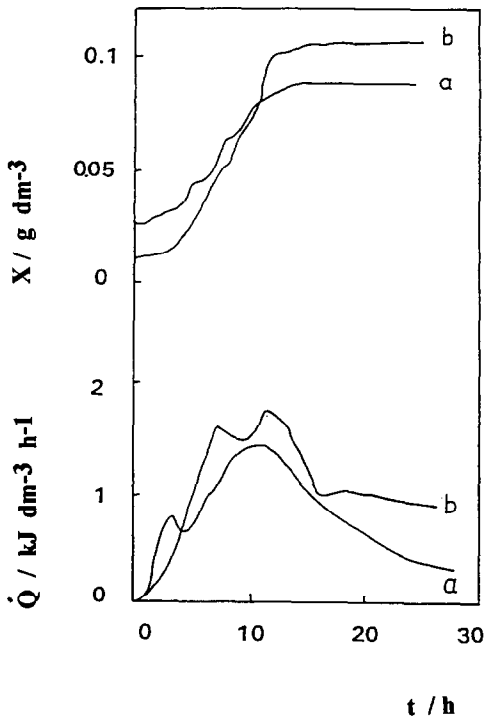

Fig. 1 Microcalorimetric measurement of batch growth of *Lodderomyces elongisporus*: a, carbon-substrate limitation; b, ammonium ion limitation.

and controlled. The reaction calorimeter was calibrated by measurement of heat flux in a stirred and aerated bioreactor containing nutrient medium without microorganisms.

As an example, calorimetric studies on budding cycle of yeasts were described, Table 1 [5]. Two different states could be observed during the budding cycle: the single-cell state and the budding state. The single-cell state was characterized by a high specific heat flux $Y_{Q/X}$ (increased external energy dissipation ψ_d) and a high carbon-substrate consumption $Y_{S/X}$ (low biomass yield $Y_{X/S}$, low enthalpic efficiency η_{enth}, and thermodynamic efficiency η)

These quantities, as well as the residence time t_r of the yeast in the single-cell state, were dependent on the carbon-substrate supply s_o (not shown). In contrast, the budding state was determinate and independent of the carbon-substrate supply s_o. Whereas the specific heat flux $Y_{Q/X}$ and specific carbon substrate consumption $Y_{S/X}$reach minimum values during budding, the biomass yield $Y_{X/S}$, the enthalpic efficiency η_{enth}, and the thermodynamic efficiency η are maximum. These results were in agreement with experiments of von Meyenburg [31] and Bley et al. [32]. They can be explained by the formation of ATP and polyphosphates in the single-cell state, with an efficiency depending on the carbon-substrate concentration and the type of limitation. Also, the

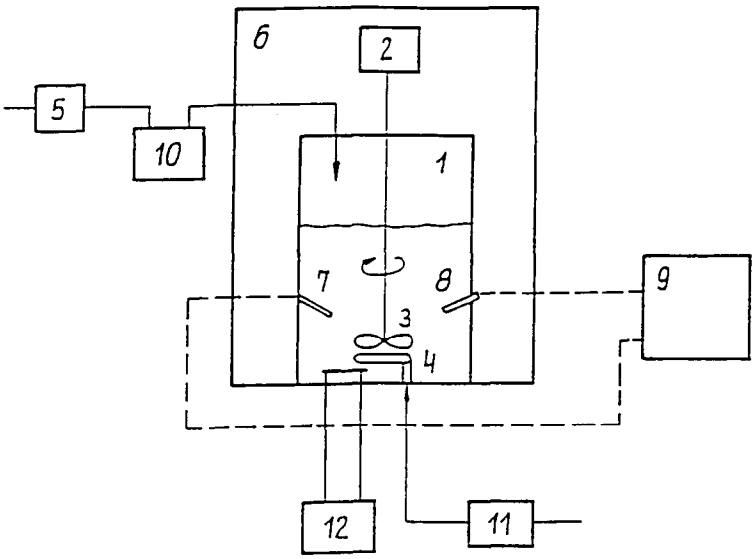

Fig. 2 Principle set up of a reaction calorimeter: 1, biorcactor with pH control; 2, motor for stirring; 3, stirrer; 4, aeration device; 5, pumps for feed medium; 6, thermostatted chamber; 7, temperature sensor; 8, heater; 9, controller; 10, thermostat for adjusting the temperature of the feed medium; 11, thermostat for adjusting temperature and humidity of air; 12, thermostatted water reservoir in contact with the culture medium.

synthesis of proteins and nucleic acids in the budding state may be coupled to hydrolysis of ATP and polyphosphates and independent of environmental factors.

The results of continuously synchronized yeast cultures characterizing the budding cycle indicate that yeast growth and proliferation should be optimized by adapting the environmental factors to the repetitive states of the budding cycle reflected by the states of such populations.

Table 1
Characterization of the budding cycle of *Lodderomyces elongisporus* grown on glucose

	Carbon limitation $S_o = 10\,g\,dm^{-3}$		Carbon excess $S_o = 12.5\,g\,dm^{-3}$	
	Single-cell state BC 20%	Budding-cell state BC 80%	Single-cell state BC 20%	Budding-cell state BC 80%
μ/h^{-1}	0.4	0.6	0.2	0.6
$Y_{S/X}/g g^{-1}$	2.8	1.9	3.9	1.9
$\rho\%$	45.8	67.5	32.9	67.5
$Y_{Q/X}/kJ\,g^{-1}$	−20.3	−8.3	−45.0	−8.5
$\psi_d/kJ\,g^{-1}\,h^{-1}$	8.1	5.0	9.2	5.1
$\eta_{enth}\%$	45.9	67.6	32.9	67.6
$t_r\%$	0.3	1.2	1.2	1.2

This could be shown by continuous cultivation of a synchronized yeast population on sucrose with a periodic adaptive carbon-substrate supply s_o, Fig. 3. The measured features, among them the specific heat flux $Y_{Q/X}$ and the specific carbon substrate consumption $Y_{S/X}$, change periodically with a period equal to the doubling time of yeast induced by the periodic carbon-substrate supply s_o. The overall energetic and material efficiencies of this controlled transient process were improved in comparison to the values of the steady-state process.

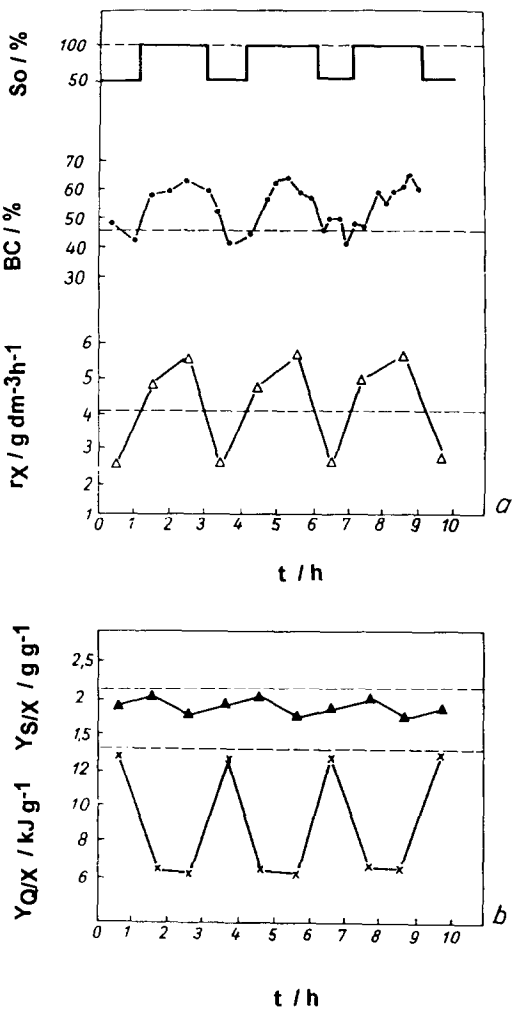

Fig. 3 Calorimetric measurement of continuous culture of *Lodderomyces elongisporus* with changing carbon-substrate supply (controlled transient state): – – –, steady state; —, controlled transient state. (a) r_X, biomass production rate; S_o, substrate concentration; BC, percentage of budding cells. (b) $Y_{S/X}$ specific substrate consumption; $Y_{Q/X}$, specific heat production. (c) external energy dissipation; ψ_u, internal energy dissipation; ψ, energy dissipation function. (d) ρ, yield; η, thermodynamic efficiency; η_{enth}, enthalpic efficiency.

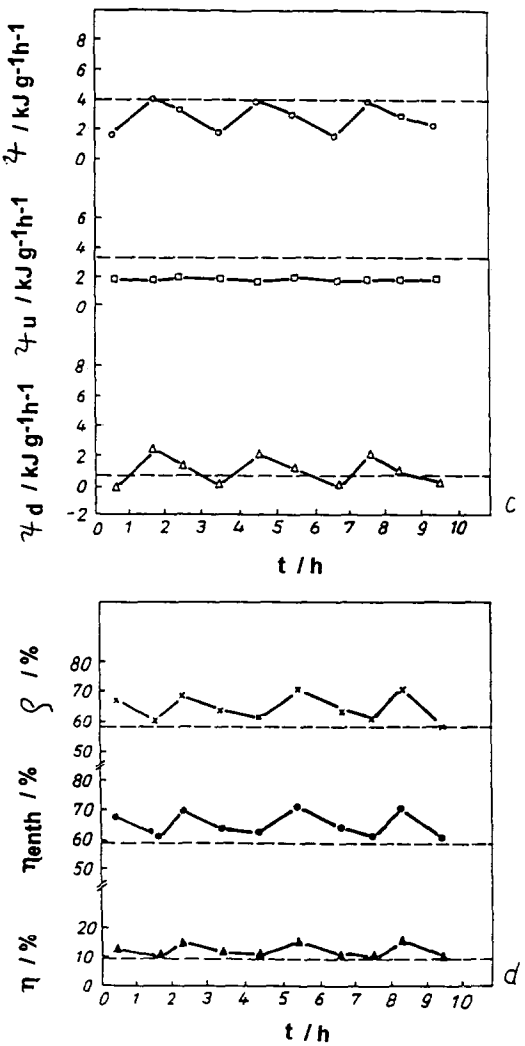

Fig. 3 (*Continued*).

3. Laser flow cytometry

Laser flow cytometry has been developed for characterization of the composition, morphology and size of microorganisms as well as physico-chemical features at the level of a single cell [33,34]. It is based on microscopy and is described in detail elsewhere [11,12, 33–43].

In contrast to microscopy, flowing microorganisms pass the laser beam at an angle of 90° to the flow direction of the suspension in a flow-through vessel, as shown in Fig. 4.

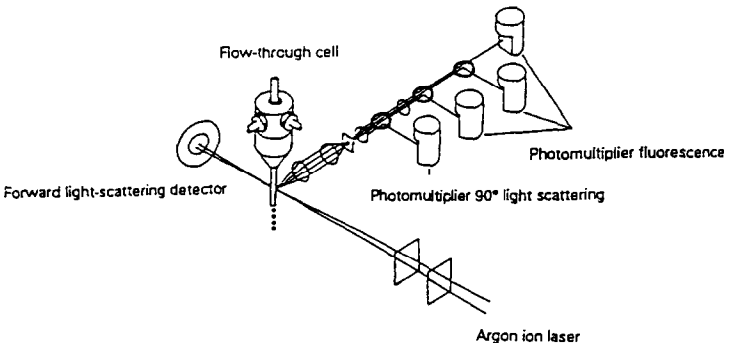

Fig. 4 Principle set-up of a laser flow cytometer.

The microorganisms in the suspension are lined up in a laminar flow due to hydrodynamic focussing under pressure or vacuum.

Thus, the features of a single cell can be measured separately. In order to calculate a distribution of the features, 5,000–10,000 microorganisms per second pass the laser beam. Electronic devices, such as a photodiode or a photomultiplier for registration of the measurement signals and a computer for measurement of signal processing, form part of the instrument.

The most important method for determination of size and morphology of microorganisms is the measurement of the forward light scattering and 90° light scattering using a photomultiplier. If the microorganisms are additionally pretreated with fluorescence dyes such as ethidiumbromide, the fluorescence per unit can be measured. Thus, the relative concentration of stained constituents, such as DNA, RNA, proteins and lipids, can be determined. The laser flow cytometers are calibrated using fluoresceinisocyanate-stained latex particles (diameter 1.47 μm).

As an example, the budding cycle has been described by flow cytometry measurements on continuously synchronized yeast cultures, Fig. 5.

At the beginning of each culture most of the microorganisms in the population were in the single-cell state. After one doubling during a time interval of 1.5 h, the yeast returned to the initial physiological state, as indicated by the light scattering signal (not shown) and the distribution of the DNA content of the yeast population, Fig. 5. These results were obtained in the experiment shown in Table 1. The sample was taken during the budding cell state (1.2 h after the beginning of the budding cycle).

4. Conclusions

Biocalorimetry and laser flow cytometry are suitable for characterizing the physiological state of microorganisms. Whereas biocalorimetry describes the state of the microorganism population, laser flow cytometry investigates both the state of a single microorganism and the state distribution. Calorimetric and cytometric data should be

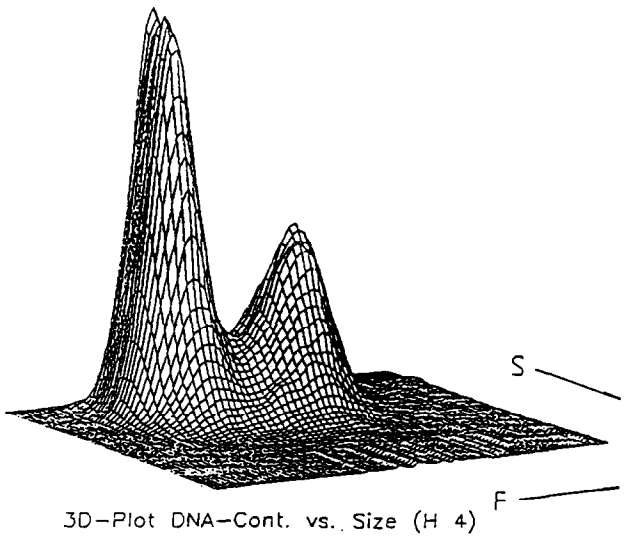

Fig. 5 Three-dimensional plot of the DNA content of *Lodderomyces elongisporus* (58% of budding cells) stained by olivomycin.

interpreted by further physico-chemical measurements using, for instance, culture fluorescence monitoring, mass spectrometry, NMR, IR and biosensors, as well as conventional bioanalytical methods, in order to obtain a unique description of the physiological state.

References

[1] I. Malek, Continuous Cultivation of Microorganisms, Pub. House of the Czechoslovak Acad. Sci., Prague, 1958.
[2] M. Novak, Continuous cultivation of microorganisms — past and future prospects, in P. Kyslik, E.A. Dawes, V. Krumphanzl and M. Novak (Eds.), Academic Press, London, 1988, p. 1–5.
[3] B. Heinritz-Zentgraf, Calorimetric studies of aerobic growth of a yeast and thermodynamic interpretation of the results, Thesis, Leipzig, 1978.
[4] T. Bley, Structured growth models—comparing studies and application to problems of technical microbiology, Thesis, Leipzig, 1980.
[5] B. Heinritz-Zentgraf, Optimization of Biological Material Conversion by Dynamic Processing, Habilitation, Leipzig, 1985.
[6] T. Bley, State-Structured Models in Biotechnology, Habilitation, Leipzig, 1990.
[7] W. Ebeling and M. Peschel, Dynamical Systems, Akademie-Verlag, Berlin, 1987.
[8] H.G. Bothe, Dynamical Models and Environmental Systems, Akademie-Verlag, Berlin, 1987.
[9] D. Rhamkrishna, Models for the dynamics of microbial growth, Dissertation, University of Minnesota, Minnesota, 1965.
[10] B. Zentgraf, G. Rogge and T. Bley, Acta Biotechnol., 12 (2) (1992) 87–97.
[11] K. Schügerl, Analytische Methoden in der Biotechnologie, Verlag Vieweg, Braunschweig, 1991.

[12] T. Scheper, Bioanalytik, Verlag Vieweg, Braunschweig, 1991.
[13] P.S.S. Dawson, J. Appl. Microbiol. Biotechnol., 22 (1972) 79–103.
[14] L. Gustafsson, Thermochim. Acta, 193 (1991) 145–171.
[15] H.D. Brown, Biochemical Microcalorimetry, Academic Press, London, 1976.
[16] J.M. Sturtevant, Ann. Rev. Biophys. Bioeng., 3 (1974) 35–51.
[17] I. Wadsö, Quart. Rev. Biophys., 3 (1970) 373–427.
[18] I. Wadsö, Calorimetric techniques, in A.M. James (Ed.), Thermal and Energetic Studies of Cellular Biological Systems, John Wright, Bristol, 1987, p. 34–67.
[19] I. Wadsö, Some problems in calorimetric measurements on cellular systems, in A.E. Beezer (Ed.), Biological Microcalorimetry, Academic Press, London, 1980, p. 247–274.
[20] I. Lamprecht and B. Schaarschmidt, Application of Calorimetry in Life Sciences, Walter de Gruyter, Berlin, New York, 1977.
[21] W. Hemminger and G. Höhne, Calorimetry. Fundamentals and Practice, Verlag Chemie, Weinheim, 1984.
[22] I. Lamprecht, Thermochim. Acta, 83 (1985) 81–88.
[23] U.v. Stockar and I. Marison, The use of calorimetry in biotechnology, in A. Fiechter (Ed.), Adv. Biochem. Eng., Vol. 40, Springer Verlag, Berlin, Heidelberg, 1969, p. 93–136.
[24] E. Gnaiger, The twin flow microrespirometer and simultaneous calorimetry, in E. Gnaiger and H. Fostner (Eds.), Polarographic Oxygen Sensors. Aquatic and Physiological Applications, Springer Verlag, Berlin, Heidelberg, New York, 1983, p. 134–166.
[25] E. Gnaiger, J.M. Shick and J. Widdows, Metabolic microcalorimetry and respirometry of aquatic animals, in C.R. Bridges and P.J. Butler (Eds.), Soc. Exp. Biol. Seminar Series, Cambridge Univ. Press, London, 1989, p. 113–135.
[26] B. Heinritz-Zentgraf, Thermochim. Acta, 172 (1990) 241–250.
[27] B. Zentgraf, Acta Biotechnol., 12 (1992) 476–474.
[28] J.H.T. Luong and B. Volesky, Heat evolution during the microbial process — estimation, measurement and application, in A. Fiechter (Ed.), Microbial Activities, Akademie-Verlag, Berlin, 1984.
[29] C.L. Cooney, D.I.C. Wang and R.I. Mateles, Biotechnol. Bioeng., 11 (1968) 269–280.
[30] M. Meier-Schneiders and G. Eigenberger, Bio Tech, 4 (1993) 6–9.
[31] K.v. Meyenburg, Catabolite repression and the budding cycle of *Saccharomyces cerevisiae*, Thesis, Zürich, 1969.
[32] T. Bley, B. Heinritz-Zentgraf, A. Steudel, E. Stichel, F. Glombitza and W. Babel, Z. Allg. Mikrobiol., 20 (1980) 11–16.
[33] H.M. Shapiro, Practical Flow Cytometry, Alan R. Liss Inc., New York, 1985.
[34] A. Degelau, R. Freitag, F. Linz, C. Middendorf, T, Scheper, T. Bley, S. Müller, P. Stoll and K.F. Reardon, J. Biotechnol., 25 (1992) 115–144.
[35] J.E. Bailey, J. Fazel-Madjlessi, D.W. Mc. Quitty, L.Y. Lee and J.A. Oroa, AIChE J., 24 (4) (1978) 570–577.
[36] P.K. Horan and L.L. Wheelees, Science, 198 (1977) 149–157.
[37] H.S. Kruth, Anal. Biochem., 125 (1982) 225–242.
[38] J.A. Steinkamp, Rev. Sci. Instrum., 55, 9(1984) 1375–1400.
[39] J.E. Bailey and D.F. Ollis, Biochem. Fundamentals, 2nd edn., McGraw Hill, New York, 1986.
[40] K.-J. Hutter and H.E. Eipel, J. Appl. Microbiol Biotechnol., 5 (1978) 203–206.
[41] K.-J. Hutter and H.E. Eipel, J. Gen. Microbiol., 107 (1978) 165–167.
[42] K.-J. Hutter, H.E. Eipel and R. Hettwer, Eur. J. Appl. Microbiol. Biotechnol., 5 (1978) 109–112.
[43] S. Müller, A. Lösche and T. Bley, Acta Biotechnol., 12 (5) (1992) 365–375.