Software sensors as a tool for optimization of animal-cell cultures.

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Software sensors as a tool for optimization of animal-cell cultures.

Proefschrift

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STELLINGEN

1   Experimenten die veel gebruikte, maar nooit aangetoonde aannames bevestigen zijn essentieel voor een goed gefundeerd onderzoek.
> Dit proefschrift.

2   Meet- en regelsystemen verschaffen zoveel informatie over het te regelen proces dat je er een proefschrift mee zou kunnen vullen.

3   Ook onderzoekers zijn gebaat bij de richtlijnen over uitvoering en verwerking van experimenten zoals die zijn opgesteld in de GMP-richtlijnen (Good Manufacturing Practice).

4   Veel experimenten zouden overbodig zijn als men de tijd zou nemen om meetresultaten gedegen te evalueren.

5   Angst voor de biotechnologie ontstaat voornamelijk door onvoldoende voorlichting en wordt versterkt door de irreële voorstelling van de biotechnologie in diverse science fiction films.

6   Het tijdstip waarop iemand 's ochtends met werken begint is omgekeerd evenredig met de afstand tussen woon- en werkplek.

7   Een vrouw moet twee keer zo goed zijn om half zo goed gevonden te worden. Gelukkig is dat niet moeilijk.
> Charlotte Whitton.

8   Door de grotere onzekerheid en de daaruit voortvloeiende stress, zal een projectmedewerker op den duur slechter functioneren dan iemand in vaste dienst.

9   Het aannemen van werknemers in tijdelijke dienst zorgt ervoor dat er onnodig veel kennis verloren gaat en dat de voortgang van langdurige projecten gevaar loopt

10   Als kruiwagens nooit waren uitgevonden, zou de wereld er totaal anders uitzien.

11   De wereld zou in een paradijs veranderen als alle gelovigen zich aan hun eigen regels zouden houden.

12   Snelheidsbeperkende maatregelen zoals verkeersdrempels en scherpe bochten verminderen de veiligheid van verkeersdeelnemers.

Stellingen behorende bij het proefschrift 'Software sensors as a tool for optimization of animal-cell cultures'.

Richard C. Dorresteijn
Maarssen, 8 december 1996.

Voor mijn vader

VOORWOORD

Promoveren is afzien. Zeker aan het begin van het promotieonderzoek is dit een veel gehoorde stelling. Natuurlijk heeft iedere promovendus de nodige tegenslagen gehad tijdens zijn of haar onderzoek. Het hoort er bij. Promoveren zonder tegenslagen is als autorijden met alleen een theorie-examen op zak. In theorie weet je precies wat je moet doen, maar je mist de ervaring om de theorie in praktijk om te zetten. Wat dat betreft heb ik veel geluk gehad. Door problemen met apparatuur heeft mijn onderzoek vooral in de beginperiode veel vertraging opgelopen. Al die problemen leverden uiteindelijk wel een publikatie op over een van de analysemethoden die ontwikkeld moest worden voor mijn onderzoek (hoofdstuk 2). Toen eindelijk alle benodigde apparatuur goed functioneerde, verliep het onderzoek voorspoedig. Helaas was er van de vier jaar nog maar de helft over. Het was natuurlijk niet zo dat er in de tussentijd niets is gebeurd. Behalve dat er drie artikelen zijn geschreven (hoofdstuk 2, 3 en 4 van het proefschrift), ben ik ook

betrokken geweest bij allerlei RIVM zaken, zoals de ontwikkeling van meet-en regelsystemen, uitbreiden van fermentrol software en oplossen van technische problemen in de produktie. Naar mijn mening is dit zowel een voordeel als een nadeel van het buiten de universiteit gestationeerd zijn. Doordat ik betrokken was bij het welzijn van het lab, investeerde ik daar zeker in de beginperiode relatief veel tijd aan.

Dankzij de financiële bijdrage van Fisons Instruments, als schadeloosstelling voor de vertraging die was ontstaan door de problemen met de massaspectrometer, werd mijn contract met drie maanden verlengd. Hierdoor heb ik de praktische werkzaamheden op tijd af kunnen krijgen ronden.

Er is ook nog een aantal mensen die ik graag wil bedanken. Als eerste natuurlijk mijn promotor, Hans Tramper en mijn co-promotoren, Kees de Gooijer en Coen Beuvery. Tijdens de besprekingen die meestal 's avonds bij Hans thuis plaatsvonden, heb ik regelmatig bruikbare adviezen van hun gekregen. Bovendien waren deze besprekingen uitermate geschikt om ervoor te zorgen dat planningen werden gehaald. Gelukkig hebben ze mij in het onderzoek veel ruimte gegeven om eigen ideeën uit te werken. Dit heb ik enorm gewaardeerd.

Iemand die ook heel belangrijk is geweest voor mij, is Gerhan Wieten. Hij heeft altijd achter mij gestaan en mij gesteund. Zijn integerheid en manier van omgaan met mensen is een goed voorbeeld voor menig leidinggevende. Inhoudelijk heeft Gerhan ook nog een bijdrage geleverd aan hoofdstuk 2 van dit proefschrift.

Luc Berwald wil ik ook heel erg bedanken. Niet alleen voor zijn vele werk aan de HPLC waarmee hij de grondlegger is geweest voor hoofdstuk 2 van dit proefschrift. Wat voor mij het belangrijkste is geweest, is de manier waarop Luc en Sohnlita mij hebben opgevangen toen ik plotseling op een nogal onvriendelijke manier uit mijn woning in de Bankastraat in Utrecht werd gezet. Ik heb bijna 9 maanden bij hun mogen wonen, totdat ik een andere woning kreeg aangeboden van de gemeente.

Ook Stef Vermeij stond in die barre tijden altijd voor mij klaar. Zijn technisch

inzicht en praktische ideeën hebben mijn werk aanzienlijk vergemakkelijkt. Verder heb ik van de zeiltochtjes met Stef en Dineke altijd enorm genoten. Bedankt hiervoor.

Marvin Philippi, Stef Vermeij en Ben van der Laan waren lange tijd mijn kamergenoten. De droge humor van Marvin kon ik altijd waarderen. Ook zijn inbreng in mijn onderzoek heb ik zeer op prijs gesteld. Helaas zijn de uitstapjes die we regelmatig organiseerden toen we nog in G10 werkten, verwaterd. Wat dat betreft, was de verhuizing naar gebouw U geen vooruitgang. Ten slotte wil ik Marvin nog bedanken voor zijn hulp bij een van mijn grootste "hobby's", namelijk verhuizen.

Ben van der Laan is bij de laatste verhuizing een enorme hulp geweest. Met wat amateuristische hulp van mij, heeft hij het hele huis behangen. Ook is hij al die jaren een hele fijne collega geweest die altijd voor mij klaar stond.

Om het rijtje 'verhuizers' maar direct af te ronden wil ik Giny Timman bedanken voor haar hulp op de Bankastraat. Ik geloof dat zo langzamerhand bij iedereen wel het besef begint te komen dat jij voor de afdeling een onmisbare kracht bent.

Marcel Thalen heeft vooral bij mijn eerste artikel (hoofdstuk 3) een belangrijke bijdrage geleverd, door de tekst kritisch door te nemen op het gebruik van nengels. Ik heb hier ook bij de volgende artikelen veel baat bij gehad.

Henk van der Kuil en Jan van de Brink hebben mij de praktische kennis van het omgaan met dierlijke cellen bijgebracht. Tiny van de Velden en Richard van der Meer wil ik bedanken voor de manier waarop zij Gebouw H beheerden. Richard van der Meer is in de laatste periode in G10 en in gebouw U mijn kamergenoot geweest. Dit heeft regelmatig voor de nodige commotie gezorgd, omdat men vaak niet wist welke Richard bedoeld werd.

Dirk Martens is -net als ik- in het avontuur gedoken om bij het RIVM het promotieonderzoek uit te voeren. Dit is hem bijzonder goed gelukt. Vooral tijdens de besprekingen in de beginperiode van ons onderzoek hebben we het nodige

kunnen bijdragen aan elkaars onderzoek.

Jos van Hertrooij is iemand waarmee ik vooral in de laatste periode van mijn werkzaamheden op het RIVM te maken heb gehad. Ik wil hem bedanken voor de prettige manier waarop wij hebben samengewerkt aan de ontwikkeling van recepten voor de automatisering van de produktiekweken.

Ook Bas Romein wil ik bedanken. Hij was altijd bereid om mee te denken en oplossingen te verzinnen voor 'wetenschappelijke' problemen.

Wie in dit lijstje van bedankjes missen, zijn de studenten. Ik heb ze helaas ook moeten missen. Door de problemen aan het begin van mijn onderzoek en door ruimtegebrek op het moment dat ik wel de gelegenheid had om delen van mijn onderzoek uit handen te geven, heb ik helaas geen gebruik kunnen maken van hun inzet.

Verder wil ik nog een speciaal iemand bedanken. Ik heb haar leren kennen toen ze via de tweede fase opleiding BODL contractresearch deed bij LGV. Al snel bleek dat we elkaar wetenschappelijk erg veel te bieden hadden. Behalve haar celbiologische kennis waar ik op dat moment om zat te springen, heeft ze mij ook geholpen een opstartmethode te bedenken voor de celkweken. Een probleem met het kweken van dierlijke cellen is, dat dit bijna niet reproduceerbaar te doen is. Door haar opstartmethode was ik in staat om celkweken volledig reproduceerbaar uit te voeren. Hierdoor kon en mocht ik de resultaten van verschillende kweken met elkaar vergelijken. Zoals het wel vaker gaat, groeide uit deze wetenschappelijke samenwerking een hele goede vriendschap en een ongelofelijk sterke liefde voor elkaar. Dit heeft er uiteindelijk toe geleid dat Karolien Harbrink Numan en ik zijn gaan samenwonen en dat wij sinds kort zijn getrouwd. Dit heeft overigens onze samenwerking alleen maar versterkt. Tot diep in de nacht zaten wij af en toe te discussiëren en ideeën uit te wisselen. Karolien heeft mede door haar celbiologische kennis een essentiële rol gespeeld in de laatste 2 artikelen (hoofdstuk 5 en 6). Naast deze bijdrage was ze ook in staat mij te motiveren op het moment dat ik het even niet meer zag zitten.

Ten slotte wil ik alle andere (ex)medewerkers van GVV, LGV en LPO bedanken voor hun praktische en sociale inbreng. Het feit dat jullie niet met naam en toenaam genoemd worden, betekent absoluut niet dat ik jullie vergeten ben.

Richard C. Dorresteijn
Maarssen, november 1996

CONTENTS

CHAPTER 1

INTRODUCTION OF SOFTWARE SENSORS

INTRODUCTION

Since the introduction of bioreactors for the cultivation of animal cells, much effort has been put in the optimization of the cultivation process. For this, information is required about the cell behaviour, the medium composition, and about the physical conditions inside the bioreactor such as dissolved-oxygen concentration, temperature, and pH. This information can be obtained from off-line analysis techniques like cell counts, from equipment coupled through on-line sampling systems like Flow Injection Analyzers[3,4,13,17] and from sensors like pH-probes.

The need for optimization of animal-cell cultures becomes clear, looking at the benefits. Animal cells, like all living organisms, have optimal physical conditions at which they perform best. If these conditions are sustained and the nutrient supply is adequate, the conditions for the cells are optimal. However, during cell growth, nutrients are utilized. Hence, to maintain optimal conditions for cell growth, these nutrients have to be supplemented to the cultivation medium. This causes more problems than might be expected. If for instance a large excess of glutamine, one of the major energy sources[14] and the major nitrogen source[20] of most animal cells, is added to the cultivation medium this will reduce the performance of the cultivation[1,8,10]. Next to the excretion of ammonia due to glutamine consumption, glutamine also spontaneously decomposes into ammonia and pyrrolidone-carboxylic acid[5,13,18]. Since ammonia inhibits cell growth[7,12,16], addition of large amounts of glutamine will also inhibit cell growth. Because of this decomposition, glutamine cannot be supplemented at once to the cultivation medium in the amount that the cells require during the whole cultivation. One way to overcome this problem is controlled addition of glutamine throughout the cultivation. This way glutamine is present during the complete cultivation while the glutamine concentration is kept to an acceptable low level. This type of control can

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significantly reduce the formation of ammonia, especially since the specific production rate of ammonia also depends on the glutamine concentration[6,21]. The same strategy can be applied to reduce the lactic-acid concentration, which is also a cell-growth inhibitor. For cell types such as hybridoma's, the specific lactic-acid production rate is proportional to the glucose concentration. Hence, reduction of the glucose concentration results in a reduced lactic-acid formation[2,4,9,10,12,21].

As already mentioned, there are several ways to gain information about the cultivation process. Off-line analysis of samples withdrawn from the cultivation broth is often used to obtain information about the cultivation process. Unfortunately, these data are not appropriate for direct control of animal-cell cultivations, since the sample frequency is low and the analysis results are obtained some time after the sample has been withdrawn from the reactor. Depending on the type of analysis, it can take up to a few days to get the results (e.g. virus titrations). Analyzers that are coupled to the bioreactor through an automated sampling device can be used for control purposes. However, in general these techniques are both expensive and prone to failures. Additionally, the sampling system can block and problems can arise with the sterility of withdrawn samples. A third option is using sensors that are placed inside the bioreactor. These provide information about the analyzed component without delay. Therefore, sensors are a priory suitable for control purposes. However, there are only a few sensors available that can withstand sterilization. Sensors for important parameters such as glucose are being developed, but they still lack reliability[15,19]. To overcome this problem, software sensors[11] can be used. Software sensors are models, implemented in the software of the measurement and control unit, that predict the course of parameters that cannot be measured on-line. They can fill up the gap that exists between the sensors that are commercially available and the sensors that are required to optimize the production process.

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DEVELOPMENT OF SOFTWARE SENSORS

The software sensors described in this thesis are developed with the intention to increase knowledge about the cultivation process. With this increased knowledge, it should be possible to improve the cultivation process in such a way that the cell density increases and that the production of toxic metabolites is minimized. This might be achieved by developing a method to control nutrient concentrations at a predefined level, throughout the cultivation. With this, both the occurrence of nutrient limitation and the production of toxic metabolites can be minimized. To achieve this, information is required about the biomass activity, about the utilization rate of glucose and glutamine, and about the production rate of the toxic waste products ammonia and lactic acid. To obtain this information, software sensors are used that are based on physiological aspects of the cell culture. This has the advantage that their responses are valid, irrespective of the type of animal cell that is cultivated. In this thesis, the volumetric oxygen uptake rate (OUR) and the volumetric acid production rate (APR) are the physiological parameters used to define the software sensors. To estimate the OUR, information about gas flow, gas composition, stirrer speed, culture volume, bioreactor dimensions and dissolved-oxygen concentration is needed. All these parameters can be measured accurately. In addition to this information however, information is required about the oxygen transfer coefficient ($k_L a$) from gas to liquid phase and about the solubility of oxygen in the cultivation medium (S_{O_2}). In general, both are considered constant throughout the cultivation. However, this might not be the case. Hence, to determine the OUR accurately, first the variation of both $k_L a$ and S_{O_2} during cultivation has to be studied. If necessary, even on-line measurement methods have to be applied to correct for changes in either of them.

The estimation of the APR is less complicated. The APR is determined from

the amount of base utilized to maintain a constant pH. If it can be shown that the build-up of a bicarbonate buffer during the cultivation has no significant effect on the base utilization rate, it can be assumed that the amount of base utilized equals the amount of acid produced. Using the assumption that lactic acid is the only acidifying component that the cells produce in significant amounts, this base utilization approximates the lactic-acid production.

From the OUR and the APR, other software sensors can be derived. In this thesis, the glucose consumption rate, the lactic-acid production rate and the biomass activity, defined as the total energy requirement of the cultivated cells, are derived from these two parameters. Furthermore, software sensors have been developed to estimate the glutamine consumption rate and the ammonia production rate during the exponential growth phase of the cell culture. The latter are only based on the APR. Contradictory to the other software sensors, these have been determined experimentally by correlating them to the acid-production rate.

OUTLINE OF THE THESIS

In this thesis the development and the application of software sensors for the on-line estimation of biomass activity, consumption rates of glucose and glutamine, and production rates of lactic acid and ammonia are discussed. Special attention is paid to the validation of the software sensors.

The first part of the thesis describes the tools that have been developed for validation of the software sensors. Chapter two presents an optimization method for HPLC analysis of primary amino acids. The HPLC method developed proved to be essential in the final part of this research. Chapter three presents an off-line method to determine the solubility of oxygen and the oxygen transfer coefficient

of cultivation media. As already mentioned, both parameters normally are assumed to be constant throughout the cultivation. However, for the verification of the software sensors, this assumption is checked first. Chapter three shows that the oxygen transfer coefficient can vary during cultivation whereas the oxygen solubility remains approximately constant. Therefore an on-line method is developed to determine the oxygen transfer coefficient during cultivation. This method is presented in chapter four.

After development of the verification tools, the software sensors are tested. Chapter five presents the development of the software sensors for oxygen uptake rate, lactic-acid production rate and biomass activity. In that chapter, the estimated oxygen uptake rate is compared to the results of a direct measurement of the oxygen uptake rate using mass spectrometry. In chapter six the software sensors developed are used for the optimization of a Vero-cell batch culture. Software sensors for glucose and glutamine consumption and for ammonia production are introduced and verified in that chapter too.

Finally, in chapter seven future trends in plant automation are discussed and put in the context of the regulations drawn up by governmental institutions like the Food and Drug Administration.

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CHAPTER 2

THE DETERMINATION OF AMINO ACIDS USING O-PHTHALALDEHYDE/2-MERCAPTOETHANOL DERIVATIZATION: EFFECT OF REACTION CONDITIONS.

ABSTRACT

This paper presents a systematic approach to determine the optimal reaction conditions, with respect to accuracy and sensitivity, of the quantitative determination of primary amino acids in a single run, using o-phthalaldehyde/-2-mercaptoethanol derivatization. For this, an experiment was designed in which the effects of reaction time, concentration of 2-mercaptoethanol and type of solvent were determined simultaneously. The response of all parameters tested was found to be interrelated: the effect of a change in one reaction condition also depended on the other reaction conditions. The reaction conditions determined in this research resulted in an accuracy better than 0.25 μM, an average reproducibility of 0.6% and an average sensitivity of 136 fmol.

INTRODUCTION

In animal cell cultivations it is common practice that large amounts of interrelated samples have to be analyzed that are taken over longer periods of time. Since trend analysis is very important, the analysis method has to obtain highly reproducible results during several days. Next to this, it is important that the method can determine all primary amino acids in a single run and is accurate in a wide concentration range. This excludes optimization methods that focus on single amino acids.

The derivatization of amino acids using o-phthalaldehyde (OPA) is the most frequently applied HPLC method for the quantitative determination of primary amino acids. This method was introduced by Roth[12] in 1971. Since then, many modifications have been proposed. These involved the composition of the mobile

phase consisting of either methanol[5,9,11,13,15] or acetonitrile[4,6,10,14], different combinations of OPA with 2-mercaptoethanol[8-15], ethanethiol[4,15] or 3-mercaptopropionic acid[5,6], and either precolumn[4-6,9-11,13-15] or postcolumn[8] derivatization. In addition, a wide range of reaction conditions is applied for the amount of 2-mercaptoethanol (0.8 - 2.5 μl/mg OPA[1,6,8,10,11,14,15]), for the reaction time (0 - 3 minutes[1,2,9-11,13-15]) and for the solvent used (dilution of the samples with water[4,6,10,13,14], borate buffer[1,10] or acid (commercial standards)). This short summary of applied derivatization conditions indicates that it is still unclear what the optimal conditions are for this derivatization method. In spite of what might be expected, only little research has been done to establish these optimal conditions[3,7,12].
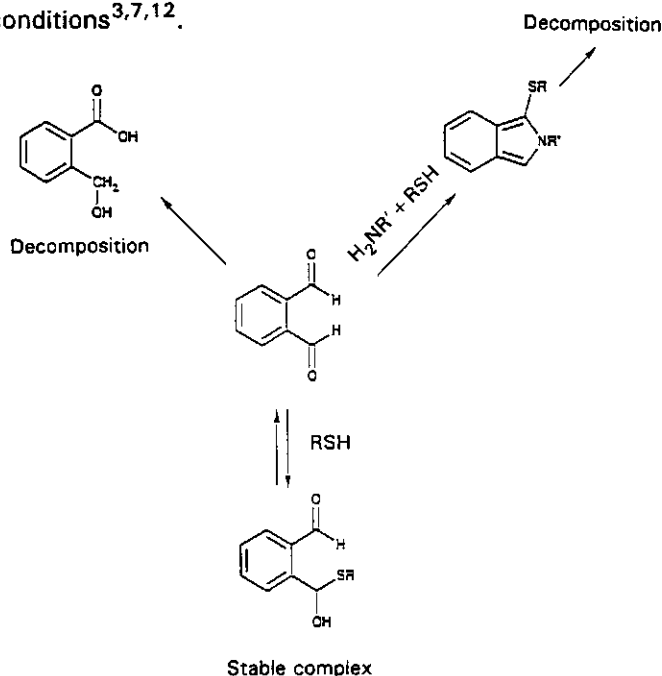
Figure 1: Simplified reaction scheme of the possible reactions of o-phthalaldehyde (OPA) with amino acids (H_2NR') and 2-mercaptoethanol (RSH): OPA hydrolyzes, it is stabilized using 2-mercaptoethanol and it reacts with 2-mercaptoethanol and amino acids, producing fluorescing derivatives that spontaneously decompose.

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The reason for the wide range of derivatization conditions used, can be found in the possible reactions of OPA with amino acids and 2-mercaptoethanol. Figure 1 shows a simplified reaction mechanism for the three possible reactions that can occur: hydrolyses of OPA, stabilization of OPA by 2-mercaptoethanol and reaction of OPA with 2-mercaptoethanol and amino acids, producing fluorescing derivatives that spontaneously decompose. The complexity of this reaction mechanism makes it difficult to predict the optimal derivatization conditions. Three conditions can be considered crucial: the pH of the reaction mixture, the concentration 2-mercaptoethanol and the reaction time. The pH is important since the reaction rates may depend on the pH. Since the pH-dependency of each reaction may be different, it can be expected that there is an optimal pH at which the production rate of the fluorescing derivatives is much higher than its decomposition rate. The 2-mercaptoethanol concentration determines the stability of the OPA/2-mercaptoethanol complex. Since this is an equilibrium reaction, addition of 2-mercaptoethanol results in a reduction of the amount of OPA that is directly available for the reaction with amino acids. Finally, the reaction time is important to allow the reaction of OPA with amino acids to complete.

This paper presents a method to determine the settings for the OPA derivatization for which the average response of all important amino acids is optimized. For this, an experimental set-up was designed in which the interaction was determined of the accuracy and the sensitivity of the analysis method with the reaction time, the 2-mercaptoethanol concentration and the pH. All three parameters were found to be essential in the optimization of the OPA method.

EXPERIMENTAL

Equipment

The HPLC system used was a Waters system (Waters, Division of Millipore, Milford, MA, USA) consisting of a Model 600E system controller with a single pump gradient system, a Model 715 Ultra WISP sample processor and a Model 470 fluorescence detector. The detector was operated using an excitation wavelength of 335 nm and an emission wavelength of 425 nm. The column was kept at 35 °C using a Model CHM column heater module and a Model TCM temperature control module. The system was coupled to a NEC Powermate 80386SX 16 MHz computer fitted with Maxima 820 Chromatography Workstation software, version 3.30 (Millipore, Milford, MA, USA).

A flow rate of 1 ml/min was used throughout all experiments. To determine the response, the peak area was used.

Column

The amino acids were separated using a 150 x 3.9 mm I.D. 85711 Waters Resolve 5 μ spherical C-18 column, protected by a Waters 15220 Nova-Pak 4 μ spherical C-18 guard-column.

Reagents

LiChrosolv quality Methanol, 2-mercaptoethanol and o-phthalaldehyde were all purchased from Merck, Darmstadt, Germany. Borate buffer (0.4 N) and a standard solution of amino acids containing Asp, Glu, Ser, Gly, Arg, Ala, Tyr, Met, Val, Phe, Ile, Leu, and Lys were purchased from Hewlett Packard, Waldbrunn, Germany. Individual amino acids were purchased from Sigma, St. Louis, MO, USA. A Milli-Q water purification system (Millipore, Milford, MA, USA) was used to obtain HPLC-grade water.

Sample preparation

Preparation of the OPA derivatization solution

To 100 ml borate buffer, 500 mg OPA and the required amount of 2-mercaptoethanol were added. The mixture was allowed to dissolve overnight, after which it was filtered through a 0.45 μm membrane filter. The OPA solution was stored in the dark at 4 °C.

Preparation of the mobile phase

The mobile phase consisted of 32 % (v/v) methanol and 20 mmol/l phosphate, dissolved in Milli-Q water (pH = 6.8).

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Preparation of the samples

A mixture containing 20 μmol/ml of Asp, Ser, Gly and Ala dissolved in 0.1 M H_2SO_4, was diluted with either borate buffer, Milli-Q water or 0.1 M H_2SO_4 until the required concentration was reached. From these samples 15 μl was injected on the column, together with 30 μl OPA reagent using the auto-addition method of the WISP. This auto-addition method involves:

- Rinsing the needle
- Acquire OPA-reagent in needle
- Rinsing the outside of the needle
- Acquiring sample in needle
- Injection of mixture on the column

Choice of experimental conditions

It was our intention to determine the optimal settings for the OPA derivatization during a routine analysis run of samples containing primary amino acids. This means that not the response of a single amino acid is important, but the average response of all primary amino acids that are to be determined. This paper presents the results of an experiment in which three reaction conditions have been varied. Since the effect of varying one reaction condition can depend on the other reaction conditions, the experiment has been set up in a matrix form. This results in 36 different combinations of the reaction conditions to be tested. Therefore, if the combined effect of the amount of 2-mercaptoethanol, the reaction time and the pH is to be determined, this will result in extensive experimental work. Side effects like decomposition of OPA in the stock solution would probably disturb these

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experiments because of this long run time. Therefore, an alternative method was applied, using samples containing only Asp, Ser, Gly and Ala in a range from 1 to 40 μM. This significantly reduced the total analysis time from 1 hour to 9 minutes. Since the overall time required for these experiments could now be limited to days rather than weeks, both the OPA solution and the mobile phase could be withdrawn from one stock solution, ensuring identical conditions throughout the experiments.
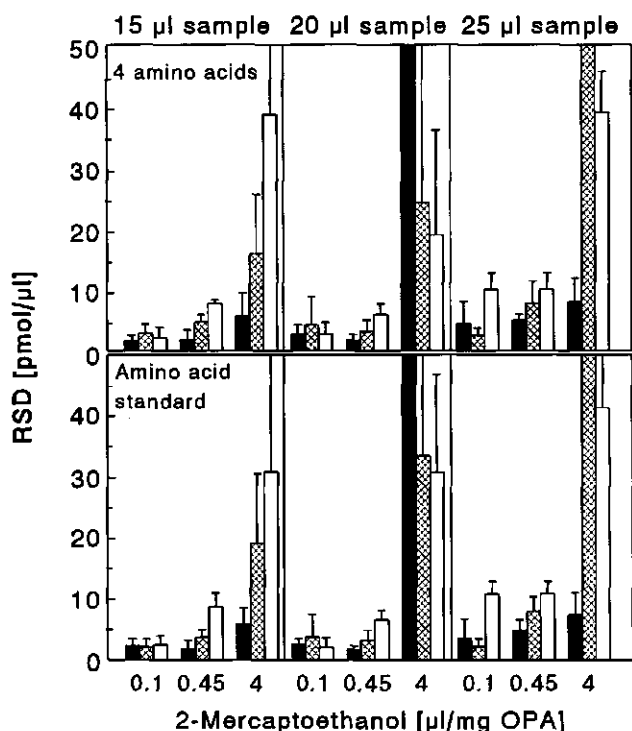
Figure 2: Comparison of the average response of a sample containing Asp, Ser, Gly and Ala with the average response of a standard solution, containing Asp, Glu, Ser, Gly, Arg, Ala, Tyr, Met, Val, Phe, Ile, Leu and Lys. In both cases Milli-Q water was used as solvent. For both sets of amino acids, the effect of reaction time, 2-mercaptoethanol concentration and injection volume was determined on the RSD. (■ Reaction time = 2 min. ⊠ Reaction time = 4 min. ☐ Reaction time = 6 min.).

RESULTS AND DISCUSSION

Selection of amino acids

An important demand for this experimental set-up is that the average behaviour of the four selected amino acids is comparable to the average behaviour of a sample containing most amino acids to be determined. Figure 2 shows that this is indeed the case. In this figure, the effect of reaction time (bars), amount of 2-mercaptoethanol (x-axis) and sample volume (three different sections in the figure) on the RSD (y-axis) is displayed for the four selected amino acids (top section) and for a commercially available amino acid standard containing Asp, Glu, Ser, Gly, Arg, Ala, Tyr, Met, Val, Phe, Ile, Leu, and Lys (bottom section). Fast degrading amino acids like Gln were not included, since their response would disturb this experiment. To compare both sets of amino acids, the residual standard deviation (RSD) was used. The RSD is a measure for the accuracy of a calibration curve, and therefore for the absolute error of a measurement. It is determined from the absolute deviation between the true concentration of a known sample and the concentration obtained with the calibration curve, using Formula 1.

$$RSD = \sqrt{\frac{residual\ sum\ of\ squares}{degrees\ of\ freedom}} = SD(Y)\sqrt{\frac{n-1}{n-2}(1-r^2)} \qquad (1)$$

In this formula, $SD(y)$ is the standard deviation of the y-value of the calibration curve, n is the number of measurement points, and r is the correlation coefficient. To determine the RSD, samples with amino acid concentrations of 10, 25, 100, 250 and 800 μM were analyzed in duplicate. In this case, Milli-Q water was used as solvent Figure 2 shows that the trend in RSD of the average response of the four amino acids does not markedly differ from the trend in RSD of the average

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response of the commercial standard containing 13 amino acids. Therefore, the mixture containing Asp, Ser, Gly and Ala can be used to determine the optimal settings for the complete set of amino acids.

Effect of 2-mercaptoethanol

In literature it is stated that the amount of 2-mercaptoethanol does not markedly influence the fluorescence of alanine[12]. Because it is assumed that this is also true for the other amino acids, small amounts of 2-mercaptoethanol are added to the OPA reagent every few days to stabilize the OPA reagent[3,9,14,15]. This assumption was verified for a sample containing 25 μM of each of the four amino acids. To be sure the reaction of OPA with the amino acids was complete, a reaction time of 3 minutes was selected. Performing the experiments with borate buffer, Milli-Q water and 0.1 M H_2SO_4, the 2-mercaptoethanol concentration was varied in a range from 0.01 to 4.8 μl/mg OPA. These three solvents were selected for two reasons. Firstly, in literature these three solvents are used most often. Secondly, applying these solvents results in a different pH of the complete reaction mixture. This way the effect of the pH on the overall reaction of OPA with amino acids and 2-mercaptoethanol can be determined. Figure 3 shows the effect of the 2-mercaptoethanol concentration on the normalized peak area of the individual amino acids. The normalization was achieved by defining the maximum peak area of the individual amino acids in the different solvents as 100 %. Figure 3 shows that the effect of the 2-mercaptoethanol concentration depends on the solvent used. Furthermore, Figure 3 clearly shows that the response is different for each amino acid. However, for each amino acid the maximum peak area is reached at a low concentration of 2-mercaptoethanol. The behaviour of the OPA derivatives
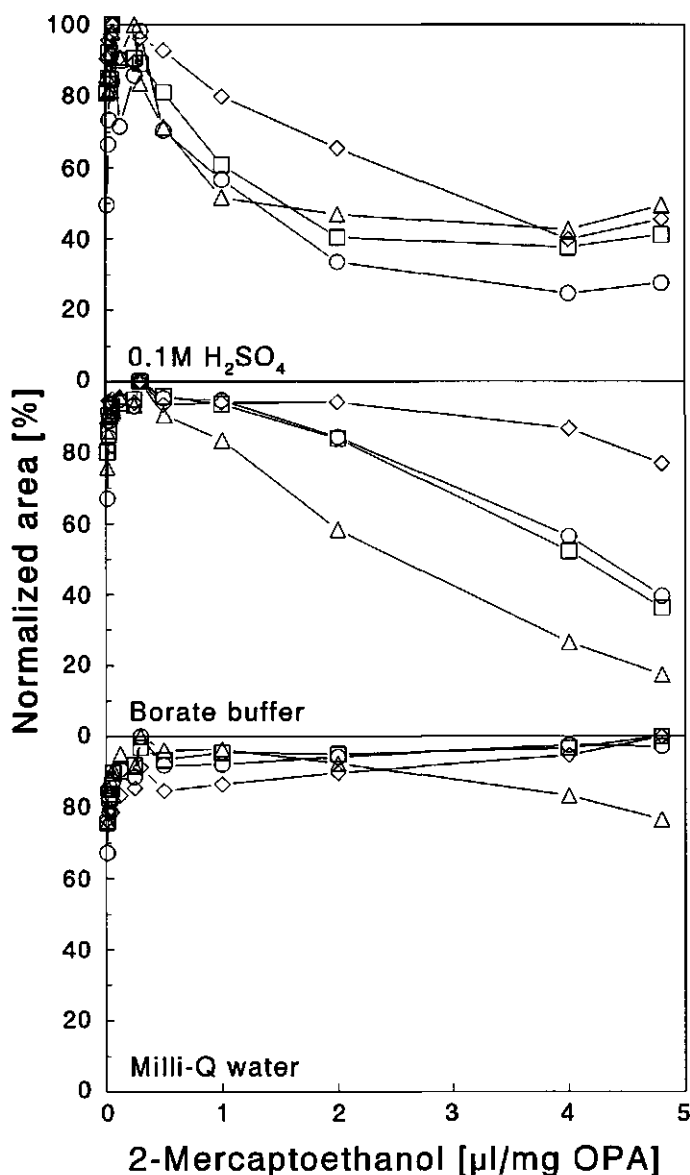
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Figure 3: Effect of the 2-mercaptoethanol concentration and the solvent on the fluorescence of the OPA-derivatives of Asp (-△-), Ser (-○-), Gly (-◇-) and Ala (-□-) at a reaction time of 3 minutes. The response has been normalized by defining the maximum peak area of the individual amino acids in the different solvents as 100%

can be explained by the stabilizing effect of 2-mercaptoethanol on OPA (Figure 1). With a large excess of 2-mercaptoethanol the equilibrium resides at the stabile OPA-mercaptoethanol complex. If an amino acid is added to this complex, OPA is not eager to react with this amino acid. Therefore, with an increasing amount of 2-mercaptoethanol, OPA reacts more readily with 2-mercaptoethanol than with the amino acid. The amount of fluorescence will therefore decrease with increasing concentration 2-mercaptoethanol. From Figure 3 three concentrations of 2-mercaptoethanol were selected to test the accuracy and the sensitivity of the analysis method: 0.14, 1.6 and 4.8 μl/mg OPA.

Determination of the sensitivity of the analysis method

Next to the variation in the 2-mercaptoethanol concentration, also the reaction time and the solvent were varied to determine the sensitivity of the analysis method. For the experiments, reaction times of 0, 1, 3 and 9 minutes were tested, where 0 minutes stands for instant injection on the column. Finally, borate buffer, Milli-Q water and 0.1 M H_2SO_4 were used as solvent. The pH-values of the reaction mixtures are shown in Table I. Figure 4 shows the relative area of a sample containing 5 μM of the 4 amino acids. This relative area is determined by defining the maximum peak area of each amino acid as 100 %. The amino acids were not normalized per solvent since this would make comparison of the sensitivity between the different solvents impossible. To check the reproducibility, the experiments were performed in sixfold. The standard deviation of these samples is presented as error bars in Figure 4. Figure 4 clearly shows that the relative area depends on the solvent, the reaction time and the amount of 2-mercaptoethanol. The solvent affects the pH of the reaction mixture (Table I), and therefore the
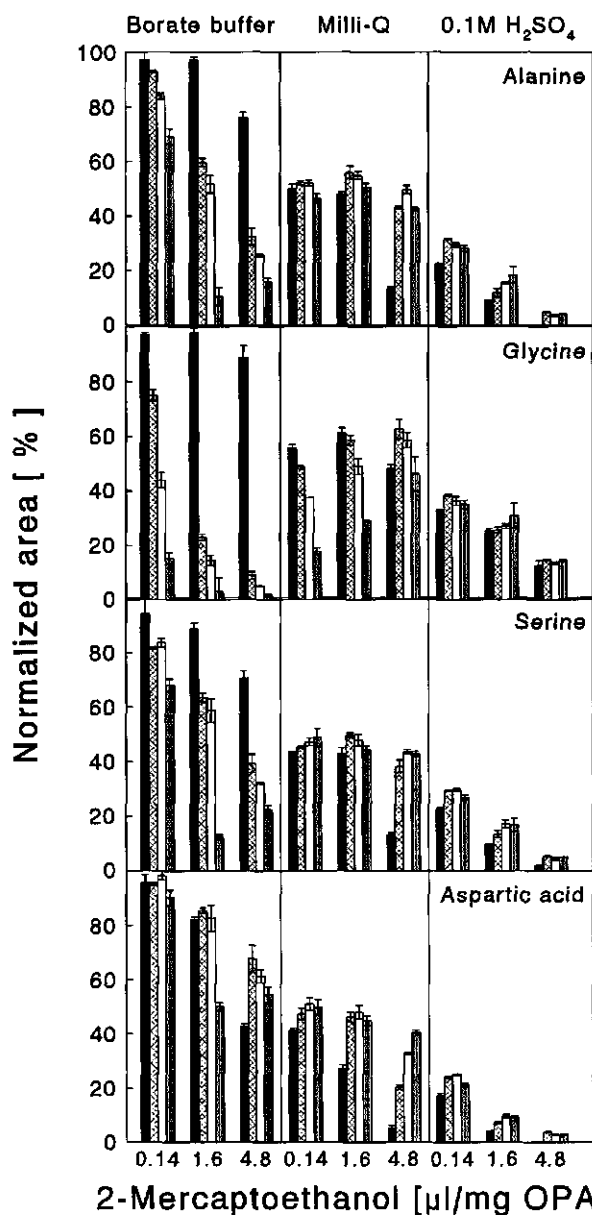
Determination of amino acids

Figure 4: Effect of reaction time, 2-mercaptoethanol concentration and solvent on the normalized area of Asp, Ser, Gly and Ala. The error bars show the standard deviation of each experiment (n = 6). (■ Reaction time = 0 min.;⊠ Reaction time = 1 min.;□ Reaction time = 3 min.;▓ Reaction time = 9 min.).

reaction conditions. Figure 4 shows that using a solvent with an increased pH results in an increase of the relative area. This is probably due to the increase in reaction rate of the reaction of OPA with amino acids. At a reaction time of 0 minutes, the reaction of OPA with amino acids in the presence of borate buffer is already completed, whereas in Milli-Q water and in acid the reaction still continues. Also the decomposition rate increases with increasing pH. This is best shown if the decrease in area with increasing reaction time in borate buffer is compared to the response in Milli-Q water and in acid. In borate buffer, a very rapid decrease of area is observed with increasing reaction time. In 0.1 M H_2SO_4 the area is less dependent on the reaction time. Finally, the stabilization reaction of OPA with 2-mercaptoethanol depends on the pH and on the 2-mercaptoethanol concentration. At a high concentration 2-mercaptoethanol, less OPA is available to react with amino acids, causing the area to decrease. Also, with decreasing pH, more OPA reacts with 2-mercaptoethanol. This also causes the area to decrease.

Table I: pH values of the reaction mixtures using 0.1 M H_2SO_4, Milli-Q water and Borate buffer as solvent.

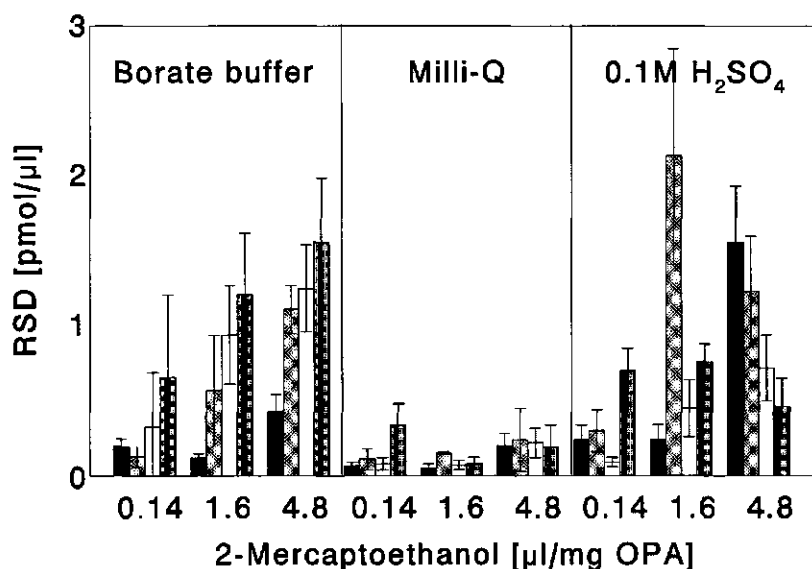| Solvent	Concentration 2-mercaptoethanol [μl/mg OPA]		
	0.14	1.6	4.8
Borate buffer	9.74	9.21	8.88
Milli-Q water	9.68	9.15	8.77
0.1 M H_2SO_4	8.32	8.17	8.02

Figure 5: Effect of reaction time, 2-mercaptoethanol concentration and solvent on the average Residual Standard Deviation (RSD) of Asp, Ser, Gly and Ala. The RSD is obtained from a quadratic curve fit. (■ Reaction time = 0 min.;⊠ Reaction time = 1 min.;☐ Reaction time = 3 min.;▦ Reaction time = 9 min.)

Determination of the accuracy of the analysis method

Figure 5 shows the average RSD of the four calibration curves as a function of the 2-mercaptoethanol concentration, the reaction time and the solvent. For this, the same conditions were used as in the previous experiment. The calibration curves were generated with a quadratic curve fit and were based on six different samples containing 1.25, 2.5, 5, 10, 20 and 40 μM of the different amino acids. All samples were analyzed in duplicate. To obtain a reliable calibration curve, the RSD should be as small as possible. Figure 5 shows that the smallest RSD values are obtained using Milli-Q water as a solvent. In borate buffer, the RSD increases with increasing reaction time and with increasing 2-mercaptoethanol concentration,

whereas in Milli-Q water the RSD is independent of reaction time. Also the 2-mercaptoethanol concentration has no significant effect on the RSD. In 0.1 M H_2SO_4 the effect on the RSD is more complicated. The RSD increases with increasing 2-mercaptoethanol concentration, but the effect of the reaction time also depends on this concentration. These effects can be explained looking at Figure 4. It shows that for a specific solvent, the RSD increases with decreasing peak area. So, to obtain a high accuracy and sensitivity, the peak area should be as high as possible, but as independent as possible from the reaction time, the 2-mercaptoethanol concentration and the solvent.

Linearity of the calibration curves

As mentioned before, Figure 5 shows the RSD values of quadratic curve fits. As can be seen in Table II, especially the calibration curve in Milli-Q water has a non-linear behaviour. In Table II, the average RSD-values for each solvent are shown, together with their standard deviation. The RSD-values are presented for both quadratic and linear curve fits. Table II shows that the calibration curves in borate buffer and in 0.1 M H_2SO_4 are linear, whereas in Milli-Q water they are quadratic. However, even the linear calibration curve through the Milli-Q data provides very accurate results. This non-linear behaviour of Milli-Q water is an important observation, since it means that the shape of the calibration curve depends on the solvent used.

Table II: Average RSD values for the quadratic and linear curve fits of the calibration curve [µM].

	Quadratic		Linear	
	RSD	SD	RSD	SD
Borate buffer	0.71	0.49	0.80	0.44
Milli-Q water	0.15	0.09	0.49	0.26
0.1 M H_2SO_4	0.74	0.61	0.81	0.66

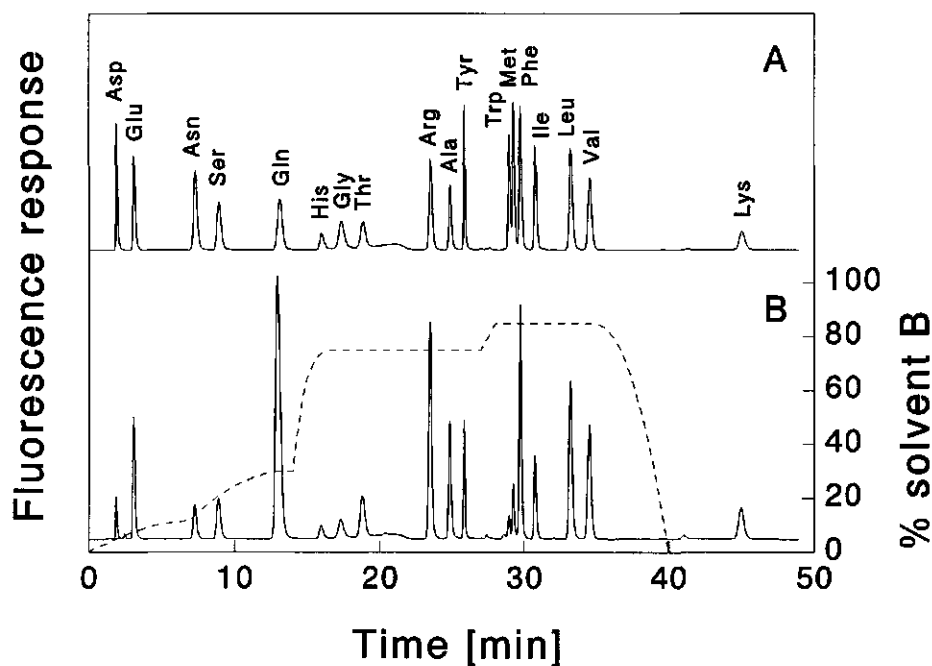**Figure 6: Typical chromatograms of a 5 µM amino acid standard (A) and DMEM cultivation medium containing 7.5 % bovine serum and 2.5% foetal calf serum (B), applying the reaction conditions defined in this paper (Reaction time 1 minute, Solvent = Milli-Q water, 2-mercaptoethanol concentration = 1.6 µg/ml OPA). Chromatographic conditions: Flow rate = 1 ml/min, temperature = 35 °C, injection volume = 15 µl sample + 30 µl reagent. Eluent A: 1/6 dilution of Eluent B with MilliQ-water, Eluent B: 65% (v/v) Methanol in 20 mM phosphate buffer (pH = 7.5). The broke lines indicate the solvent gradient composition.**

Real-life test

From the data presented, the optimal settings for our analysis method could be determined accurately. Milli-Q water was preferred because the results were, compared to borate buffer and H_2SO_4, relatively independent of the amount of 2-mercaptoethanol and the reaction time. A 2-mercaptoethanol concentration of 1.6 μl/mg OPA was selected because the OPA reagent is more stable with an increased concentration of 2-mercaptoethanol. However, since the reaction of OPA with amino acids is slowed down by 2-mercaptoethanol, a further increase of the amount of 2-mercaptoethanol would mean a loss of sensitivity. Stability experiments showed that an OPA-stock solution containing 1.6 μl 2-mercaptoethanol/mg OPA is stable for at least one month, provided it is kept dark and cool. Finally a reaction time of 1 minute was selected, because after 1 minute all amino acids have reached their maximum fluorescence. Selecting a reaction time of 0 minutes would mean that some amino acids have not finished their reaction with OPA. On the other hand, selecting 3 or 9 minutes reaction time would mean that the amino acid-OPA derivatives have more time to decompose. These setting lead to an accuracy better than 0.25 μM (Figure 5).

Figure 6 shows two typical chromatograms applying the reaction conditions defined in this paper. The reproducibility and sensitivity of the method were determined using standard solutions of 5 and 1.25 μM respectively. Results are shown in Table III. It shows that the average limit of sensitivity (signal to noise ratio of 3) and reproducibility of this method are 136 fmol and 0.6% respectively.

Table III. Limit of sensitivity determined on a 1.25 µM sample (S/N = 3) and Confidence Interval of a stock solution containing 5 µM of each amino acid (n = 14)

Amino Acid	Limit of sensitivity (fmol)	Confidence interval (µM)
Asp	49	5 ± 0.010
Glu	71	5 ± 0.011
Asn	89	5 ± 0.015
Ser	125	5 ± 0.025
Gln	136	5 ± 0.033
His	426	5 ± 0.023
Gly	224	5 ± 0.092
Thr	266	5 ± 0.040
Arg	78	5 ± 0.016
Ala	107	5 ± 0.017
Tyr	47	5 ± 0.012
Trp	64	5 ± 0.046
Met	50	5 ± 0.012
Val	50	5 ± 0.013
Phe	70	5 ± 0.027
Ile	71	5 ± 0.016
Leu	101	5 ± 0.016
Lys	426	5 ± 0.111

CONCLUSIONS

The determination of amino acids using HPLC in combination with OPA as a derivatization agent provides a very useful method. However, to obtain a reliable method, care must be taken with the treatment of the samples and the standard solutions, because the linearity of the calibration curve depends on the solvent being used. The use of linear calibration curves does not always coincide with the real response of the OPA amino-acid derivatives. If Milli-Q water is used as solvent,

a quadratic calibration curve is required to produce accurate results. All three parameters tested - the 2-mercaptoethanol concentration, the reaction time and the solvent used - were found to be crucial for the optimization of the amino acid analysis method. Therefore, a regular addition of 2-mercaptoethanol to the OPA solution should be avoided. This addition will lower the peak area which will result in a decrease of the sensitivity. Since the calibration curves will also change by this addition, the reproducibility of the analysis method will also decrease.

Applying the optimal reaction conditions for the quantitative determination of amino acids as determined in this research results in an accuracy better than 0.25 μM. The average limit of sensitivity (signal to noise ratio of 3) and reproducibility of this method are 136 fmol and 0.6% respectively. Table IV shows a summary of sensitivity and reproducibility data obtained from the literature cited, together with the results presented in this paper. Compared to the literature cited, the method developed in this research is both sensitive and reproducible.

Table IV. Comparison of sensitivity and reproducibility obtained in this research and extracted from cited literature.

	Sensitivity (pmol)		Reproducibility (%)	
Literature (S/N = 2-2.5)	0.2-10	(avg = 2.30)	0.4-8	(avg = 4.8)
This method (S/N = 3)	0.05-0.43	(avg = 0.14)	0.2-2.2	(avg = 0.6)

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CHAPTER 3

A METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE SOLUBILITY AND THE TRANSFER COEFFICIENT OF OXYGEN IN AQUEOUS MEDIA USING OFF-GAS MASS SPECTROMETRY.

ABSTRACT

A method was developed to determine simultaneously the solubility of oxygen and the oxygen-transfer coefficient in aqueous media in a stirred bioreactor. The method was based on a system developed by van Sonsbeek et al for the determination of the static ka in a liquid-impelled loop reactor. This system was improved in order to determine both parameters measuring only the physical properties of the liquid with the aid of a mass spectrometer. As data are available in literature about the solubility of oxygen in water, we tested water to make a comparison of published data and our data possible. No significant differences have been observed.

The oxygen-transfer coefficient determined using the measurement system was found to be similar to the oxygen-transfer coefficient determined using the dynamic method. Temperature effects on the oxygen-transfer coefficient were checked using the Arrhenius equation. Finally Trypticase Soy Broth (TSB) was used to verify that the measurement system was capable of determining both parameters in cultivation media without the composition of the medium disturbing the measurement.

INTRODUCTION

In animal-cell technology a strong tendency exists of developing advanced control algorithms in order to govern the conditions inside the bioreactor. In these developments, the solubility of oxygen in cultivation media and the oxygen-transfer coefficient are physical parameters that play an important role[3,10]. The determination of both parameters is, however, troublesome. Many of the published

methods for the determination of the solubility of oxygen in cultivation media or in water are disturbed by glucose or other chemical components[11,14,16,19,24]. Also several authors refer to literature values for the solubility of oxygen in water in order to calibrate their method[15,18,19]. This introduces a systematic error since the values for the solubility of oxygen in water differ depending on the method of determination[11,14,20]. Only a few papers have been presented in which the solubility of oxygen is determined using a physical method[18]. A physical method has the advantage that measurements are independent of the composition of the liquid being sampled. However, even though Schneider and Moser[18] use a method based upon the physical properties of the medium determined with a mass spectrometer, they still require the solubility of oxygen in water as a calibration factor.

In contrast to the relatively small number of papers presented about the determination of the solubility of oxygen, numerous papers have been published about the determination of the oxygen-transfer coefficient. The vast majority of them is based on the calculation of the oxygen-transfer coefficient using the dynamic method[2,5,8,9,12,23]. Others are based on the sulfite method[6], but only a few articles have been published using the static method to determine the oxygen-transfer coefficient[13,21]. The dynamic method is very often preferred due to the simple measurement technique, although at high oxygen-transfer rates, the effect of the response time of the dissolved oxygen probe cannot be neglected[4,5,7,8,22]. The static method is more reliable because it is independent of this response time of the dissolved oxygen probe.

This paper presents a method for the simultaneous determination of the solubility of oxygen and the oxygen-transfer coefficient in cultivation media using only the physical properties of the liquid. The method is based on a system developed by van Sonsbeek et al[21] for the determination of the static ka in a liquid-

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impelled loop reactor. This system has been improved in such a way that it became possible to determine both the solubility of oxygen and the static oxygen transfer coefficient. This was achieved using the combined measurement of the amount of oxygen saturation of the liquid using dissolved oxygen probes and the oxygen-transfer rate using an off-gas mass spectrometer. In this way chemical components like glucose could not disturb the analysis method and calibration of the system was not required. The oxygen-transfer coefficient could be determined for head-space aeration as well as for aeration using a sparger, provided that no air bubbles were transported from the bioreactor to the strip vessel. The reliability of the individual measurements was usually better than 95 %.

To check whether the measurement results were valid, the measurements were carried out using water and Trypticase Soy Broth (TSB). The solubility of oxygen in TSB is determined by Slininger et al[19] and literature data about the solubility of oxygen in water are readily available in the selected temperature range[11,14,20]. Although all papers present different solubilities for oxygen at the same temperature, using these data, insight could be obtained about the reliability of the measurement system.

MATERIALS AND METHODS

A system was set up that could be discerned into three parts: the bioreactor, the strip vessel and the tubing between both vessels (Figure 1). The complete system was designed in such a way that it was autoclavable.

The bioreactor (diameter 0.2 m.) had a volume of $7 \cdot 10^{-3}$ m^3 and contained $3 \cdot 10^{-3}$ m^3 distilled water. It was equipped with a marine impeller (4 bladed, diameter 0.10 m), a polarographic dissolved oxygen probe (Ingold, Urdorf,

Switzerland), a Pt100 platinum resistance temperature detector (SDL, Southport, England) for temperature measurement and piping for liquid transport and aeration. Surface aeration was used for the oxygen supply. Temperature, air flow and stirrer speed were controlled using an CF500 measurement and control unit (Applikon Dependable Instruments, Schiedam, The Netherlands).

The strip vessel consisted of a $1 \cdot 10^{-3}$ m^3 glass vessel filled with approximately 20 metres of thin-wall silicon tubing (Watson- Marlow, Falmouth, England) with an inner diameter of $3 \cdot 10^{-3}$ m. The deoxygenation of the water passing through the tubing was done using nitrogen as a strip gas.

The tubing between the bioreactor and the strip vessel was made of stainless steel, except for the parts that had to be flexible (e.g. inside the pump). These parts were made of Norprene (Cole-Parmer, Chicago, USA). The tubing was insulated using a combination of cotton wool and aluminium foil which was adequate up to a temperature difference between the measurement system and the environment of approximately 20 °C. The liquid was circulated between the two vessels using a peristaltic pump (504U; Watson Marlow, Falmouth, England). A second dissolved oxygen probe was fitted in the liquid flow between strip vessel and bioreactor.

The composition of all gas flows was measured using an off-gas mass spectrometer (Prima 600; Fisons Instruments, Middlewich, England). All gas flows were checked using calibrated rotameters (Fischer & Porter, Horsham, USA). The air pressure was measured using a barometer (R.Fuess, Berlin-Steglitz, Germany).

For checking the measurement system with cultivation medium, Trypticase Soy Broth (30 g/l TSB; BBL Microbiology Systems, Cockeysville, USA) was selected.
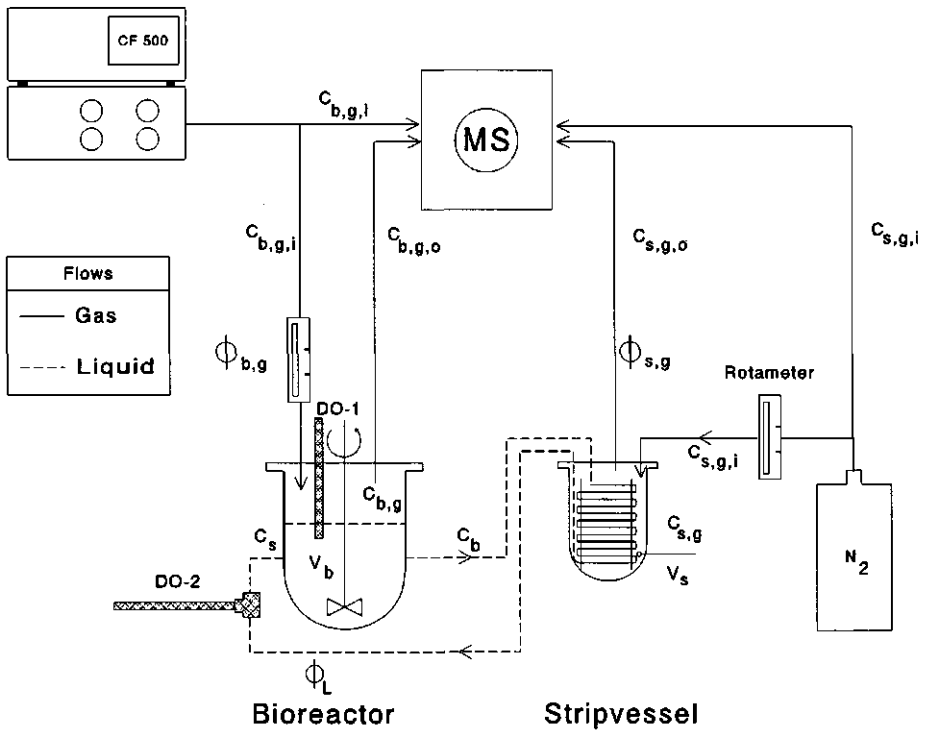
Figure 1: Measurement system in which a continuous transport of oxygen from the bioreactor to the strip vessel takes place. This oxygen transport was used to determine S_{O_2} and $k_l a$ simultaneously.

RESULTS AND DISCUSSION

Determination of the solubility of oxygen in water

To check whether the measurement conditions affected the measurement results, the conditions were varied in three ways: change of temperature inside the system, change of liquid-flow rate between bioreactor and strip vessel and change of stirrer speed inside the bioreactor. Changing the stirrer speed or changing the liquid-flow
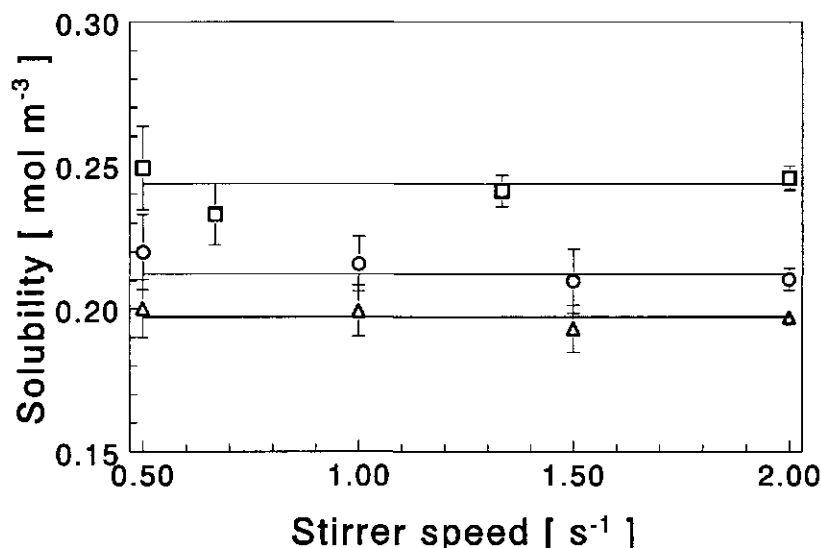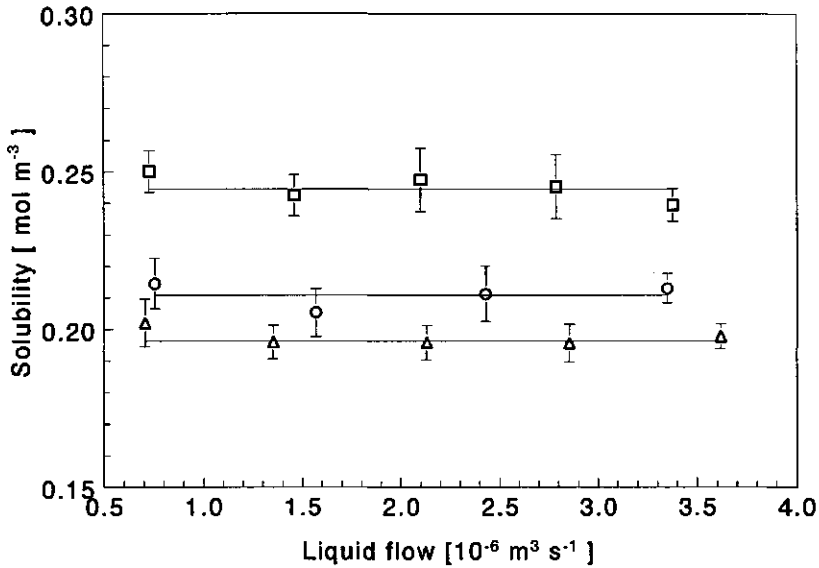
Figuur 2: Effect of the stirrer speed on the determination of the solubility of oxygen in water (□ - 29°C, O - 37°C, △ - 40°C **).**

rate should not have a statistical significant effect on the results. To check this, several experiments were carried out in which stirrer speed and liquid-flow rate were varied at a constant temperature. The stirrer speed was varied between 0.5 and 2 s^{-1}. If the stirrer speed exceeds 2 s^{-1}, air will be entrained into the liquid and causes shear forces which damage the cells[1]. The entrained air can also be transported to the strip vessel which results in disturbing the equilibrium between bioreactor and strip vessel.

Figure 2 shows the relationship between the stirrer speed and the measured solubility of oxygen in water. This solubility as well as the oxygen-transfer coefficient were calculated according to the method described in the appendix. The average measurement error in the determination of the S_{O_2} was 2.5 %. The horizontal lines in Figure 2 represent the average of the measurements. In order to calculate this average the variation (SD^2) of the measurements was used as weight

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Figure 3: Effect of the liquid-flow rate between bioreactor and stripvessel on the determination of the solubility of oxygen in water (□ - 29°C, O - 37°C, Δ - 40°C).

factor[17]. The data showed no significant effect of the stirrer speed on the solubility of oxygen.

Figure 3 shows the relationship between the liquid-flow rate between bioreactor and strip vessel and the solubility of oxygen in water. In this graph the horizontal lines represent the average of the measurements using their variation as weight factor. The range for the liquid-flow rate was selected in such a way that no significant build up of pressure inside the system could be detected. From Figure 3 no significant effect of the liquid-flow rate could be detected within this measurement range.After having validated the system as described, it was used to determine the solubility of oxygen in water. In figure 4 both experimental results and results obtained from literature have been combined. A temperature range of 25 - 40 °C was selected because the cultivation of animal cells is usually
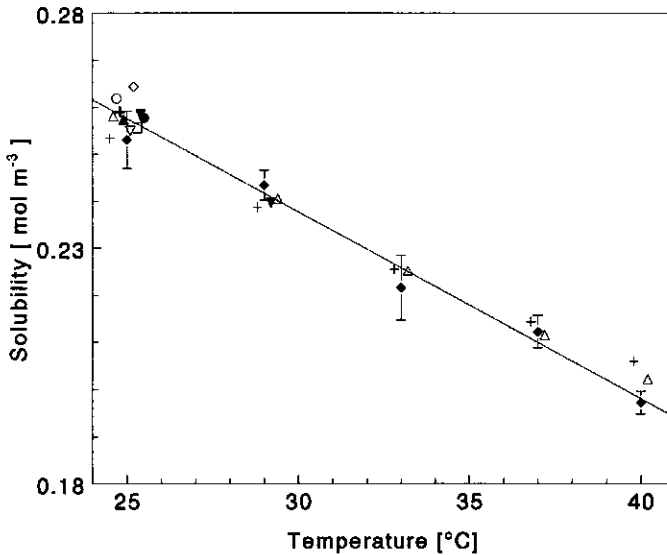
Figure 4: Comparison of the measured solubility of oxygen in water and values for oxygen solubility in water found in the literature[11,14,20] as a function of temperature.

performed within these ranges and because data about the solubility of water is readily available in this temperature regime. It shows that measured data and data found in literature are comparable. Only at 40 °C the measured data seem to deviate from data found in literature. The standard deviation of the measurements at 40 °C show that this deviation is indeed statistical significant. However, the standard deviations of the literature values are not incorporated in this graph because usually the measurement error is not included in the articles. When a measurement error is presented, this value roughly lays between 1 % and 10 % of the measured value. A likely explanation for the deviation between measured and literature values at 40 °C was that the system experienced problems measuring at higher temperatures. This was most likely due to the decrease of the temperature of the liquid outside the bioreactor. In addition to this, diffusion of oxygen through the tubing increases with increasing temperature.
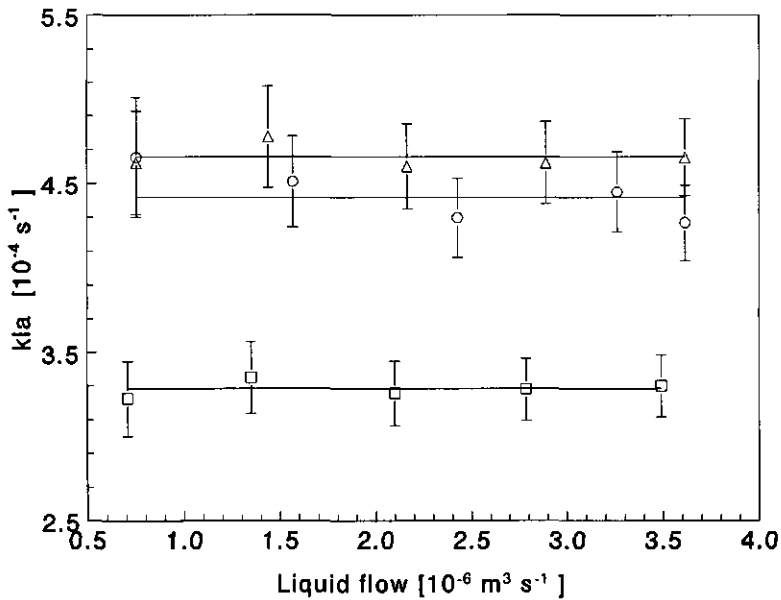
Figure 5: Effect of liquid-flow rate between bireactor and stripvessel on the determination of the oxygen-transfer coefficient of the bioreactor. (□ – 29°C, o – 37°C, △ – 40°C)

Determination of the static k_La

The k_La is influenced by both stirrer speed and temperature inside the bioreactor. Altering the liquid-flow rate however, should not have a statistical significant effect on the k_La. To test this, experiments were carried out at constant stirrer speed and temperature. The liquid-flow rate during these experiments was varied between $0.5 \cdot 10^{-6}$ and $4 \cdot 10^{-6}$ $m^3 \cdot s^{-1}$. From the data in figure 5 it can be concluded that the liquid-flow rate did not have a significant effect on the k_La. This implied that the measurement system could be used - within the specified regimes for stirrer speed, liquid-flow rate and temperature - to determine both the static k_La and the solubility of oxygen in water.
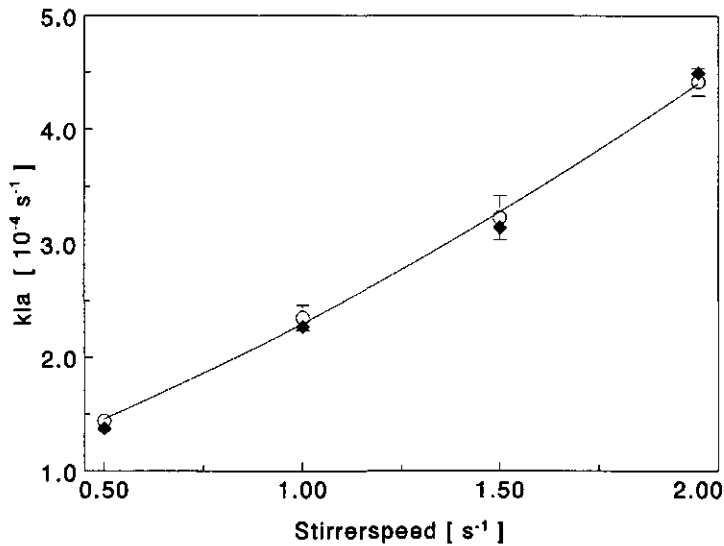
Figure 6: Effect of stirrer speed inside the bioreactor on the oxygen-transfer coefficient of the bioreactor at 37°C using the static method as well as the dynamic method

(○-*Static*, ◆-*Dynamic*)

The $k_L a$ was determined at different temperatures and at different stirrer speeds. Figure 6 shows the correlation between the $k_L a$ determined using the measurement system and the stirrer speed. Also the $k_L a$ determined using the dynamic method[22] is shown in this graph. In this stirrer-speed range, the $k_L a$ determined using the static method equalled the $k_L a$ determined using the dynamic method. This was to be expected because the response time of the DO-probe can be neglected at this stirrer-speed range.

By plotting the $k_L a$ against temperature using an Arrhenius plot, a linear graph was obtained from which the activation energy required for oxygen diffusion from gas phase to water phase could be calculated (Figure 7). The activation energy (E_a) for the diffusion of oxygen in water was found to be 18.0 kJ/mol.
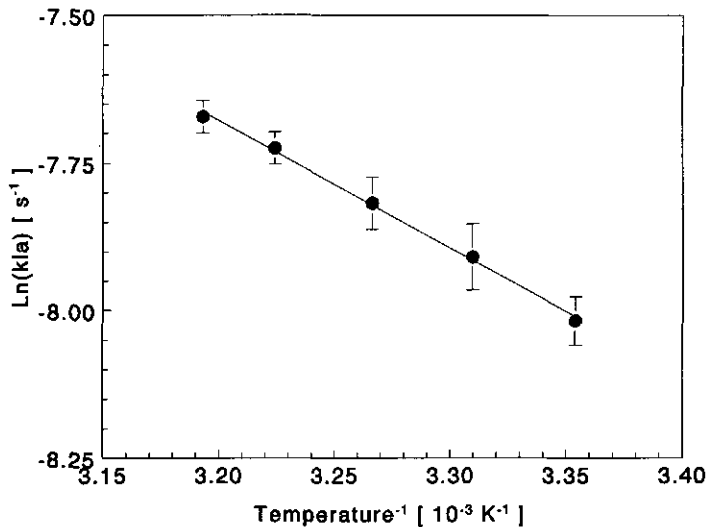
Figure 7 : Determination of the activation energy for diffusing oxygen into water at a stirrer speed of 2 s^{-1} from the slope of the Arrhenius plot in which Ln(k$_l$a) is plotted against temperature^{-1}.

Because this value is a constant for water, it is independent of the bioreactor used and also independent of the stirrer speed. Determining the activation energy for diffusing oxygen in water therefore enables one to predict the oxygen-transfer coefficient in water at any temperature within the specified range, in any type of bioreactor doing only one measurement.

Checking the system with cultivation medium

In order to prove that the system is indeed capable of determining the S_{O_2} and the $k_L a$ in cultivation medium this was verified for one type of cultivation medium. TSB was selected for this purpose because it could be compared to results found in

literature[19]. Using our measurement system the solubility of oxygen in TSB at 30 °C was found to be 0.209 ± 0.006 mol·m^{-3} under 760 mm. Hg of air (31.9 ± 1.0 mg·l^{-1} under 760 mm. Hg of oxygen) based on two measurements. According to Slininger et al the solubility of oxygen in TSB at 30 °C is 34.4 ± 1 mg·l^{-1} at an oxygen pressure of 760 mm Hg. However, on the basis of the information provided by Slininger et al (an uncertainty of 0.02 absorbance units in their measurements and the quantitative addition of 0.020 ± 0.0005 ml. sample) the real uncertainty in the solubility is approximately 11.5%. Furthermore, they used the solubility of oxygen in water as a calibration factor which caused the measurement to suffer from an additional systematic error. The solubility of oxygen found by Slininger et al, corrected for the solubility of oxygen in water found by our measurement method is 33.3 ± 4 mg·l^{-1}.

CONCLUSIONS

The measurement system described in this paper was able to determine S_{O_2} and k$_L$a simultaneously. Because only physical properties of the liquid were measured, the system will be able to determine both parameters in water as well as in all other aqueous solutions (e.g. cultivation media for animal cells) without chemical components disturbing the analysis. This was verified for TSB.

The average measurement error in the determination of the S_{O_2} was 2.5 %, based on a minimum of two measurements per experiment. This measurement error included errors in the readout of the equipment used. For a single measurement the error was 4.5 %. The average measurement error in the determination of the k$_L$a was 5 %. The measurement error for a single measurement was 6 %.

The k$_L$a can be predicted in any type of bioreactor for a temperature range

X_{O_2} = Mole fraction of oxygen present in the gas phase. [-]

In order to calculate the amount of oxygen present in the liquid, the signal of the DO probe is used :

$$C_l = S_{O_2} \cdot \frac{DO}{100} \tag{7}$$

where S_{O_2} [mol·m^{-3}] stands for the solubility of oxygen in the liquid and DO [%] for the amount of oxygen saturation of the liquid. Combination of equation 5,6 and 7 gives :

$$(x_{O_2,b,i} - x_{O_2,b,o}) \cdot \frac{P}{R \cdot T} \cdot \phi_{b,g} = (\frac{DO_b}{100} - \frac{DO_s}{100}) \cdot S_{O_2} \cdot \phi_l \tag{8.1}$$

or rewritten to S_{O_2} :

$$S_{O_2} = \frac{(x_{O_2,b,i} - x_{O_2,b,o})}{(\frac{DO_b}{100} - \frac{DO_s}{100})} \cdot \frac{P}{R \cdot T} \cdot \frac{\phi_{b,g}}{\phi_l} \tag{8.2}$$

From this equation the solubility of oxygen can be calculated. The $k_L a$ can be calculated using equation 1. A combination of equation 1, 6 and 7 gives :

$$(k_l a)_b = \frac{(x_{O_2,b,i} - x_{O_2,b,o}) \cdot \frac{P}{R \cdot T} \cdot \phi_{b,g}}{V_b \cdot [\frac{x_{O_2,b} \cdot \frac{P}{R \cdot T}}{H} - \frac{DO_b}{100} \cdot S_{O_2}]} \tag{9}$$

In this equation the solubility of oxygen, obtained using equation 8.2, can be used.

NOMENCLATURE

C	=	Oxygen concentration	[mol·m^{-3}]
DO	=	Amount of oxygen saturation of the liquid	[%]
E_a	=	Activation energy	[J·mol^{-1}]

H	$=$	Henry constant	[-]
k_La	$=$	Oxygen transfer coefficient	[s^{-1}]
n	$=$	Number of gas moles present	[-]
P	$=$	Pressure of the gas phase	[Pa]
R	$=$	Gas constant	[8.314 $J \cdot mol^{-1} \cdot K^{-1}$]
S_{O_2}	$=$	Solubility of oxygen in the liquid	[$mol \cdot m^{-3}$]
T	$=$	Temperature of the gas	[K]
V	$=$	Volume	[m^3]
X_{O_2}	$=$	Mole fraction of oxygen present in the gas phase.	[-]
ϕ	$=$	Flux	[$m^3 \cdot s^{-1}$]

Subscripts

b	$=$	Bioreactor
g	$=$	Gas phase
i	$=$	Incoming
l	$=$	Liquid phase
o	$=$	Outgoing
s	$=$	Strip vessel

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CHAPTER 4

A SIMPLE DYNAMIC METHOD FOR ON-LINE AND OFF-LINE DETERMINATION OF K_LA DURING CULTIVATION OF ANIMAL CELLS

ABSTRACT

A new, fast method is described to determine $k_L a$ either off-line, or on-line during animal-cell cultivation. Since it does not need the equilibrium concentration of oxygen in the liquid phase, it is not required to await a new steady state. Furthermore, the results do not depend on the calibration value of the dissolved-oxygen probe. The method yielded accurate values for $k_L a$, both for an oxygen-consuming and a non-consuming system.

INTRODUCTION

In the control of animal-cell cultivations, an increased use is made of models[7,9]. If models are applied, knowledge about changes that occur during the cultivation process is essential and, in batch cultivations, the composition of the cultivation medium varies with time. The volumetric oxygen transfer coefficient of a bioreactor ($k_L a$) largely depends on the composition of the medium. Fluctuations in $k_L a$ of more than 50%, caused by a change in the medium composition, are therefore no exception[2,8]. If $k_L a$ is to be implemented in a model, its correct value must be known. Sometimes this value can only be obtained from an on-line method for $k_L a$-determination during the cultivation process.

There are several papers that describe a method to determine $k_L a$ on-line[5,8,11,12]. Mignone and Ertola[5] used an elegant method based on a change in agitation rate. In animal-cell cultivations however, shear forces have to be minimized and low stirrer speeds are used. Since applying their method results in an increase of shear forces, it cannot always be used for this case. Mukhopadhyay and Ghose[8] also developed a simple technique to determine $k_L a$ on-line. They use

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a paramagnetic oxygen analyzer to determine the volumetric oxygen transfer rate. However, since this type of equipment is rarely available, their method is often not applicable. Votruba and Guthke[11] described a method, specially designed for bioreactors with a high aeration capacity. The method described by Yang *et al.*[12] is based on aeration using a sparger. Therefore, these methods are also not suitable for this case.

This paper presents a simple method to determine $k_L a$ during the cultivation of animal cells. It is based on a step change of the oxygen concentration in the headspace of the bioreactor. The method can be used both on-line and off-line. Its main advantages are that it is both fast and reliable. Furthermore, off-line application of this method provides an on the spot calculation method that can easily be incorporated into a programmable pocket calculator. The increased speed of this method is obtained by the new way in which $k_L a$ is calculated. Using this method, the equilibrium concentration of oxygen (C^*) is not needed. Therefore, it is no longer required to wait for a new steady state to establish. In addition to this, the method is independent of the calibration value of the dissolved-oxygen probe. Since this probe drifts with changes in atmospheric pressure, this is an important demand for an on-line method to determine $k_L a$. Finally, because in many animal-cell cultivations surface aeration is used, low values for $k_L a$ are obtained. Since this results in large characteristic times compared to the response time of the dissolved-oxygen probe, no correction for the response time of this probe has to be made in our case[3].

THEORY

The change in dissolved-oxygen concentration caused by a step change of the

oxygen concentration in the headspace of the bioreactor, is described by the following Equation:

$$\frac{d\,C_L}{d\,t} = k_L a\,(\ C^* - C_L\ ) - rX \tag{1}$$

In this Equation, C_L represents the dissolved-oxygen concentration [mol·m^{-3}], C^* is the dissolved-oxygen concentration in the liquid phase at equilibrium with the oxygen concentration in the gas phase [mol·m^{-3}], $k_L a$ is the volumetric oxygen transfer coefficient [s^{-1}] and rX is the oxygen consumption rate of the biomass [mol·m^{-3}·s^{-1}]. To determine $k_L a$, it is assumed that for the duration of the measurement, rX remains constant. Compared to the growth rate of the cells, a relative short time is needed to determine $k_L a$. For the Vero cells used in our experiments, a maximum growth rate of 0.021 h^{-1} was observed. The maximum increase of the cell density (X) during a $k_L a$-measurement of 40 minutes therefore becomes 1.4%. Compared to the error of the $k_L a$-measurement, the effect of cell growth during a $k_L a$-measurement on the outcome of the measurement, can therefore be neglected. Hence, if r also remains constant during the measurement, it is admitted to assume rX constant. The value of r is constant in a certain oxygen-concentration range. Outside this range, either oxygen limitation or oxygen inhibition occurs. For the Vero cells used in this study, this range is not known. For other cell lines however, some data have been published. Kilburn et al.[4] report a Monod constant of 0.39% air saturation for the oxygen-limited growth rate of mouse LS cells. Miller et al.[6] report a Monod constant of 0.50% air saturation for hybridoma cells, and data from Balin et al.[1] show an inhibition constant for WI-38 cells of 400% air saturation. For the $k_L a$-measurements during the cultivation of Vero-cells, the dissolved-oxygen concentration varied between 50 and 80% air saturation. If it is assumed that the behaviour of Vero cells regarding the effect of the oxygen concentration on r is comparable to the behaviour of the cell lines

described above, no variation of r is to be expected in this concentration range. Assuming a constant rX, the rise in C_L is solely due to the excess of oxygen applied to the system. The solution of Equation (1) therefore becomes:

$$k_L a \cdot t = -\ln \frac{C^* - \dfrac{rX}{k_L a} - C_{L,t}}{C^* - \dfrac{rX}{k_L a} - C_{L,0}} \tag{2}$$

Since rX, k_La and C^* are constant, the equilibrium concentration at stationary conditions can be described by:

$$C_{L,\infty} = C^* - \frac{rX}{k_L a} \tag{3}$$

From Equation (2), $C_{L,\infty}$ can be isolated:

$$C_{L,\infty} = \frac{C_{L,0} \cdot e^{-k_L a \cdot t} - C_{L,t}}{e^{-k_L a \cdot t} - 1} \tag{4}$$

If the dissolved-oxygen concentration is measured at points of time t_1 and t_2, $C_{L,\infty}$ can be eliminated:

$$\frac{C_{L,0} \cdot e^{-k_L a \cdot t_1} - C_{L,t_1}}{e^{-k_L a \cdot t_1} - 1} = \frac{C_{L,0} \cdot e^{-k_L a \cdot t_2} - C_{L,t_2}}{e^{-k_L a \cdot t_2} - 1} \tag{5}$$

Equation (5) can be rewritten to:

$$e^{-k_L a \cdot t_1} \cdot (C_{L,0} - C_{L,t_2}) + e^{-k_L a \cdot t_2} \cdot (C_{L,t_1} - C_{L,0}) + (C_{L,t_2} - C_{L,t_1}) = 0 \tag{6}$$

This Equation can be solved for k_La if:

$$t_2 = 2 \cdot t_1 \tag{7}$$

K_La can now be calculated using:

$$k_L a = \frac{-1}{t_1} \ln \frac{C_{L,t_2} - C_{L,0} \pm \sqrt{C_{L,0}^2 + 4 C_{L,t_1}^2 + C_{L,t_2}^2 - 4 C_{L,0} \cdot C_{L,t_1} + 2 C_{L,0} \cdot C_{L,t_2} - 4 C_{L,t_1} \cdot C_{L,t_2}}}{2 (C_{L,t_1} - C_{L,0})} \tag{8}$$

In Equation (8), the $\pm$ sign is negative when C_L increases and positive when C_L

decreases during the measurement. Using Equation (8), only the dissolved-oxygen concentrations at the points of time t_0, t_1 and t_2 are required to determine $k_L a$.

ERROR PROPAGATION

In order to assess the accuracy of this method, the error propagation was investigated. The error in the $k_L a$-measurement can be ascribed to three parameters: the amount of noise on the signal of the dissolved-oxygen probe (ν, depends on the measurement system), the total increment of C_L during the $k_L a$-measurement ($C_{L,t_2} - C_{L,0}$) and the total duration of the measurement (t_2). To study the individual effects, simulations were done in which one parameter was varied while the others were kept constant. These simulations showed that the absolute error in the $k_L a$-measurement (ε) was directly proportional to ν and inversely proportional to t_2 and to $C_{L,t_2} - C_{L,0}$. The overall effect of these parameters on $k_L a$ can be described by:

$$\varepsilon = \frac{\sqrt{18.58 + \dfrac{9.216 \cdot 10^5}{(t_2)^2}}}{C_{L,t_2} - C_{L,0}} \cdot \nu \tag{9}$$

For a $k_L a$ of $2.1 \cdot 10^{-4}$ s^{-1}, a noise (ν) of 0.03% air saturation ($6 \cdot 10^{-5}$ mol·m^{-3}) and an absolute measurement error (ε) of 2.5, 5 and 10% respectively, Figure 1 shows the settings for and t_2. Figure 1 can be used to determine the time needed for a $k_L a$-measurement if a certain accuracy is required. For example, if an accuracy (ε) of 5% is required and a ΔC_L of 30% air saturation is feasible, then a measurement will take at least 1600 seconds. However, if an accuracy of 2% is required, the measurement will take 4000 seconds.
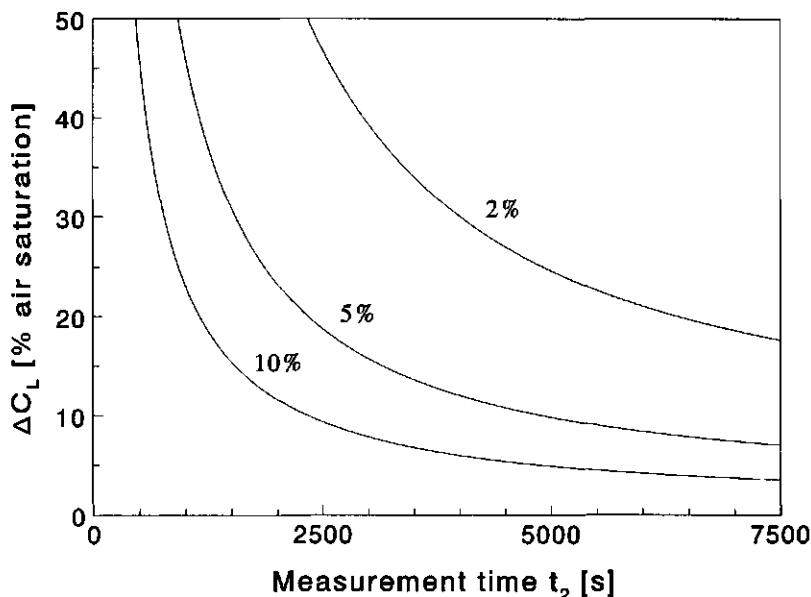
Figure 1: Settings for ΔC_L and t_2 at $\nu = 6 \cdot 10^{-5}$ mol$\cdot$m^{-3} and $k_L a = 2.1 \cdot 10^{-4}$ s^{-1}, obtaining an absolute measurement error (ε) of 2.5, 5 and 10% respectively.

MATERIALS AND METHODS

The bioreactor (diameter 0.2 m) had a volume of $6 \cdot 10^{-3}$ m^3 and contained $3 \cdot 10^{-3}$ m^3 cultivation medium. It was equipped with a marine impeller (4 bladed, diameter 0.1 m), a pH electrode (Ingold, Urdorf, Switzerland), a polarographic dissolved-oxygen probe (Ingold, Urdorf, Switzerland) and a Pt100 platinum resistance temperature detector (SDL, Southport, England). A CF500 measurement and control unit (Applikon Dependable Instruments, Schiedam, The Netherlands) was used to control pH, dissolved-oxygen concentration, temperature, gas flow and stirrer speed. The algorithm to determine $k_L a$ was incorporated into the software of the CF500.

Vero cells were cultivated in MEM Hank's medium (Gibco BRL Life Technologies B.V., Breda, The Netherlands) with additional 7.5 % bovine serum

(Bocknek Laboratories Inc, Canada), 2.5 % foetal calf serum (Sanbio, Uden, The Netherlands), 2 mM L-glutamine, 10 mM glucose and 1 % antibiotics (SVM, Bilthoven, The Netherlands). The cells were cultivated at a temperature of 37 °C, a pH of 7.20, a dissolved-oxygen concentration of 50 % air saturation and a stirrer speed of 50 rpm.

RESULTS AND DISCUSSION

First, the new algorithm was compared to the commonly applied dynamic gassing-in method[10]. This method uses Equation (2) to determine $k_L a$. Since the same data were used in both cases, they should result in the same $k_L a$. This was verified for a non-consuming system. The results of this experiment are shown in Table I. In this table, the measurement error for the on-line $k_L a$ originates from Equation (9). For the manually determined $k_L a$ no measurement error can be determined. Only the confidence interval of the curve fit can be provided, but this value does not provide information about the confidence interval of the $k_L a$-measurement. Figure 2 shows a representative example of one of the experiments presented in Table I. For this experiment, C_L was measured over a period of forty minutes with an interval of one minute. Furthermore, $C_{L,\infty}$ was measured to be able to determine $k_L a$ manually. The measured $C_{L,\infty}$ as well as the predicted $C_{L,\infty}$ using Equation (3) are also displayed in Table I. It shows that both methods result in the same values for $C_{L,\infty}$ and $k_L a$. This is hardly surprising, since both methods of determination originate from Equation (2). A previous paper already confirmed that in the stirrer-speed range tested, $k_L a$ determined using the dynamic method equalled $k_L a$ determined using a steady-state method[3].
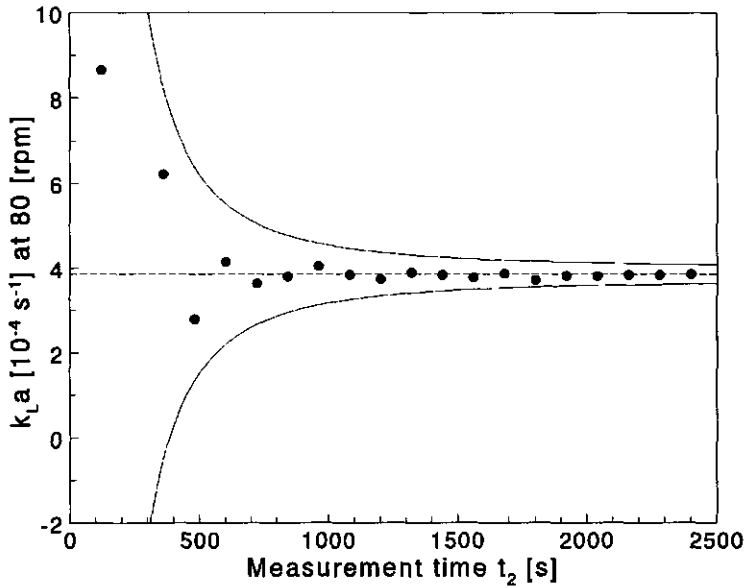
Figure 2: Comparison of manual vs. automated k_La-measurement ($\Delta C_{L,t=40min} = 4\cdot10^{-5}$ mol·m^{-3}; $\nu = 6\cdot10^{-5}$ mol·m^{-3}): (●) Automated k_La, (——) Measurement error of automated k_La, (– –) Manually determined k_La.

Table I. k_La-values obtained using the on-line method and Equation (2).

Stirrer speed	On-line k_La		Manual k_La	
[rpm]	k_La [10^{-4} s^{-1}]	$C_{L,\infty}$ [% DO]	k_La [10^{-4} s^{-1}]	$C_{L,\infty}$ [% DO]
40	2.33 ± 0.11	53.2	2.28	53.7
60	3.06 ± 0.14	91.3	2.97	91.6
80	3.78 ± 0.14	35.5	3.86	35.3
100	4.56 ± 0.14	68.0	4.61	67.9
120	5.64 ± 0.17	94.2	5.89	94.1

The method was also tested during a batch culture of Vero cells. K_La was determined regularly during this cultivation. Figure 3 shows k_La and the cell density during this experiment. The error bar in this graph represents the measurement error of k_La, calculated using Equation (9). The k_La-values obtained during

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cultivation were verified manually. For this, the data were fitted using Equation (2), thus acquiring an estimate for both $C_{L,\infty}$ and k_La. Since two parameters have to be estimated, curve fitting is not very accurate. For the same data, the estimate for the k_La-value can vary up to 20%, depending on the estimate for $C_{L,\infty}$. However, verification using curve fitting still provides useful information about the reliability of the measurement method developed. Results from this verification are presented in Table II. It shows that the on-line method to determine k_La can also be used in an oxygen consuming system.

Table II shows that during the cultivation presented here, k_La varied from 1.97 to $2.75 \cdot 10^{-4}$ s^{-1}. The variation of k_La during this cultivation indicates that k_La cannot be considered constant during a cultivation. Therefore, if a k_La-value is required as a model parameter, this value has to be determined regularly.
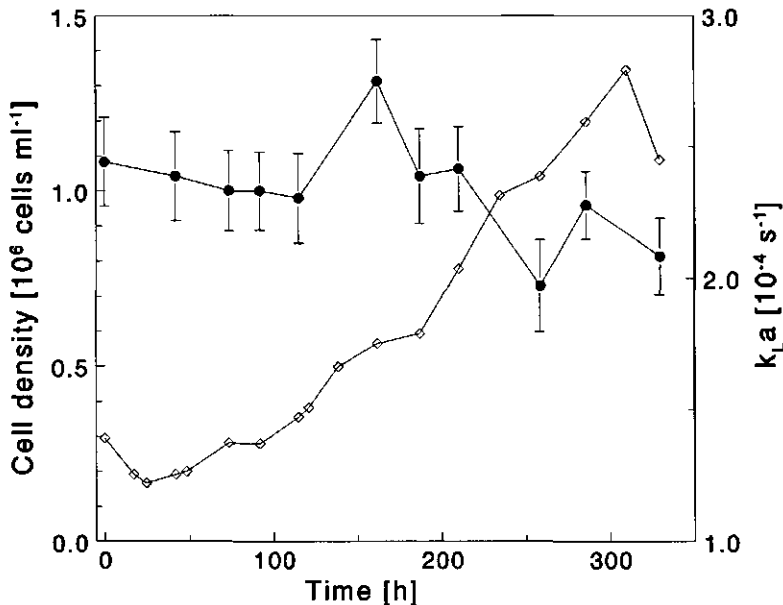

Figure 3: Course of the k_La during a batch cultivation of Vero cells: (●) k_La, (◇) Cells

Table II. $k_L a$-values obtained during cultivation and by curve fitting of the data using Equation (2).

Cultivation time [h]	On-line $k_L a$ [10^{-4} s^{-1}]	Manual $k_L a$ [10^{-4} s^{-1}]
0	2.44 ± 0.16	2.36
42	2.39 ± 0.16	2.17
74	2.33 ± 0.16	2.39
92	2.33 ± 0.13	2.31
115	2.31 ± 0.16	2.31
162	2.75 ± 0.16	2.69
187	2.39 ± 0.16	2.36
210	2.42 ± 0.16	2.47
259	1.97 ± 0.16	1.78
286	2.28 ± 0.13	2.25
330	2.08 ± 0.13	2.14

CONCLUSIONS

The method presented for the on-line determination of $k_L a$ in animal-cell cultivations, can be used for both an oxygen-consuming and a non-consuming system. This was verified for a batch culture of Vero cells and for cultivation medium without cells. The measurement results are comparable to results obtained using the commonly applied dynamic method. Because of the low values for $k_L a$ of surface aerated bioreactors, no adaptations have to be made to compensate for the response time of the dissolved-oxygen probe. This method provides an easy-to-use, fast and reliable way to determine $k_L a$ on-line. The calculation method can be programmed on-line into a measurement and control unit as well as off-line into a programmable pocket calculator. The need for an on-line method to determine $k_L a$ is indicated by the overall change in $k_L a$ of 30% during a batch cultivation of Vero cells.

NOMENCLATURE

C_L - Dissolved-oxygen concentration $[\text{mol}\cdot\text{m}^{-3}]$

C^* - $C_{L,\infty}$ in equilibrium with the oxygen concentration in the

       gas phase $[\text{mol}\cdot\text{m}^{-3}]$

$C_{L,\infty}$ - Equilibrium oxygen concentration at stationary conditions $[\text{mol}\cdot\text{m}^{-3}]$

$k_L a$ - Volumetric oxygen transfer coefficient $[\text{s}^{-1}]$

r   - Specific oxygen consumption of biomass $[\text{mol}\cdot\text{cell}^{-1}\cdot\text{s}^{-1}]$

X   - Cell concentration $[\text{cells}\cdot\text{m}^{-3}]$

t   - Time $[\text{s}]$

ν - Noise of dissolved-oxygen probe $[\text{mol}\cdot\text{m}^{-3}]$

ε - Absolute error of $k_L a$-measurement $[\text{s}^{-1}]$

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ABSTRACT

A software sensor was developed to determine the volumetric biomass activity of animal cell cultivations on-line. It was based on the on-line estimation of the ATP-production rate from the oxygen uptake rate and the lactic-acid production rate. The sensor was verified for a batch culture of Vero cells, and a batch and a continuous culture of hybridoma cells. For the hybridoma cells, the sensor showed a good correlation with the biomass concentration. However, this was not the case for the Vero cells. As soon as glutamine was exhausted, the biomass activity stabilized, whereas the amount of biomass almost doubled. Since the sensor developed responds to nutrient limitations much faster than becomes visible through cell density measurements, and since the volumetric biomass activity can be related to the volumetric consumption rates and production rates of important metabolites, it shows excellent possibilities for control purposes.

INTRODUCTION

In classic cultivation technology, one of the most important off-line analyses is the biomass determination. Regardless of the cell type being cultivated, biomass is always determined. Although it is considered very important, on-line measurement of the biomass concentration still is a scarce phenomenon. On-line determination of the biomass activity is seldom found too. Yet, it offers an increased insight in the cultivation process. It can be used as an indicator for nutrient limitation, to detect possible contaminations, or to signal the initialization of process steps like virus inoculation. Moreover, it can be used in control algorithms or as a tool in the optimization of the cultivation process.

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In the past two decades much work was devoted to develop a method to obtain on-line information about the cell behaviour. For this, two strategies have been followed. The first uses different types of sensors or other analytical tools to determine the biomass concentration on-line. The tools developed are based on a wide range of techniques. These involve Flow Injection Analysis[11], biosensors for ATP-determination[11], fibre-optic sensors[2,11], acoustic resonance densitometry[9,11,30], calorimetry[11], fluorometry[11,30], turbidity[11,26,34], filtration methods[11,30], optical density[30], electrical properties (Bugmeter)[30] and mass spectrometry[17]. These direct measurement methods all have their disadvantages. In some cases, irrelevant substances interfere or falsification occurs due to the physical or chemical environment (e.g., pressure, gas bubbles, pH,..). Additionally, some in-situ measurement techniques use equipment that is difficult or impossible to sterilize and therefore prone to contamination[11]. Besides, many above-mentioned methods have been designed to operate for bacterial or yeast cultivations. Their sensitivity is often too low to determine the biomass concentration during animal-cell cultivations.

The second strategy is to use software sensors to estimate either the biomass concentration or the biomass activity on-line[15,18,31,32,36]. A software sensor can be defined as a "sensor" implemented in the software, which uses information already available, to predict parameters that are not measured on-line. Two types of software sensors can be distinguished[15]. The first one uses macroscopic models, derived from mass balances and kinetic equations. This type of model requires input parameters like Monod constants, which are often difficult and laborious to determine experimentally. The implementation of a complete working model can therefore become quite troublesome. Even if the input parameters have been determined accurately, the model still suffers from inaccuracy, caused by the unavoidable simplifications that have to be made to

define such a model. Yet, if one of the estimated parameters can be measured off-line, these data can be used to correct the model inaccuracies. With this off-line correction of the model, it can be used to predict the course of modelled parameters on-line.

The second option uses general-structured models. These are models, based on techniques like Kalman filters or artificial neural networks. Since this type of model is able to adapt to the behaviour of the process, application of such a model can result in a significant reduction of development time. However, it does not contribute to the acquisition of knowledge about the process. Besides, the learning time of the model can become a major part of the total runtime. Especially for batch experiments this is a major drawback.

As an alternative to the above-mentioned sensor types, this paper presents a model to estimate the biomass activity, based on general knowledge about the physiology of the cells. In this case, biomass activity is defined as the total energy requirement of the cultivated cells. This type of software sensor is applicable for any type of animal cell or cultivation type, without adaptation of the model. Input parameters for this sensor can be made available in any computerized measurement and control unit, since only gas flow, gas composition, stirrer speed and added amount of base are required. No additional model parameters have to be determined experimentally. The physiological model is based on a paper by Glacken et al.[8] in which they propose a simple model for biomass estimation based on the oxygen uptake rate (OUR) and the lactic-acid production rate (LPR).

To evaluate the results of the software sensor, the individual parts of the sensor were verified. For the estimation of the OUR, both the oxygen solubility of cultivation medium (S_{O_2}) and the oxygen transfer coefficient ($k_L a$) during the cultivation of animal cells are required. Literature provides little information about the behaviour of both parameters. Usually, they are assumed to be constant

throughout the cultivation. To determine the S_{O_2}, a few methods have been proposed[4,20,22,24,29]. Nevertheless, no papers have been published about its course during cultivation. Also, several methods have been developed to determine the $k_L a$ on-line[7,10,13,16,35], but up to now only one paper has been published providing information about the course of the $k_L a$ during animal-cell cultivation[5].

To obtain information about the behaviour of the S_{O_2} and the $k_L a$ during cultivation, experiments have been done in which the effect of some major medium components on both parameters was determined. These experiments were carried out using an earlier developed method for the simultaneous determination of S_{O_2} and $k_L a^4$. The results of the present study show that the S_{O_2} does not vary significantly during cultivation. To account for possible changes in the $k_L a$, an on-line measurement method was used to determine the $k_L a$ during cultivation[5].

The results obtained using the software sensor provide a good insight in the behaviour of the animal cells tested. They show that cell counts cannot be used to visualize the true response of the cells with respect to nutrient limitation. Especially in the last part of batch cultivations a distinct difference between the biomass concentration and the biomass activity was observed, indicating that the biomass activity responds much faster to changes in environmental or cell conditions than becomes visible through cell counts.

THEORY

The software sensor is based on a simple model developed by Glacken et al.[8]. In their paper it is stated that after a short lag phase, the specific ATP-production rate is constant. Hence, the biomass activity can be defined as the volumetric ATP-production rate or the volumetric energy production rate (EPR). The EPR can be

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estimated by calculating the energy generation associated with the volumetric oxygen uptake rate (OUR) and the volumetric lactic-acid production rate (LPR)[8]:

$$EPR = 6\,OUR + LPR\,(F_{L/C}) \tag{1}$$

In this equation, $F_{L/C}$ is the ratio of lactic acid produced from glucose and the total lactic acid produced (range of 0-1). The value of $F_{L/C}$ is unknown, but Glacken showed that only a small error is introduced if $F_{L/C}$ is assumed to be 1. This assumption is confirmed by data presented by Reitzer et al.[21]. They show that for cultured HeLa cells, about 80% of the glucose and 13% of the glutamine is converted to lactic acid. With initial concentrations as used in our study for glucose and glutamine of 10 mM and 2 mM respectively, this means that 98% of the lactic acid originates from glucose. With this assumption, only the OUR and the LPR are required to estimate the biomass activity.

On-line determination of the OUR and the LPR

The OUR can be determined from the oxygen balance over the bioreactor. For the cultivation medium, the oxygen balance is:

$$OUR = k_L a\left(\frac{[O_2]_G}{H} - [O_2]_L\right) + \frac{\phi_L}{V_L}\left([O_2]_{L,\,in} - [O_2]_{L,\,out}\right) - \frac{d[O_2]_L}{dt} \tag{2}$$

In this equation, $[O_2]_G$ is the oxygen concentration in the head space of the bioreactor [mol·m^{-3}], $[O_2]_L$ is the oxygen concentration in the liquid [mol·m^{-3}], H is the Henry coefficient [-], ϕ_L is the liquid flow rate [m^3·s^{-1}], V_L is the liquid volume [m^3] and t is the time [s]. $[O_2]_G$ can be determined from the oxygen balance

over the head space:

$$\frac{\phi_G}{V_G} [O_2]_{G,in} = \frac{\phi_G}{V_G} [O_2]_{G,out} + k_L a \left(\frac{[O_2]_G}{H} - [O_2]_L \right) + \frac{d[O_2]_G}{dt} \qquad (3)$$

Here, ϕ_G is the gas flow rate $[m^3 \cdot s^{-1}]$, V_G is the head-space volume $[m^3]$, $[O_2]_{G,in}$ the oxygen concentration of the incoming gas $[mol \cdot m^{-3}]$, and $[O_2]_{G,out}$ is the oxygen concentration of the outgoing gas $[mol \cdot m^{-3}]$. Since the oxygen concentration in the liquid is controlled, it can be assumed constant. With this assumption, and with the assumption that the head space of the bioreactor is ideally mixed ($[O_2]_{G,out} = [O_2]_{G,in}$), the solution of equation (3) becomes:

$$[O_2]_{G,t} = \frac{k_L a [O_2]_L + \frac{\phi_G}{V_G} [O_2]_{G,in}}{\frac{\phi_G}{V_G} + \frac{k_L a}{H}} + \left([O_2]_{G,t_0} - \frac{k_L a [O_2]_L + \frac{\phi_G}{V_G} [O_2]_{G,in}}{\frac{\phi_G}{V_G} + \frac{k_L a}{H}} \right) e^{-\left(\frac{\phi_G}{V_G} + \frac{k_L a}{H} \right) t} \qquad (4)$$

The LPR equals the volumetric acid production rate (APR) if it is assumed that the only acidifying product excreted by the cells is lactic acid. This assumption is reasonable, since Glacken et al.[8] showed that in their case, the only titratable acid was lactic acid. The APR can be determined from the amount of base added to maintain a constant pH.

MATERIALS AND METHODS

The bioreactor used in all experiments (diameter 0.2 m) had a volume of $6 \cdot 10^{-3}$ m^3 and contained $3 \cdot 10^{-3}$ m^3 cultivation medium. It was equipped with a marine impeller (4 bladed, diameter 0.1 m), a pH electrode (Ingold, Urdorf, Switzerland),

a polarographic dissolved-oxygen probe (Ingold, Urdorf, Switzerland) and a Pt100 platinum resistance temperature detector (SDL, Southport, England). A CF500 measurement and control unit (Applikon Dependable Instruments, Schiedam, The Netherlands) was used to control pH, dissolved-oxygen concentration, temperature, gas flows and stirrer speed. The amount of base added was monitored using a balance (4800P; Sartorius GMBH, Göttingen, Germany) coupled to the measurement and control unit through RS232. The algorithms to determine the $k_L a$ and the biomass activity were incorporated into the software of the CF500. During the cultivation experiments, the cells were grown at a temperature of 37 °C, a pH of 7.20, a dissolved-oxygen concentration of 50% air saturation and a stirrer speed of either 50 rpm (Vero cells) or 100 rpm (hybridoma cells). The pH was controlled using 0.3 M sodium hydroxide to be able to determine the acid production of the cells.

Off-line analysis techniques

Glutamine was determined on an HPLC system (Waters, Division of Millipore, Milford, MA, USA). The amino acids were separated with a 150 x 3.9 mm. I.D. 85711 Waters Resolve 5 μ spherical C-18 column, using o-phthalaldehyde with 2-mercaptoethanol as derivatizing agent[3]. Lactic acid and glucose were determined using a YSI 2300 STAT analyzer (Yellow Springs Instruments, Ohio, USA). Osmolality was determined using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). For Vero cells, the cell density was determined by counting their nuclei after treatment with crystal violet (1 g/l in 0.1 M citric acid; SVM, Bilthoven, The Netherlands). Hybridoma cells were counted using the trypan-blue exclusion method.

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Determination of S_{O_2} and $k_L a$

The measurement system and the calculation method used to determine both S_{O_2} and $k_L a$, have been described extensively in a previous paper[4]. Briefly, it consists of a $3 \cdot 10^{-3}$ m^3 bioreactor and a strip vessel filled with approximately 20 meters of thin-wall silicon tubing (I.D. = 0.003 m.; Watson-Marlow, Falmouth, England). The liquid passing through the tubing is deoxygenated by flushing the strip vessel with nitrogen. From the amount of oxygen transport from the bioreactor to the strip vessel, both S_{O_2} and $k_L a$ can be determined. Two dissolved-oxygen probes (Ingold, Urdorf, Switzerland) and an off-gas mass spectrometer (Prima 600; Fisons Instruments, Middlewich, England) are used to determine this oxygen transport.

The assumption that S_{O_2} can be considered constant was verified for changes in the concentrations of five medium components: glucose, lactic acid, glutamine, bovine serum and sodium bicarbonate. Glucose and glutamine were purchased from SVM (Bilthoven, The Netherlands), lactic acid was purchased from Boehringer (Mannheim, Germany), sodium bicarbonate was purchased from Merck (Darmstadt, Germany) and bovine serum was purchased from Bocknek Laboratories Inc. (Canada). The effect of a single medium component was determined by testing cultivation medium with and without this component.

Determination of the $k_L a$ during cultivation

For this, an earlier developed method was used as well[5]. The method uses the response of the dissolved-oxygen probe on a step change of the oxygen concentration in the head space of the bioreactor to determine the $k_L a$. Details

about the calculation method are described extensively in the paper mentioned[5].

Determination of the OUR with the mass spectrometer

The dissolved-oxygen concentration is controlled by controlling the oxygen concentration in the head space of the bioreactor. This is done by varying the oxygen concentration of the incoming gas and applying a constant flow rate. If the cell density increases, the total oxygen demand of the cells (OUR) increases too. Because of this, more oxygen is transported from the head space to the liquid. Therefore, the oxygen concentration in the head space will decrease. If the system is in a steady state, the difference between the oxygen concentration of the incoming gas and the outgoing gas equals the amount of oxygen consumed by the cells. However, a steady-state situation never occurs during a batch culture. To determine the OUR with a mass spectrometer, the theoretical oxygen concentration in the head space of the bioreactor has to be known. Because of the low flow rate, the residence time of the gas in the head space of the bioreactor is approximately 3000 seconds. Therefore, the theoretical oxygen concentration in the head space does not equal the oxygen concentration of the incoming gas. However, it can be calculated from the composition of the inlet gas, assuming ideal mixing of the head space and assuming no oxygen transport to the cultivation medium. The OUR can now be calculated by subtracting the measured oxygen concentration of the exit gas from this theoretical oxygen concentration in the head space of the bioreactor. The resulting value of the subtraction is the amount of oxygen transported to the cultivation medium. From this value, the oxygen transfer rate is calculated. If it is assumed that the dissolved-oxygen concentration is constant, the oxygen transfer rate equals the OUR.

Cultivation of Vero cells

Vero cells (WHO seeds, ECACC) were cultivated in a batch culture using MEM Hank's medium without sodium bicarbonate (Gibco BRL Life Technologies B.V., Breda, The Netherlands) but with 7.5% bovine serum (Bocknek Laboratories Inc., Canada), 2.5% foetal calf serum (Sanbio, Uden, The Netherlands), 2 mM L-glutamine, 10 mM glucose and 1% antibiotics (SVM, Bilthoven, The Netherlands). The cells were cultivated on 2 g/l Cytodex I microcarriers (Pharmacia, Uppsala, Sweden). The cell attachment on the microcarriers took place in a spinner flask containing MEM Hank's medium supplemented with sodium bicarbonate. This was done because the cell attachment in bicarbonate-free medium is poor. The cells were allowed to attach for five hours, after which the medium was replaced by medium without sodium bicarbonate and the bioreactor was inoculated. This startup method was used to be able to inoculate the bioreactor in a reproducible way. This was important to allow comparison of the different cultivations.

Cultivation of hybridoma cells

Hybridoma type C1a (kindly provided by Centocor, Leiden, The Netherlands) was cultivated in a batch culture and MN12 (kindly provided by Dr. J. Poolman, National Institute of Public Health and the Environmental, Bilthoven, The Netherlands) in a continuous culture. The cultivation medium was Iscove's modified Dulbecco's medium without sodium bicarbonate (Gibco Laboratories, Grand Island, NY), supplemented with 0.25% (w/v) Primatone RL (Sheffield Products, Norwich, NY), 5% heat-inactivated foetal calf serum (Sanbio, Uden, The Netherlands) and 1%

antibiotics.

RESULTS AND DISCUSSION

Determination of S_{O_2} and $k_L a$

For the estimation of the OUR it is essential to know if both the S_{O_2} and the $k_L a$ can be considered constant during cultivation. Any variation in either one of these parameters will cause a systematic error in the estimation of the OUR. Therefore, experiments were done in which the effect of glucose, lactic acid, glutamine, bovine serum and sodium bicarbonate on both the S_{O_2} and the $k_L a$ was determined. The range in which these medium components were tested, was derived from the maximum concentration expected during cultivation. To obtain information about the effect of the osmolality of the medium on both parameters, a high lactic-acid concentration was tested, since lactic acid affects the osmolality substantially. The results of the experiments are shown in Table I. It shows that the components tested do not significantly change both parameters. It can therefore safely be assumed that the S_{O_2} is constant throughout the cultivation. However, literature states that the $k_L a$ can change significantly[5,16]. Therefore the $k_L a$ was determined regularly during the cultivations.

Table I : Effect of medium components on the S_{O_2} and the k_La

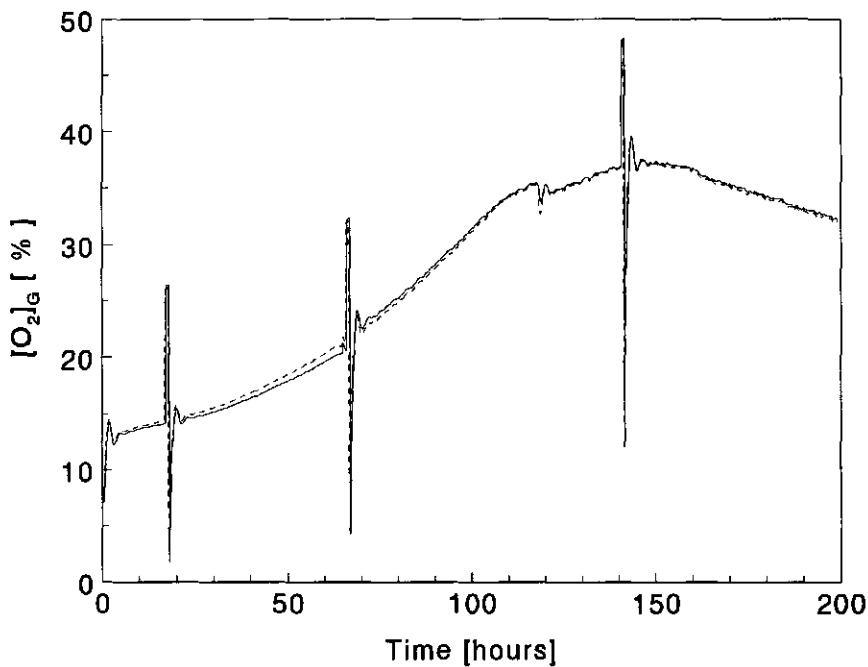| Component added	Concentration added		Osmolality [mOsmol·kg^{-1}]	S_{O_2} [mol·m^{-3}]	k_La [10^{-4} s^{-1}]
Default medium	-		272	0.200 ± 0.002	3.84 ± 0.01
+ Glucose	12	[mM]	295	0.200 ± 0.002	3.76 ± 0.02
+ Lactic acid	150	[mM]	551	0.193 ± 0.001	3.74 ± 0.04
+ Glutamine	7.75	[mM]	272	0.204 ± 0.009	3.75 ± 0.27
+ Bicarbonate	5.0	[mM]	289	0.206 ± 0.002	3.87 ± 0.13
+ Bovine serum	10	[%]	279	0.195 ± 0.004	4.01 ± 0.15

Figure 1: Estimated (————) and measured (- - -) oxygen concentration in the head space of the bioreactor during a batch culture of Vero cells
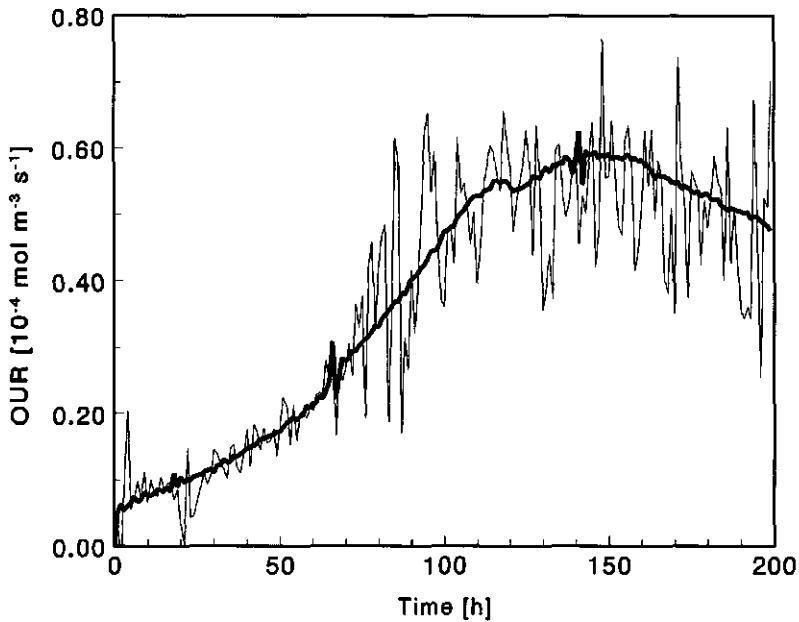
Figure 2: Estimated (———) and measured (——) OUR during a batch culture of Vero cells

Experimental verification of the biomass-activity sensor

To determine the practical use of the biomass-activity sensor, it was tested for two cell types, Vero cells and hybridoma cells, each having its own characteristics. Vero cells are anchorage dependent, whereas hybridoma cells are cells that grow in suspension. To test the sensor for different cultivation types, both batch and continuous cultivations were performed. Vero cells and hybridoma C1a cells were cultivated in batch mode, and hybridoma MN12 cells were cultivated in a continuous mode.

To check the reliability of the individual parts of the software sensor, the batch culture of Vero cells was monitored with an off-gas mass spectrometer (MS). This
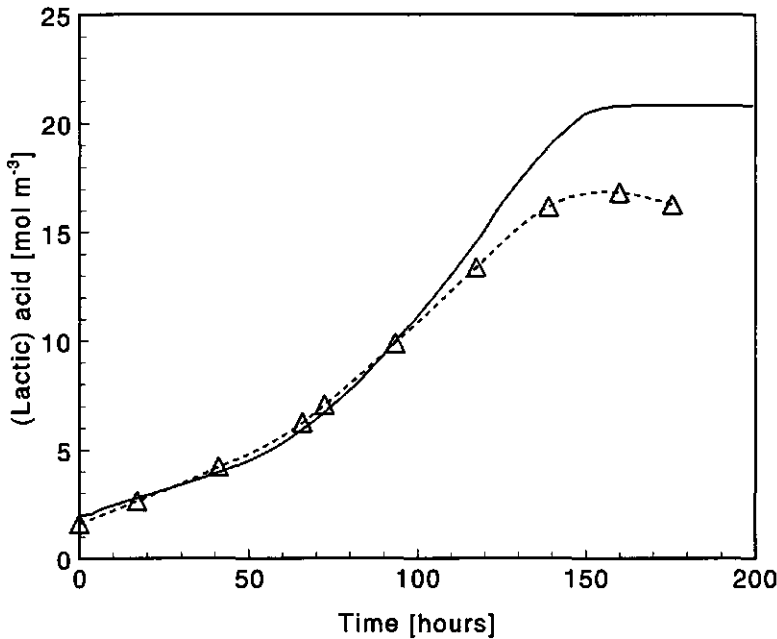
Figure 3: Course of the acid production (——) and the measured lactic-acid concentration (···△···) during a batch culture of Vero cells.

way, both the estimated values for the oxygen concentration in the head space of the bioreactor and the OUR could be verified by the MS-data. The results of this verification are presented in Figures 1 and 2. Figure 1 shows the measured (MS) and the estimated oxygen concentration in the head space ($[O_2]_G$, equation 4). The spikes in Figure 1 are due to on-line k_La-measurements during the batch culture[5]. Between 30 and 70 hours, a small deviation between both values occurs. This is caused by a small calibration error of the nitrogen mass-flow controller. Figure 1 clearly shows that equation (4) provides a very good estimate of the $[O_2]_G$.

Figure 2 presents the measured (MS) and the estimated value for the OUR (equation 2). The measured OUR contains much noise. This is caused by the differential method with which the OUR is determined. The difference in noise

before and after 70 hours of cultivation is caused by a small decrease in the stability of the dissolved-oxygen controller (from 50.0 ± 0.05 to 50.0 ± 0.1 % air saturation). This affects the OUR determined with the mass spectrometer, but does not affect the estimated OUR, since the estimator corrects for these fluctuations in the dissolved-oxygen concentration (equation 2). The spikes in the estimated OUR at 20, 70 and 140 hours are again caused by on-line $k_L a$-measurements. During this cultivation experiment, the $k_L a$ remained approximately constant at $2.2 \cdot 10^{-4}$ s^{-1} and was therefore not adjusted in the calculation of the OUR. As with the $[O_2]_G$, the measured and the estimated OUR show a very good correlation.

To be sure that the software sensor provides a good estimate for the EPR, the LPR has to be verified as well. Figure 3 shows the measured lactic-acid concentration, together with the total amount of acid produced. Until the moment that the OUR starts to stabilize (Figure 2), the acid production determined from the amount of base added, and the measured lactic-acid concentration, are comparable. After that, the measured lactic-acid concentration is lower than the acid production. A possible cause for this difference is that the cells utilize energy through a different metabolic route, suddenly producing more carbon dioxide. This again could increase the accumulation of carbon dioxide in the medium, which would lead to acidification of the medium. If this is the case, the respiratory quotient (RQ = CER/OUR) will increase after 100 hours of cultivation. This however, is not the case. The RQ remains constant up to the moment that the cell density starts to decrease (data not shown). Next to this, accumulation of carbon dioxide in the cultivation medium is not likely to cause the difference between acid production and lactic-acid production: Assuming a $(k_L a)_{CO_2}$ of $0.89 \cdot (k_L a)_{O_2}$ [23] and assuming pseudo steady state, the mass balance across the head space of the bioreactor can be solved. Applying these assumptions, the maximum concentration

CO_2 in the cultivation medium was found to be 0.21 mol·m^{-3}. A significant amount of the carbon dioxide is present in the cultivation medium as HCO_3^-. The concentration HCO_3^- can be calculated according to:

$$[HCO_3^-] = [CO_2]_L \frac{10^{-pKa}}{10^{-pH}} \tag{5}$$

Using a pH of 7.20 and a pKa of 6.35, the total amount of carbon dioxide acidifying the cultivation medium now becomes 1.5 mol·m^{-3}. This is 27% of the difference of 5.5 mol·m^{-3} between estimated and measured lactic-acid concentration. Therefore, carbon dioxide accumulation cannot account for the total difference between both concentrations.
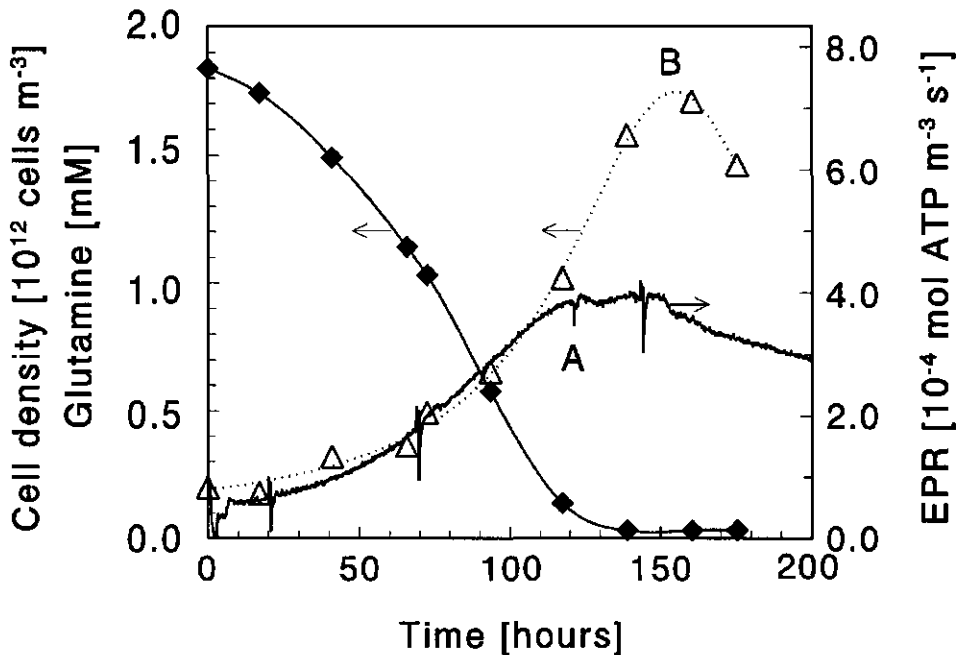

Figure 4: Course of cell density (···△···), EPR (——) and glutamine concentration (♦) during a batch culture of Vero cells. Points A and B are explained in Table II.

Another possible explanation can be found examining Figure 3. As can be seen at 170 hours, the lactic-acid concentration starts to decrease. This effect can be seenin all batch cultures of Vero cells. So, next to production, also consumptionof lactic acid takes place[1]. Lactic acid uptake is an active process that requires a transporter. At least two types of transporters are described in literature. The first type is a lactate-H^+-cotransporter[25]. Since with each lactic-acid molecule a proton is transported into the cell, no H^+ is left behind in the cultivation medium when lactic acid is taken up by the cells. However, there is a report[25] indicating an inhibition or substitution of the H^+ cotransport by K^+ of 36%. If this is true in our case, this can in theory account for the difference between measured and estimated lactic-acid concentration. Next to this type of transporter, a Na^+-dependent lactic-acid transporter has been found in kidney cells[28]. In this case no cotransport of H^+ occurs. Therefore, if lactic acid is taken up by the cell using the Na^+-dependent lactic-acid transporter, H^+ is left behind in the cultivation medium. In that case, the predicted lactic-acid concentration will be higher than the real one. In addition to this, the estimation of the EPR will be affected too. Under the stated hypotheses, a proton is left behind in the cultivation medium when a lactate ion is taken up by the cell. Therefore the glycolysis step is counted twice in equation 1. However, only a very small error arises from this. The total difference between estimated and measured lactic-acid concentration is 5.5 $mol \cdot m^{-3}$ (Figure 3). In total, 20 $mol \cdot m^{-3}$ of lactic acid was estimated to be produced. If the total difference can be explained by lactic acid consumption, 27.5% of the lactic acid produced, is consumed again. Next to this, Table II shows an average EPR/OUR ratio of 7.2. From this and using equation 1, it can be calculated that the LPR/EPR ratio is 1/6. Applying these numbers in equation 1, a maximum error in the EPR of 4.6% is obtained. This error is small enough to be neglected.

Table II : Comparison of Vero batch cultures

Description	Culture 1	Culture 2	Culture 3
Stabilization of EPR (A in Fig. 4)			
Time [h]	120	113	109
Time at μ_{max} [h]	116	108	108
EPR [10^{-4} mol·m^{-3}·s^{-1}]	4.12	4.24	4.08
Cell density [10^{12} m^{-3}]	1.08	1.16	1.18
[Glutamine] [mol·m^{-3}]	0.13	0.22	0.25
Maximum Cell density (B in Fig. 4)			
Reached at time [h]	160	162	161
Cell density [10^{12} m^{-3}]	1.71	1.76	1.72
Cell doublings after stabilization EPR	0.66	0.60	0.55
EPR / OUR	7.1 ± 0.1	7.4 ± 0.2	7.2 ± 0.3

After evaluation of the complete estimator, it can be concluded that the individual parts of the software sensor provide accurate values for $[O_2]_G$, OUR and EPR. The LPR provides accurate results as long as there is no nutrient limitation.

Figure 4 shows the cell density of the Vero-cell batch culture, in combination with the biomass-activity sensor and the glutamine concentration. The first 120 hours, the sensor shows a good correlation with the cell density. In that part, the specific EPR is 4.1 ± 0.5·10^{-16} mol·cell^{-1}·s^{-1}. During the last part of the cultivation, the EPR starts to stabilize, whereas the cell density continues to increase. The stabilization of the EPR seems to coincide with the exhaustion of glutamine. This phenomenon has been observed during all batch cultures of Vero cells. This is shown in Table II, in which a summary of three Vero-cell batch cultures is presented that have been cultivated under comparable conditions. An explanation for this observation might be that glutamine limitation causes the cells to shift their metabolism towards maintenance, requiring less energy. Another
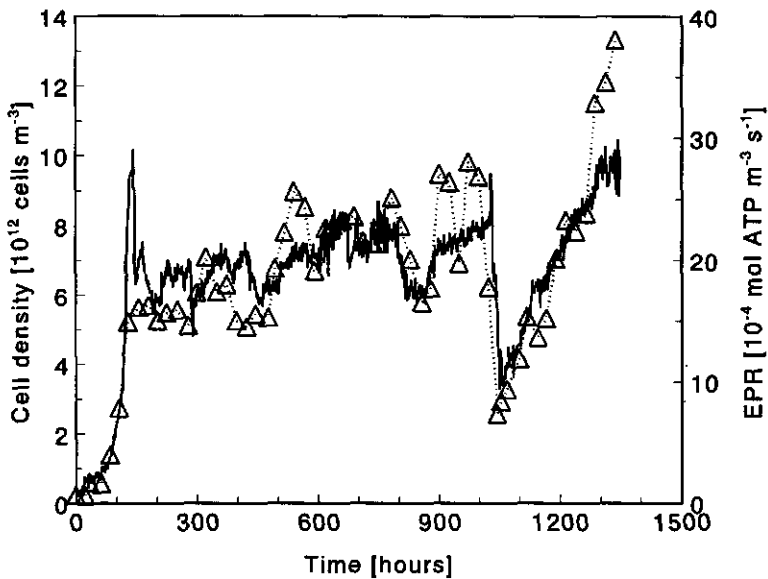
Figure 5: Course of cell density (··Δ···) and EPR (——) during a continuous culture of MN12 hybridoma cells. At 65 hours, the dilution rate was set to 0.4 volumes/day, at 1050 hours it was set to 0.8 volumes/day. At 1000 hours, 50% of the cells were removed from the bioreactor.

explanation is that during the cultivation the cell size decreases, and consequently the amount of biomass per cell. Since volumetric biomass activity is estimated instead of cell density, the result of the software sensor deviates from the cell density. The decrease in cell size is probably caused by glutamine limitation. When glutamine becomes limiting, it becomes more difficult for the cells to take up glutamine. Cells that have just entered the G_1-phase cannot take up enough glutamine, resulting in cell-cycle arrest. Since cell-cycle events become largely independent of extracellular factors after cells enter the S-phase[19], cells that are beyond the restriction point in the G_1-phase will finish the cell cycle. Because of the cell-cycle arrest, the growth rate gradually decreases. At a certain point in time, most cells will be arrested in the G_1-phase. At this point the cells decide whether

they stay in the G_1-phase, or enter the G_0-phase. Since cells in the G_1-phase have an unduplicated DNA content, they are smaller than dividing cells. Furthermore, G_0-cells are even smaller than G_1-cells[19]. Table II clearly shows that the stabilization of the EPR during the exponential growth phase is characteristic for this cell type. The way in which this occurs, is very reproducible. The stabilization occurs when the cells reach the maximum growth rate (μ_{max}). The amount of cell doublings after this stabilization occurs, is also constant (about 0.6). Finally, the EPR-OUR ratio shows that the LPR does not significantly alter the response of the EPR (equation 1). In this case the OUR provides the same information as the EPR.

To check the biomass-activity sensor for other cell types, hybridoma cells were used. Table I already showed that during cultivation, no significant changes are to be expected in the S_{O_2}. During the hybridoma cell cultures, also no significant changes in the k_La were observed. Figure 5 shows a continuous cultivation of MN12 hybridoma cells. In this figure, both the cell density and the result of the software sensor, the EPR, are displayed. Contradictory to the behaviour of the EPR during the batch cultivation of Vero cells, in this case a very good correlation exists between cell density and EPR both during the exponential growth phase and during the steady states. The changes in dilution rate at 65 hours (0 to 0.4 volumes/day) and at 1050 hours (0.4 to 0.8 volumes/day) and additionally the removal of 50% of the cells after 1000 hours of cultivation are described well by the EPR. During this cultivation, the specific EPR was 3.0 $\pm$ $0.2 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}. This value correlates well with the specific EPR for hybridoma cells of $3.5 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}, found by Miller et al.[14]. Glacken et al.[8] found a specific EPR of $2.1 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}. This indicates that the software sensor provides realistic values for the EPR. Since the specific EPR was constant during this cultivation, in this specific case the software sensor could be used as a biomass sensor as well. A close examination of the data showed that, as might

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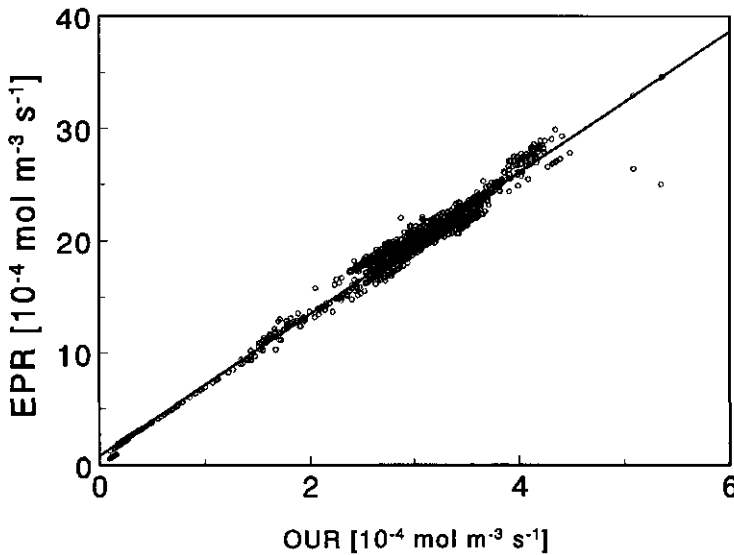
Figure 6: Relationship between the EPR and the OUR during the continuous culture of MN12 hybridoma cells

be expected from equation 1 and from the results of the experiments with the Vero cells, the LPR plays only a minor role in the estimation of the EPR. Hence, in these cases the biomass activity can be described using only the OUR. Figure 6 shows that leaving out the LPR does not affect the behaviour of the estimator, since a linear relationship exists between the OUR and the EPR. Literature confirms this observation[27]. It can therefore be concluded that the role of the LPR in this software sensor for biomass activity is negligible.

Figure 7 shows a representative example of a batch culture of hybridoma C1a cells, together with the OUR. Again, during the exponential growth phase the OUR shows a very good correlation with the cell density. After that, the OUR drops radically, whereas the drop in cell density is gradual. During the exponential growth, the specific OUR was $0.65 \pm 0.04 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1} in. As for the EPR,
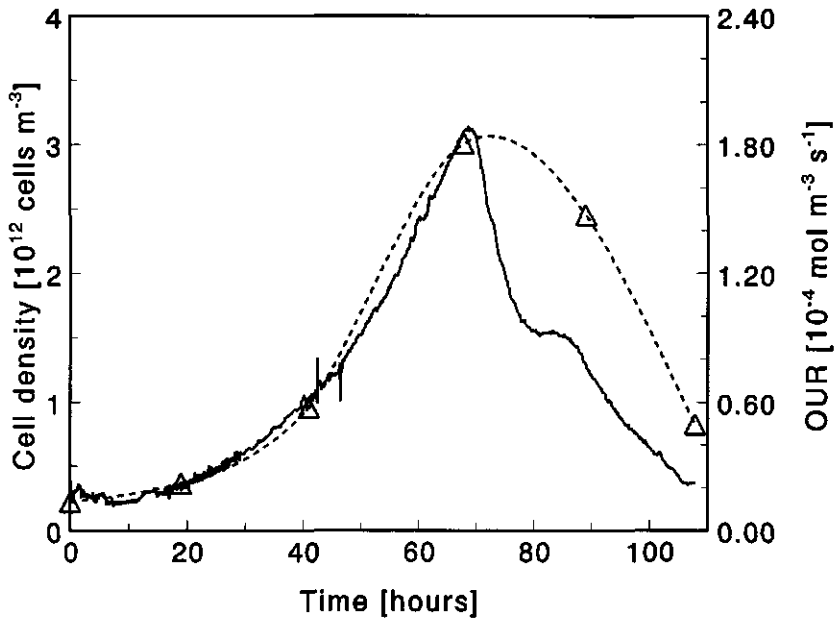
Figure 7: Course of cell density (···△···) and OUR (——) during a batch culture of C1a hybridoma cells.

this value is comparable with values found in literature. Miller *et al.*[14] found a specific OUR of $0.53 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}, whereas Fleischaker and Sinskey[6] and McLimans *et al.*[12] showed that for most mammalian cells, the specific OUR lies between 0.14 and $1.4 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}.

CONCLUSIONS

The results of this paper showed that on-line estimation of the OUR and the LPR can be done by means of information already available in most measurement and control units. This was verified for the OUR using an off-gas mass spectrometer, and for the LPR using off-line measured lactic-acid concentrations. The EPR can be

estimated from the OUR and the LPR using equation (1). This was tested for hybridoma MN12 cells and for Vero cells. For the MN12 hybridoma cells, the EPR showed a very good correlation with the cell density. The specific EPR was found to be $3.0 \pm 0.2 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}. For the Vero cells this value was $4.1 \pm 0.5 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}. These values are comparable with values found in literature. The results of these experiments showed that the contribution of the LPR to the biomass-activity sensor was not significant. The biomass activity could be described with the OUR just as well as with the EPR. Since for the determination of the OUR no adaptations have to be made on the pH-control and on the cultivation medium used, using the OUR instead of the EPR is beneficial. The use of the OUR as a measure for the biomass activity was confirmed during a batch culture of hybridoma C1a cells. The specific OUR for C1a cells was found to be $0.65 \pm 0.04 \cdot 10^{-16}$ mol·cell^{-1}·s^{-1}.

At the last part of the batch cultivation of Vero cells, a distinct difference between biomass activity and cell density occurred. This was probably due to glutamine limitation. It shows that the biomass-activity sensor can detect changes in the cell metabolism before these become visible through cell counts. It can therefore be used for automatic adjustment of the cultivation conditions. The sensor can be used for any type of animal cell, without adapting the model, since there are no model parameters that depend on the cell type. Care has to be taken however, interpreting the results being directly proportional to the biomass concentration[33]. The EPR is proportional to biomass activity, and not to biomass concentration. However, for control purposes the total activity of the biomass present in the bioreactor is more important than the exact amount of biomass since it can be related to the consumption rates and production rates of important metabolites.

NOMENCLATURE

APR	Volumetric acid production rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
CER	Carbon-dioxide evolution rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
EPR	Volumetric ATP production rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
$F_{L/C}$	Ratio of lactic acid produced from glucose and the total lactic acid produced	$[-]$
H	Henry coefficient	$[-]$
$k_L a$	Volumetric oxygen transfer coefficient	$[s^{-1}]$
LPR	Volumetric lactic-acid production rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
$[O_2]$	Oxygen concentration	$[mol \cdot m^{-3}]$
OUR	Volumetric oxygen uptake rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
S_{O_2}	Solubility of oxygen in cultivation medium	$[mol \cdot m^{-3}]$
t	Time	$[s]$
V	Volume	$[m^{-3}]$
ϕ	Liquid flow rate	$[m^3 \cdot s^{-1}]$

Subscripts

0	Zero
CO_2	Carbon dioxide
G	Gas phase
in	Incoming
L	Liquid phase
out	Outgoing
O_2	Oxygen
t	Time

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CHAPTER 6

APPLICATION OF SOFTWARE SENSORS FOR THE CONTROL OF GLUCOSE AND GLUTAMINE IN VERO-CELL CULTIVATIONS

ABSTRACT

This paper presents software sensors for the estimation of biomass activity and of glucose, glutamine, lactic acid and ammonia concentrations. The software sensors have been used to control glucose and glutamine levels in batch cultivations of Vero cells. The biomass activity sensor could be used to detect nutrient limitations instantaneously. Application of the software sensors, in combination with off-line control of the concentrations of methionine and serine, resulted in a doubling of the cell density. The total ammonium production could be reduced by 62%.

INTRODUCTION

The control of substrate levels plays an important role in the optimization of animal-cell cultivations. Glutamine and glucose levels determine the production rates of ammonium and lactic acid[6,10,11,14,15,20,26]. Since the latter can inhibit cell growth[10,13,16,19], and ammonium also can inhibit virus production[4,7], lowering the production rate of these metabolites will probably enhance both cell growth and virus production. Glacken et al.[6] showed for Madin-Darby canine kidney cells (MDCK) that the ammonium production could be reduced by controlling the glutamine concentration at a fixed low level. For human diploid foreskin cells (FS-4), they showed that the lactic-acid production could be reduced by controlling the glucose concentration at a fixed low level. Xie and Wang[26] showed the same effect for hybridoma cells and in addition to this, they showed that their feeding strategy resulted in a higher cell density and a higher monoclonal-antibody production.

To develop a feeding strategy for important nutrients, on-line measurement

methods for these nutrients have to be available. Due to the sterile barrier, on-line analysis of the cultivation medium is difficult to accomplish. Methods have been developed to overcome this problem such as on-line HPLC, flow injection analysis and biosensors that are often placed outside the sterile barrier[1,5,22,25]. Alternatively, software sensors can be used to predict and control the substrate concentrations on-line. In general, software sensors are models, either based on neural networks, fuzzy logic, or on mass balances and kinetic models in combination with Kalman filtering techniques[12,17,18,26,27].

This paper presents software sensors for the estimation of biomass activity and of glucose, glutamine, lactic acid and ammonia concentrations. These software sensors can be divided into two categories. The first is based on physiological properties of the cells. Since it requires only the oxygen uptake rate and the lactic-acid production rate, no additional model parameters have to be determined. Biomass activity and both the glucose and the lactic-acid concentration are estimated this way. The second type assumes a link between glycolysis and glutamine utilization[8,21,28,29]. Using this assumption, both the concentrations of glutamine and ammonium can be estimated from the lactic-acid production.

The software sensors were used to determine the effect of controlled addition of glucose and glutamine on the production of lactic acid and ammonium during batch cultivations of Vero cells. Furthermore, using off-line amino-acid analysis in combination with information about the biomass activity, limitation of other nutrients was avoided. The combination of these software-sensor applications, resulted in a doubling of the cell density and a reduction of the ammonium concentration of 62%.

MATERIALS AND METHODS

For the experiments, a $6 \cdot 10^{-3}$ m^3 bioreactor was used, containing $3 \cdot 10^{-3}$ m^3 cultivation medium. The medium was stirred with a four-bladed marine impeller (diameter 0.1 m). A pH electrode, a polarographic dissolved-oxygen probe (Ingold, Urdorf, Switzerland) and a Pt100 platinum resistance temperature detector (SDL, Southport, England) were used to measure the conditions inside the bioreactor. These conditions were controlled with a CF500 measurement and control unit (Applikon Dependable Instruments, Schiedam, The Netherlands). The software of the CF500 was supplemented with the software-sensor algorithms for glucose, glutamine, ammonium, lactic acid and biomass activity. The amount of sodium hydroxide added (0.3 M, Merck, Darmstadt, Germany) was monitored with a balance (4800P; Sartorius GMBH, Göttingen, Germany) coupled to the CF500 measurement and control unit through RS232. The cultivation conditions inside the bioreactor were kept constant at a temperature of 37 °C, a pH of 7.20, a dissolved oxygen concentration of 50% air saturation and a stirrer speed of 50 rpm.

Off-line analysis techniques

Amino acids were determined on a HPLC system (Waters, Division of Millipore, Milford, MA, USA). The amino acids were separated with a 150 x 3.9 mm. I.D. 85711 Waters Resolve 5 μ spherical C-18 column, using o-phthalaldehyde with 2-mercaptoethanol as derivatizing agent[3]. Lactic acid and glucose were determined using a YSI 2300 STAT analyzer (Yellow Springs Instruments, Ohio, USA). The cell density was determined by counting their nuclei after treatment with crystal violet (1 g/l in 0.1 M citric acid; SVM, Bilthoven, The Netherlands).

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Cultivation of Vero cells

Vero cells were cultivated in a batch culture, using MEM Hank's medium without sodium bicarbonate (Gibco BRL Life Technologies B.V., Breda, The Netherlands) but with 7.5% bovine serum (Bocknek Laboratories Inc., Canada), 2.5% foetal calf serum (Sanbio, Uden, The Netherlands), $0.5 - 4$ $mol \cdot m^{-3}$ L-glutamine, $0.25 - 10$ $mol \cdot m^{-3}$ glucose and 1% antibiotics (SVM, Bilthoven, The Netherlands). The cells were grown on 2 $kg \cdot m^{-3}$ Cytodex I microcarriers (Pharmacia, Uppsala, Sweden). The cell attachment on the microcarriers took place in a spinner flask containing MEM Hank's medium supplemented with sodium bicarbonate. This was done because the cell attachment in bicarbonate-free medium is poor. The cells were allowed to attach for five hours, after which the medium was replaced by medium without sodium bicarbonate and the bioreactor was inoculated. This startup method was used to inoculate the bioreactor in a reproducible way, which was important to allow comparison of the different cultivations.

Estimation of the biomass activity

The biomass activity, defined as volumetric ATP-production rate or volumetric energy-production rate (EPR), was estimated from the volumetric oxygen uptake rate (OUR) and the volumetric lactic-acid production rate (LPR), using the equation derived by Glacken et al.[6]:

$$EPR = 6 \ OUR + LPR \qquad (1)$$

This software sensor has been evaluated extensively in a previous paper[2].

The biomass activity was shown to be an important tool for the detection of

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nutrient limitations in Vero-cell batch cultures. When a limitation occurs, the biomass activity stabilizes instantaneously whereas the biomass concentration continues to increase[2].

Estimation of the lactic-acid concentration

The lactic-acid concentration was determined from the volumetric lactic-acid production rate (LPR) and the initial lactic-acid concentration. The LPR was estimated assuming that the only acidifying product excreted by the cells is lactic acid[2]. With this assumption, the amount of sodium hydroxide required to control the pH at a fixed level equalled the amount of lactic acid produced. Results using this software sensor are presented in a previous paper[2]. In that paper it is shown that the assumption is valid until nutrient limitations occur. After that, the estimated lactic-acid concentration is too high.

Estimation of the glucose concentration

If it is assumed that the glycolysis is solely responsible for both the oxygen consumption and the lactic-acid production, the volumetric glucose uptake rate (GlcUR) can be calculated from the OUR and the LPR. Glucose can be converted to carbon dioxide and to lactic acid:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$
$$C_6H_{12}O_6 \rightarrow 2\ C_3H_6O_3$$
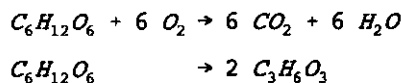
In the first case, 6 oxygen molecules are consumed for 1 glucose molecule,

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whereas in the second case two lactic-acid molecules are produced from 1 glucose molecule. Therefore, the total GlcUR can be described by:

$$GlcUR = \frac{1}{6} \ OUR + \frac{1}{2} \ LPR \tag{2}$$

RESULTS AND DISCUSSION

Determination of the decomposition rate of glutamine

Glutamine spontaneously decomposes into ammonium and pyrrolidone-carboxylic acid. To determine the true glutamine uptake rate and ammonium production rate of the cells, these values have to be corrected for this decomposition. Literature[6,9,23,24] provides a wide range of decomposition rates, varying between $1.4 \cdot 10^{-7}$ and $14 \cdot 10^{-7}$ s^{-1}. This indicates that environmental conditions like temperature, serum concentration and pH affect the decomposition rate of glutamine significantly. To obtain the decomposition rate of glutamine in our case, the glutamine concentration in fully prepared cultivation medium was monitored over a period of ten days using HPLC. During this period of time, the medium was kept at standard cultivation conditions (pH = 7.2, T = 37°C, 10% serum). The decomposition rate of glutamine in cultivation medium at cultivation conditions, was $8.14 \cdot 10^{-7}$ s^{-1}. This value is comparable to the values found in literature.

Table I. Comparison of glucose-controlled cultivations of Vero cells during the exponential growth phase.

Exp.	μ_{max}	$[cell]_{max}$	$[Glc]$	$[Gln]_{init}$	$[Lac]_{prod.}$	$[NH_4^+]_{prod.}$	$\bar{q}_{glc}$	$\bar{q}_{gln}$[2]	$\bar{q}_{lac}$	$\bar{q}_{amm}$[2]
	$[10^{-6}\,s^{-1}]$	$[10^{12}\,cells \cdot m^{-3}]$			$[mol \cdot m^{-3}]$			$[10^{-17}\,mol \cdot cell^{-1} \cdot s^{-1}]$		
1	4.78	1.71	NC[1]	2	14.7	1.37	-5.0	-0.68	7.4	0.65
2	4.59	1.72	10	2	11.1	1.58	-3.5	-0.77	5.8	0.57
3	4.55	1.76	5	2	15.2	1.16	-4.8	-0.69	7.9	0.50
4	5.40	2.11	2	4	16.8	2.27	-4.6	-0.94	7.7	0.36
5	5.72	1.98	1	4	17.0	2.40	-4.9	-0.73	7.2	0.40
6	5.20	2.03	0.5	4	18.7	2.47	-3.9	-0.85	7.1	0.54
7	5.92	2.22	0.25	4	17.0	2.71	-4.1	-1.07	7.5	0.62

[1] NC = No Control, Initial $[Glc] = 10\ mol \cdot m^{-3}$

[2] Corrected for glutamine decomposition

Control of glucose level

The glucose software sensor was tested in six batch cultivations of Vero cells. For that, the glucose concentration was controlled at fixed levels between 0.25 and 10 mol·m^{-3}. Results of these cultivations are shown in Table I, experiments 2-7. Experiment 1 is a reference culture, in which neither glucose nor glutamine was controlled. The initial glucose concentration of this reference culture was 10 mol·m^{-3}. In Table I the maximum growth rate (μ_{max}), the maximum cell density, the total amount of lactic-acid and ammonium produced and the average specific production rates during the exponential growth phase ($\bar{q}$) of glucose, glutamine, lactic acid and ammonium are presented. Both $\bar{q}_{gln}$ and $\bar{q}_{amm}$ have been corrected for glutamine decomposition.

As stated in the introduction, lactic acid is a known inhibitor for the growth of animal cells. A successful way to minimize lactic-acid production, is growing animal cells at a fixed low glucose concentration[6,11,15,26]. For hybridoma cells, this approach has led to a reduction of the lactic-acid production up to 26 times, compared to a conventional batch cultivation[26]. Therefore, it is to be expected that for Vero cells this approach will also lead to a reduction of the lactic-acid production. Table I shows that this is not the case. Reduction of the glucose concentration down to 0.25 mol·m^{-3} does not result in a decrease in lactic-acid production in our case. This means that glucose is still present in excess at a concentration of 0.25 mol·m^{-3}. For Vero cells, the Monod constant for glucose must therefore be much lower than 0.25 mol·m^{-3}. Determination of the Monod constant would therefore require that the cultivation is done at glucose concentrations of 0.1 mol·m^{-3} or less. This means that the glucose concentration should be controlled with an accuracy of 0.01 mol·m^{-3}. Since this software sensor is not accurate enough to establish this, accurate determination of the Monod

constant of glucose for Vero cells was not possible.

Verification of the glucose software sensor

As glutamine utilization also results in lactic-acid production and oxygen consumption, the maximum error in the GlcUR depends on the glutamine uptake rate of the cells. This error in the GlcUR can be calculated if the conversion rates for glucose and glutamine are known. These conversion rates are not known for Vero cells. Since determination of these rates would require extensive research, based on analysis of radioactive labeled carbon molecules from glucose and glutamine, results from other cell types have to be used to estimate the error in the GlcUR. Reitzer et al.[21] have determined conversion rates for glucose and glutamine into lactic acid and carbon dioxide for HeLa cells. Like Vero cells, these cells are also anchorage dependent. Furthermore, since both are continuous cell lines originating from carcinomas of primates, their origin is comparable. Reitzer et al.[21] showed that HeLa cells convert 80% of the glucose consumed to lactic acid, and 7-10% to carbon dioxide. For glutamine these values are 13% and 35% respectively. If it is assumed that for each carbon-dioxide molecule produced, one oxygen molecule is consumed, the oxygen consumption is also known. Table I shows that glucose is consumed at least 4.5 times faster than glutamine ($\bar{q}_{glc}/\bar{q}_{gln}$). From this table it can also be derived that, if it is assumed that all of the lactic acid originates from glucose, approximately 88% of the glucose is converted into lactic acid ($\frac{1}{2}\bar{q}_{lac}/\bar{q}_{glc}$). This value is comparable to the results found for HeLa cells[21]. Applying the data of Reitzer et al.[21] to equation (2), a maximum error for the GlcUR of 9% is obtained. Since they claim that glutamine is the main energy source of the
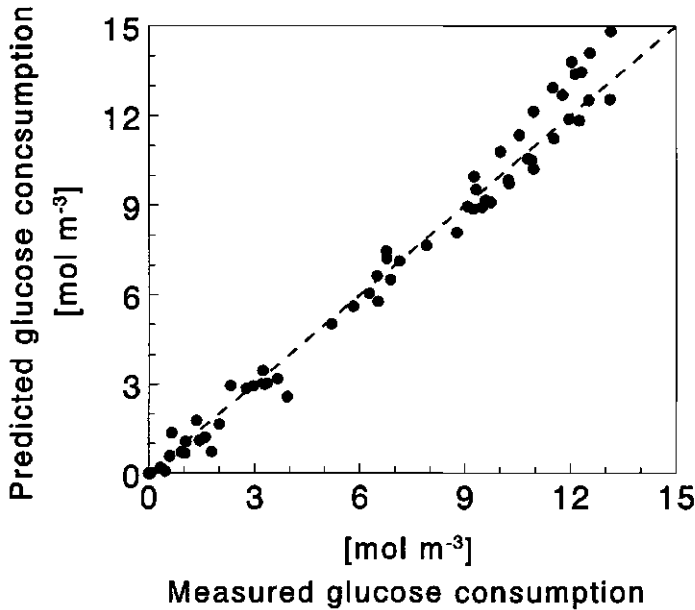
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Figure 1: Relationship between measured and estimated glucose consumption during glucose-controlled Vero-cell batch cultures (cultures 2 - 7).

cells, it is not likely that the contribution of glutamine in the GlcUR is underestimated.

For cultures 2 to 7, Figure 1 shows the relationship between measured and estimated amount of glucose consumed. It shows that the software sensor provides a good estimate of the glucose consumption, independent of the glucose concentration. During the cultivations presented in Table I, the overall error in the estimation of the glucose uptake rate was 6%. This is less than the maximum error of 9% that was expected. Therefore, in our case, the assumptions made to develop the glucose sensor are likely to be valid.
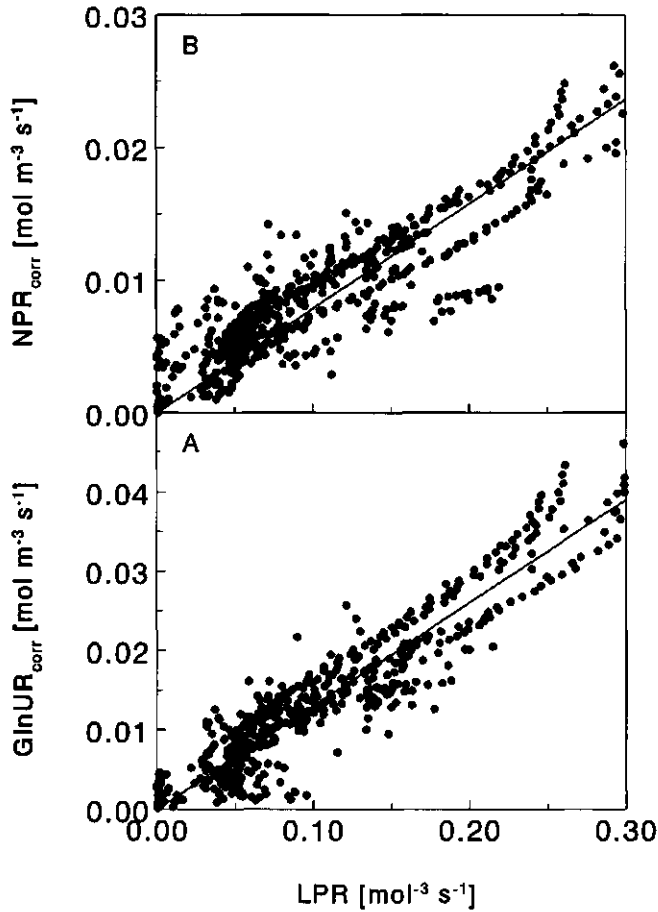
Figure 2: Relationship between the corrected glutamine consumption rate (GlnUR$_{corr}$) and the lactic-acid production rate (LPR) (part A) and between the corrected ammonium production rate (NPR$_{corr}$) and the lactic-acid production rate (LPR) (part B)

Estimation of the concentration of glutamine and ammonium

In several papers, a link is suggested between the glycolysis and the glutamine utilization[8,21,28,29]. If this link exists, the metabolic activity of glutamine and ammonium can be predicted from the LPR. This has been verified during the

exponential growth phase of the seven batch cultivations of Vero cells presented in Table I. To obtain the true relationship between the LPR, the glutamine uptake rate and the ammonium production rate, first the volumetric glutamine uptake rate (GlnUR) and the volumetric ammonium production rate (NPR) had to be corrected for the spontaneous decomposition of glutamine. Figure 2 shows the relationships between both $GlnUR_{corr}$ and NPR_{corr} and the LPR. As can be seen, both correlated well with the LPR ($r = 0.94$ for $GlnUR_{corr}$ vs. LPR and $r = 0.92$ for NPR_{corr} vs. LPR). During the exponential growth phase, the GlnUR and the NPR can therefore be calculated using:

$$GlnUR = 0.130 \ LPR + 8.14 \cdot 10^{-7} \ [Gln] \tag{3}$$

$$NPR = 0.0787 \ LPR + 8.14 \cdot 10^{-7} \ [Gln] \tag{4}$$

Control of glucose- and glutamine levels

To test the glutamine software sensor, two batch cultures have been executed in which both glucose and glutamine were controlled at fixed levels. The glucose concentration was controlled at 2 mol·m^{-3} in both cases. Glutamine was controlled at 4 and 0.5 mol·m^{-3} respectively. However, since the initial glutamine concentration of the basal cultivation medium was 2 mol·m^{-3}, during the first part of the cultivation the glutamine concentration was higher than 0.5 mol·m^{-3}. Results of these cultures are presented in Figure 3 and in Table II, cultures 8 and 9. Again, culture 1 is the reference culture in which neither glucose nor glutamine is

Table II: Comparison of glucose and glutamine controlled cultivations of Vero cells during the exponential growth phase.

Exp.	μ_{max}	$[cell]_{max}$	[Glc]	[Gln]	$[Lac]_{prod.}$	$[NH_4^+]_{prod.}$	$\dot{q}_{glc}$	$\dot{q}_{gln}^2$	$\dot{q}_{lac}$	$\dot{q}_{amm}^2$
	$[10^{-6}\,s^{-1}]$	$[10^{12}\,cells \cdot m^{-3}]$			$[mol \cdot m^{-3}]$			$[10^{-17}\,mol \cdot cell^{-1} \cdot s^{-1}]$		
1	4.78	1.71	NC^1	NC^1	14.7	1.37	-5.0	-0.68	7.4	0.65
8	5.04	2.12	2	4	17.7	6.48	-6.2	-0.90	8.1	0.72
9	5.58	2.18	2	0.5	14.7	2.44	-5.2	-0.61	6.9	0.45

[1] NC = No Control, Initial [Glc] = 10 mol·m^-3, initial [Gln] = 4 mol·m^-3
[2] Corrected for glutamine decomposition
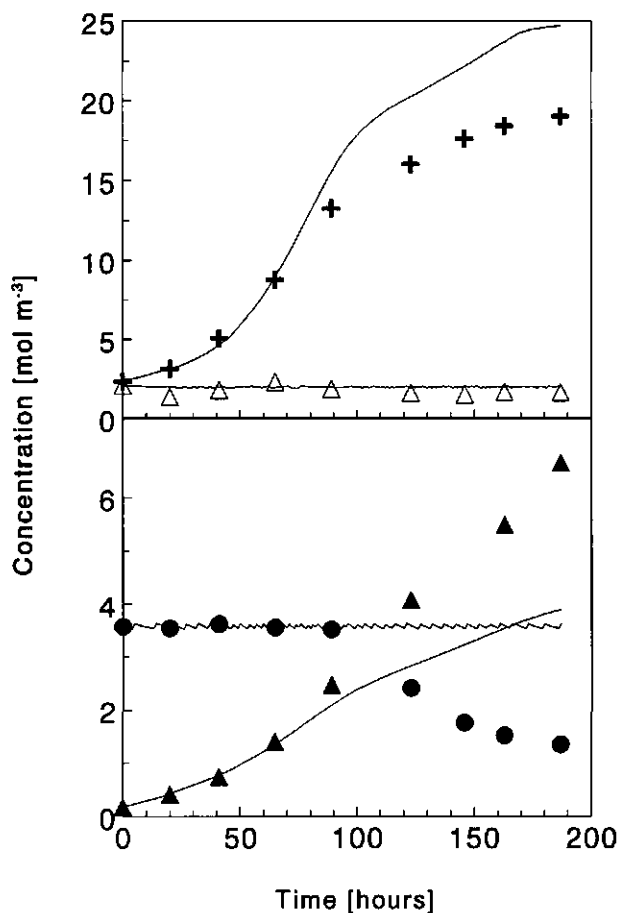
Figure 3: Cultivation 8. Glucose (2 mol·m^{-3}) and glutamine (4 mol·m^{-3}) controlled culture of Vero cells. -●- Glutamine, -▲- Ammonium, -△- Glucose, -+-Lactic acid.

controlled. Figure 3 shows the course of both the measured and the estimated concentrations of glucose, glutamine, lactic acid and ammonia of cultivation 8. During the exponential growth phase, all concentrations are estimated well. After that time, only the glucose software sensor produces reliable estimates. The glutamine consumption rate and the ammonium production rate are both underestimated, whereas the lactic acid production rate is overestimated. Since

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both the glutamine and the ammonium software sensor have been developed for the exponential growth phase only, it is hardly a surprise that these sensors do not function properly after that period. Obviously some kind of limitation occurred, forcing the cells to consume relatively more glutamine, thereby producing more ammonium. This change in metabolism is not accounted for in both software sensors.

Next to lactic acid, also ammonium is a known inhibitor. Animal cells are even more susceptible to ammonium than to lactic acid. To minimize ammonium production, in literature the same strategy is used as for minimizing the lactic acid production[6,10,14,26]. Lowering the ammonium production by controlling the glutamine concentration at a low level, will always result in a decrease of the ammonium production, since the decomposition rate of glutamine depends on the glutamine concentration. However, next to this decrease in glutamine decomposition, also the specific ammonium production is expected to decrease because of a more efficient utilization of glutamine. This hypothesis was verified for cultures 8 and 9. Indeed, a distinct difference is observed between the amount of ammonium produced in both cases (Table II). Both the $\bar{q}_{gln}$ and the $\bar{q}_{amm}$ increase with increasing concentration of glutamine, confirming the hypothesis. So, applying a controlled, low glutamine level will lead to a significant drop in total ammonium production since both the ammonium excreted by the cells and the ammonium resulting from glutamine decomposition decrease.
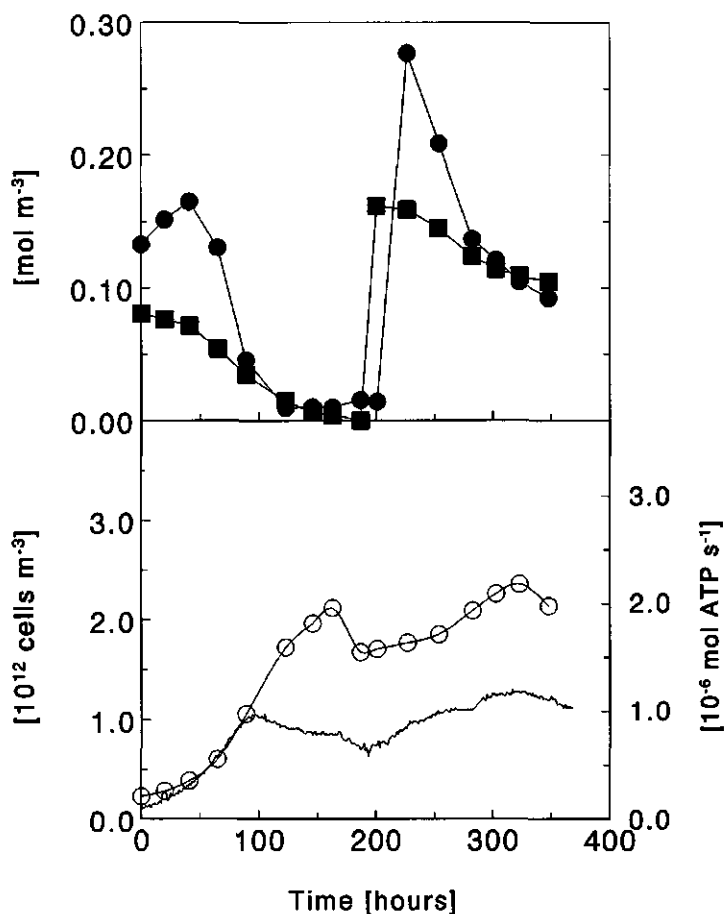
Figure 4: Cultivation 8. Glucose (2 mol·m^{-3}) and glutamine (4 mol·m^{-3}) controlled culture of Vero cells. In the second part, successively methionine and serine were added. -●- Serine, -■- Methionine, -○- Cell density, — Biomass activity.

Effect of addition of limiting nutrients

Figure 3 shows that after approximately 100 hours of cultivation, a sudden change in the cell behaviour occurs. This is probably due to a limitation of one or more amino acids. To determine the primary limiting components, the amino-acid

composition of batch cultures 8 and 9 was determined daily using off-line HPLC. Figure 4 shows the results of cultivation 8. At the moment that the growth rate starts to decrease and the biomass activity stabilizes, two amino acids are exhausted, serine and methionine. To test if these were indeed the growth limiting components, they were successively added to the cultivation medium at the end of the cultivation. First, methionine was added. This immediately resulted in an increase in biomass activity. This increase was even accelerated when serine was added the next day. Also the cell density increased from the moment that methionine was added, although the final cell density was only 11% higher than the cell density reached in the first part of the cultivation. This cultivation showed that exhaustion of methionine, one of the essential amino acids, is indeed causing the growth rate to decrease. However, it still is unclear if limitation of serine, not an essential amino acid, also contributed to this decrease. Hence, at the end of cultivation 9, the experiment was repeated. This time however, the amino acids were added in reverse order. The results of this experiment are shown in Figure 5. The addition of serine after 170 hours did not result in the expected recovery of the cultivation. Neither the biomass activity, nor the growth rate responded to the serine addition. Contradictory to this, addition of methionine the next day immediately resulted in an increase of the biomass activity. This time, the increase was even more pronounced. The final cell density reached after addition of the amino acids was 33% higher than the first maximum of this cultivation. The difference in cell density between culture 8 and 9 can be explained by the ammonium formation. In cultivation 8, the maximum ammonium concentration was 10.6 mol·m^{-3}, compared to 4.9 mol·m^{-3} in cultivation 9. Since ammonium is a known growth inhibitor[6,10,14,26], lowering the ammonium concentration will lead to a higher growth rate.
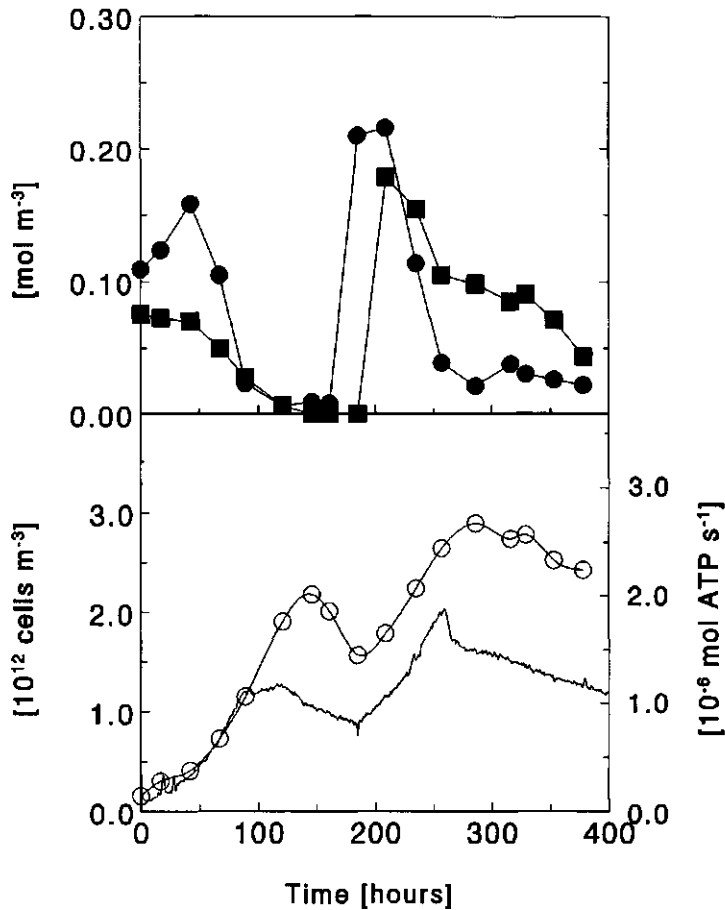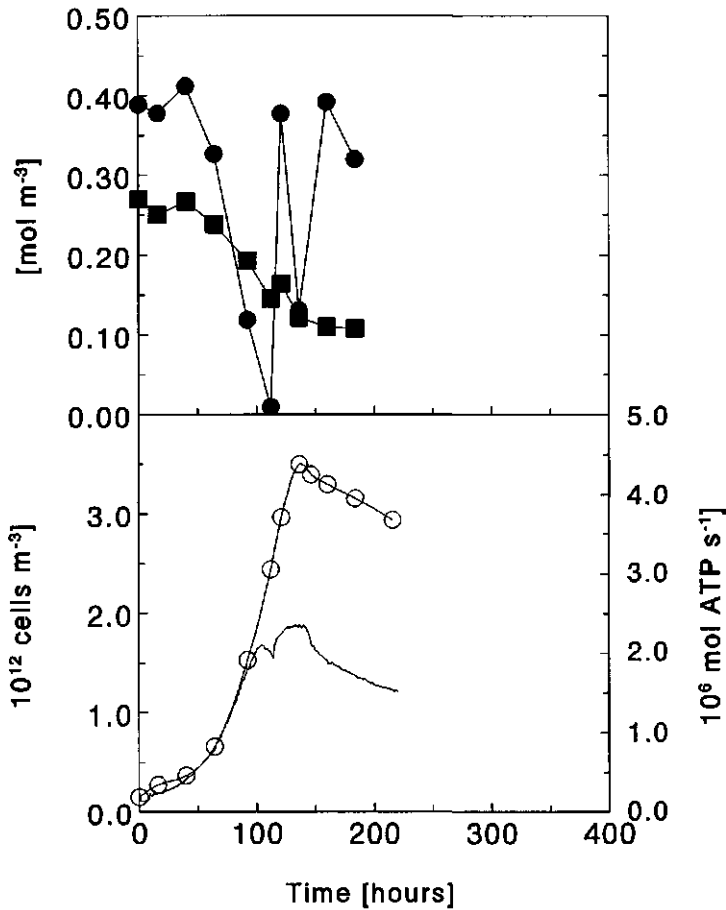
Figure 5: Cultivation 9. Glucose (2 mol·m^{-3}) and glutamine (0.5 mol·m^{-3}) controlled culture of Vero cells. In the second part, successively serine and methionine were added. -●- Serine, -■- Methionine, -○- Cell density, — Biomass activity.

To optimize the batch culture, a final experiment was executed in which the initial cultivation medium was supplemented with both serine and methionine. The other cultivation conditions were kept identical to culture 9. Figure 6 shows the results of culture 10. It shows that, in spite of the additional serine added to the cultivation medium, serine is depleted after 110 hours. The biomass activity

Figure 6: Cultivation 10. Glucose (2 mol·m⁻³) and glutamine (0.5 mol·m⁻³) controlled culture of Vero cells. Both methionine and serine were supplemented to the cultivation medium at the start of the culture. -●- Serine, -■- Methionine, -○- Cell density, — Biomass activity.

momentarily stabilizes at that point. The addition of serine after 113 hours only results in a partial recovery of the biomass activity. In spite of this short serine limitation, the cell density increases up to $3.5 \cdot 10^{12}$ cells·m⁻³. This means that the cell density doubled compared to the reference culture.

CONCLUSIONS

During the exponential growth phase, it is possible to estimate the consumption rate of glutamine and the production rate of ammonium, and therefore the concentrations of glutamine and ammonium in the cultivation medium. Both rates can be estimated from the lactic-acid production rate, which is again estimated from the utilization rate of sodium hydroxide[2]. This lactic-acid production rate can also be used in combination with the oxygen uptake rate (OUR), to estimate the glucose consumption rate and the biomass activity. The OUR can be determined from parameters like gas flow, gas composition in the head space of the bioreactor and stirrer speed[2]. So, using equipment that is generally available and applying relatively simple assumptions, it is possible to obtain on-line information about the most important metabolites and nutrients. Furthermore, nutrient limitations are detected instantaneously by the biomass activity sensor. If a limitation is detected, off-line HPLC can be used to determine the limiting component. This approach has led to a doubling of the cell density compared to the reference culture. In addition to this, lowering the glutamine concentration resulted in a decrease in ammonium formation of 62%.

NOMENCLATURE

OUR	-	Volumetric oxygen uptake rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
LPR	-	Volumetric lactic-acid production rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
NPR	-	Volumetric ammonium production rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
EPR	-	Volumetric ATP production rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
GlnUR	-	Volumetric glutamine uptake rate	$[mol \cdot m^{-3} \cdot s^{-1}]$

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GlcUR	-	Volumetric glucose uptake rate	$[mol \cdot m^{-3} \cdot s^{-1}]$
$\bar{q}$	-	Average specific production rate	$[mol \cdot cell^{-1} \cdot s^{-1}]$
μ	-	Growth rate	$[s^{-1}]$
r	-	Correlation coefficient	[-]

Subscripts

corr	-	corrected for glutamine decomposition
Amm	-	Ammonium
Glc	-	Glucose
Gln	-	Glutamine
Lac	-	Lactic acid
Init	-	Initial concentration
Prod	-	Total produced during cultivation
Max	-	Maximum

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CHAPTER 7

CURRENT GOOD MANUFACTURING PRACTICE IN PLANT AUTOMATION OF BIOLOGICAL PRODUCTION PROCESSES

ABSTRACT

The production of biologicals is subject to strict governmental regulations. These are drawn up in current good manufacturing practices (cGMP), a.o. by the U.S. Food and Drug Administration. To implement cGMP in a production facility, plant automation becomes an essential tool. For this purpose Manufacturing Execution Systems (MES) have been developed that control all operations inside a production facility. The introduction of these recipe-driven control systems that follow ISA S88 standards for batch processes has made it possible to implement cGMP regulations in the control strategy of biological production processes. Next to this, an MES offers additional features such as stock management, planning and routing tools, process-dependent control, implementation of software sensors and predictive models, application of historical data and on-line statistical techniques for trend analysis and detection of instrumentation failures. This paper focuses on the development of new production strategies in which cGMP guidelines are an essential part.

INTRODUCTION

For many years, biologicals are produced in bioreactor systems. From bioreactor point of view, no major differences can be observed between the present production processes and the way the biologicals were produced thirty years ago. However, in the control of these production processes, major improvements have been achieved. The introduction of computerized measurement and control units has made it possible to apply on-line modeling of the production process, thereby improving both the yield and the consistency of the process. Recently,

developments in the measurement and control focus on recipe-driven control systems[3,25,46]. These enable the development of tailor-made control strategies, allowing current good manufacturing practice (cGMP) rules to be implemented. Already in 1985 the introduction of recipe-driven control systems which were allowed to make decisions concerning plant operation was predicted[14]. Although it has been technically feasible for years, this type of control systems still is a scarce phenomenon. In the past five years, especially Locher *et al.* have paid much attention to advanced bioprocess automation[24-28]. Their research clearly showed that the reproducibility of bioprocesses is mainly dependent on the reproducibility of the environmental conditions for the cells and, hence, on the quality of the equipment[26]. They showed that ruling out operator actions by means of extensive automation resulted in identical batch cultures of *Saccharomyces cerevisiae*. The need for identical cultivation conditions is again emphasized by Kell and Sonnleitner[18]. They claim that 'one should not aim to seek improvements by constructing a predictor-controller to cope with fuzzy biology. Rather an appropriate Standard Operating Procedure (SOP) should be devised, or better equipment used'.

With the increasing governmental regulations, it has become more interesting to develop recipe-driven control systems. This paper presents the most recent advancements in the development of new production strategies for biological production processes in which cGMP guidelines are an essential part.

INTRODUCTION OF CURRENT GOOD MANUFACTURING PRACTICE IN CONTROL SYSTEMS

Biological production processes are obliged to obey to strict governmental regulations. Amongst others, the U.S. Food and Drug Administration has designed

the rules for cGMP[11]. These include requirements related to the methods used in, and the facilities and controls used for, design, purchasing, manufacture, packaging, labeling, storage, installation, and servicing of all finished products intended for human use. As can be seen, these rules cover a broad range of operations that are directly or indirectly linked to the production process. Amongst these rules, validation of the production process and equipment as well as the use of SOPs are essential. SOPs prescribe in detail how the production process has to be executed. Not only the bioreactor process, but all of the facilities used are subjected to the cGMP rules. This implies a huge amount of registration activities and therefore a lot of additional work. Fortunately, computers can take over most of these tasks. This is done in so-called Manufacturing Execution Systems (MES). These are dedicated systems that allow one to monitor and control all product and information flows, the production environment and all production processes in the facility. In case of batch production processes, an MES is generally built according to the ISA S88 standard for batch processes[16,46]. This standard defines reference models for batch control as used in the process industries, and terminology that helps explain the relationships between those models and terms. This software standard was developed in conjunction with IEC and ISO standards groups. Figure 1 shows a typical plant structure with the MES operating as the heart of the plant. It is clear that the MES operates on four levels. The production management is provided with tools for planning and routing of the production process, and is able to extract real-time and historical data from the database in order to generate batch records, (semi) annual reports and quality control charts. On the level of plant operation, MES controls humidity, pressure household and temperature inside the production facility. Additionally, all incoming and outgoing stocks are registered and the inventory of the storehouse is kept up to the mark. On the process floor, MES controls all processes, varying from the bioreactor production process to

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cGMP in plant automation of biological production processes
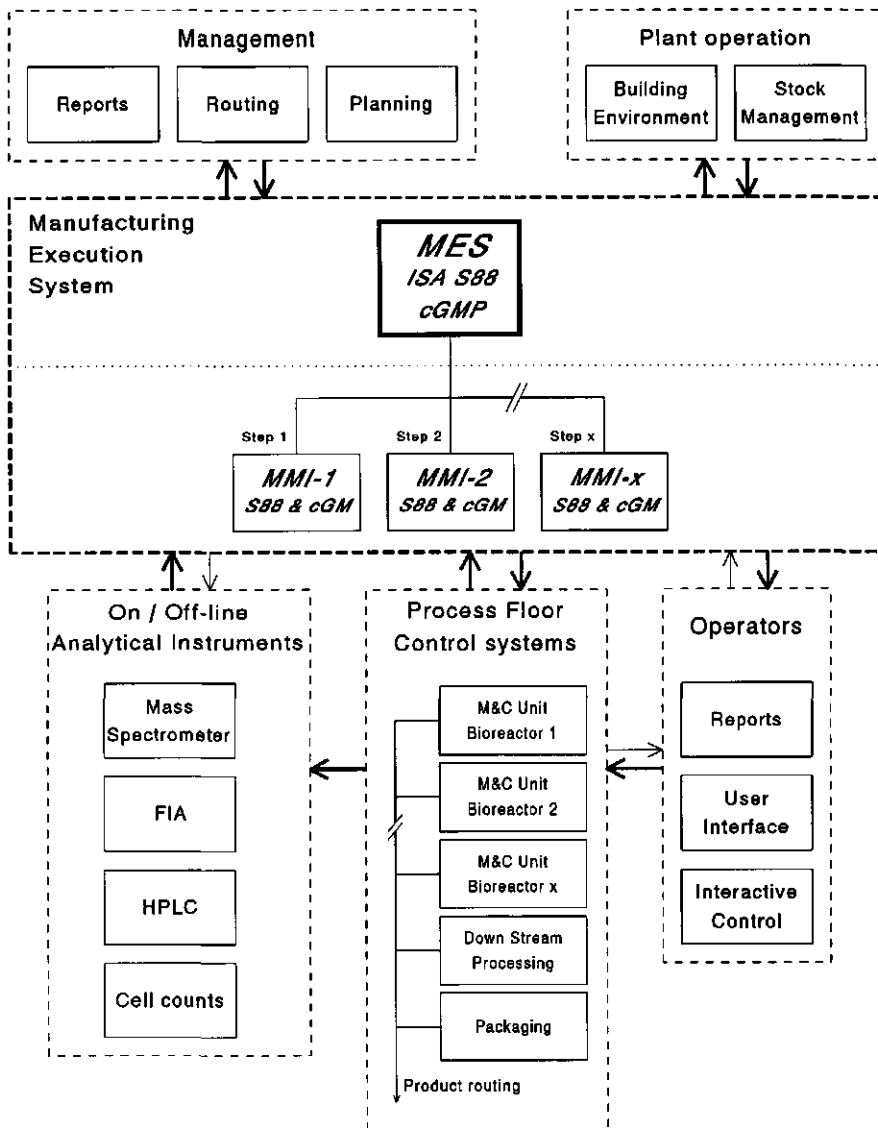
Figure 1: Typical plant design with the Manufacturing Execution System serving as the heart of the production facility. (cGMP = current Good Manufacturing Practice; (ISA) S88 = Software standard for batch processes; MES = Management Execution System; MMI = Man Machine Interface; M&C = Measurement and Control).

downstream processing and packaging. Depending on the integration level, these processes are either controlled by a general MES, or by Man Machine Interfaces (MMI) that are directly linked to an MES. For all process equipment, log-books and calibration reports are archived. As soon as a new production run is started, the reliability of the equipment to be used is verified using these data. The operator uses the MES to generate daily reports, to look at real-time data, and to operate the measurement and control unit. The latter is done using a graphical user interface that interacts with the operator by means of instructions and questions. Finally, the MES collects and if necessary verifies all data generated by on- and off-line analytical equipment. If required, the MES can adjust the production process based on the outcome of these analyses.

An important part of the production process of most biologicals is done in bioreactors. Since the measurement and control unit that is attached to the bioreactor controls every action regarding the bioreactor, it makes sense to implement the SOPs concerning this part of the process in the software that controls the measurement and control unit. This way it is assured that all instructions described in the SOP are carried out. It can be verified whether or not a production run is done according to the SOP by logging all operator actions by the computer. Additionally, the data obtained by the measurement and control unit has an increased value, since each dataset is derived under identical conditions. Comparison of different batches becomes relatively easy this way. Finally, the use of SOPs ensures that all procedures are executed identically. This way highly consistent production results can be obtained that mainly depend on the quality of the raw materials. Therefore the batch-to-batch variation caused by small variations in the operations during the production period will be minimized. This is clearly shown in a paper by Locher *et al.*[26]. They show that in six repetitive batch cultures of *Saccharomyces cerevisiae* differences in batch cultivations with respect to

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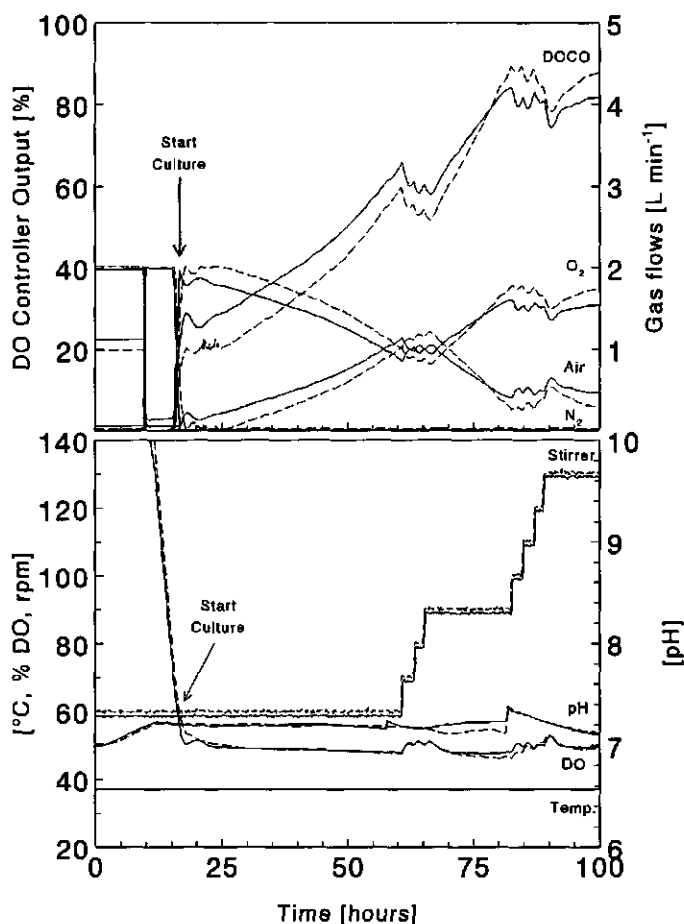
Figure 2: Example of two simultaneously performed batch cultures of Vero cells that followed the same recipe. The inoculum of both cultures originated from the same primary Vero-cell culture. The cultures were performed in two different bioreactors, using two different measurement and control units.

duration and amplitudes of CO_2 did not exceed 5%. The main reason for this small deviation could be found in the irreproducible way the amount of inoculum was weighed. Figure 2 shows the results of two Vero-cell batch cultures, run simultaneously in different cultivation units. Both were cultivated according to the

same recipe. The inoculum originated from a primary batch culture of Vero cells. During both cultivations, operator actions like sampling were still done. This figure shows that, although the cultures were executed in two different cultivation units, their characteristics are virtually identical. This concerns not only the controlled parameters such as pH, DO (dissolved oxygen) and stirrer speed. Even the controller action of the dissolved-oxygen controller (DOCO) that is required to maintain setpoint is almost identical.

THE MAN-MACHINE INTERFACE

MMI can be seen as the interface that translates existing SOPs into instructions for both the measurement and control unit and the operator. It is either embedded or directly linked to an MES, or it operates stand alone. Just as an MES, an MMI-based system is built according to ISA S88 standards for batch processes[16,46]. This enables the validation of the software code, including that of the implemented features (software sensors, event-based control,..). The basis is formed by the Recipe. A schematic representation of the linkage between the different levels of the Recipe is presented in Figure 3. It shows that a recipe consists of four different parts. The Header contains information about the authors, the tags used, the history of modifications and the version number. The Procedure contains the control algorithms, the software code for the different production phases, and the safety interlock system. The latter prevents the occurrence of hardware conflicts such as simultaneous heating and cooling of the bioreactor. Formula is used to store process constants like setpoints of controllers and trigger values for certain
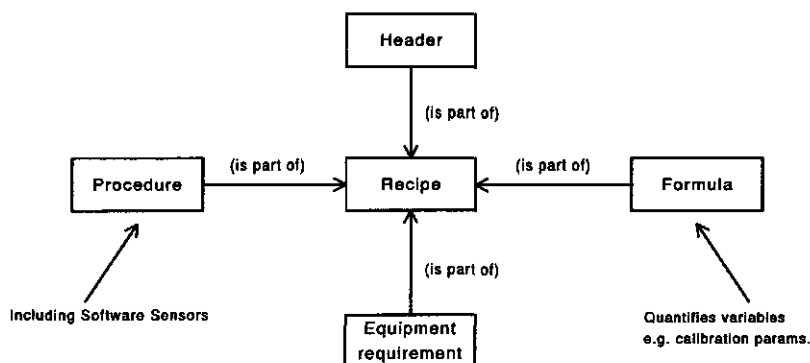
Figure 3: Internal structure of ISA S88-based control systems.

event-based procedures such as taking a sample every 5 hours. The Equipment requirement contains information about the hardware components. For a bioreactor system, this includes the measurement and control unit and the service unit (water and steam supply). In the Recipe all parts are combined to form a complete set of information that specifies the control requirements for manufacturing a batch.

Recipes can be built that focus on specific steps of the production process. Therefore, in each production step, the response of the measurement and control unit can be adapted to the current needs. For example, the temperature inside the bioreactor can be controlled at a fixed value during the cultivation procedure, whereas during the inactivation of the product, the temperature is forced to follow a predefined profile. MMI can also be given the authority to decide at what point actions have to be taken. The cultivation process can for instance be stopped as soon as the biomass activity starts to decrease[12]. This ensures identical cell conditions at the end of each cultivation. Depending on the time at which the culture ends (e.g. midnight), MMI then decides if the harvest has to be cooled until

the next day, or if an operator has to be warned that the cultivation has ended. In fully automized production facilities, the harvest will be pumped to a clarification or a concentration unit, where the next step in the production procedure will be started automatically. From cGMP point of view, MMI-based control systems offer increased control over the operator actions. It is for instance possible to command an operator to finish a complete calibration sequence before the next step of the process is executed. On the other hand, MMI can provide assistance by informing the operator about the actions that have to be taken during important operations. Additionally, the presence of different access levels enables decisions to be made at the right level of authority. An operator is only allowed to strictly follow the recipe, whereas the production manager is allowed to change setpoints, start and stop controllers, design his own recipes, etcetera.

DATA HANDLING IN ISA S88-BASED MMI-SYSTEMS

The use of MMI-based control systems enables the use of sophisticated features. For instance software sensors and advanced stat-based control strategies can be implemented easily. Stats, such as pH-stats or chemostats, combine control and physiological parameters to regulate the feed rate of substrates in fed-batch bioreactors or the dilution rate in chemostats. Software sensors are models, implemented in software of the MMI, that use information already available to predict parameters that are not measured on-line[5]. This information generally consists of common parameters like stirrer speed, dissolved-oxygen concentration and pH. The use of software sensors is far from a new idea. As early as in 1978 papers about software sensors have been published[49]. In literature different

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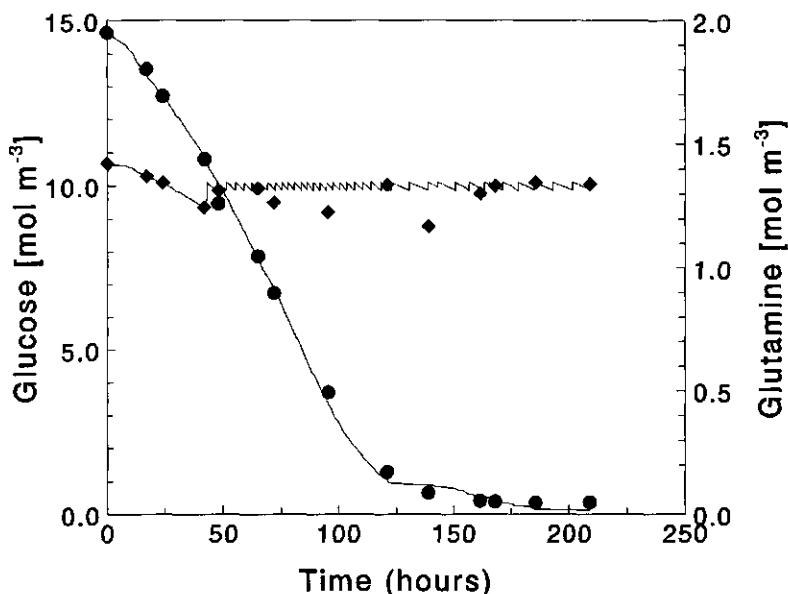
Figure 4: Example of a glucose-controlled batch culture of Vero cells [♦ Glucose; ● Glutamine].

applications of software sensors have been described. Roughly they can be subdivided into three categories. Software sensors based on metabolic activity are mostly based on the measurement of the oxygen uptake rate, carbon-dioxide evolution rate, acid production rate and redox potential[2,5,6,7,9,10,13,20,34,36,38,44,47,48]. Another type of software sensors uses artificial neural networks, fuzzy logic and knowledge-based supervision systems[8,19,22,30,32,35,41]. These do not require specific knowledge of the characteristics of the organism that is being cultivated. Instead, they rely on historical data of previous production runs and experience of process experts. Finally, software sensors have been developed based on component balancing and predictive models, often in combination with Extended Kalman Filtering techniques[1,4,23,33,37,40,42,43].

With the aid of these software sensors it is possible to estimate parameters

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like biomass concentration or activity, and the consumption and production rates of important metabolites like glucose, glutamine, ammonium and lactate on-line. Control algorithms based on these software sensors are possible too. Figure 4 shows an example of the course of glucose and glutamine during a glucose-controlled batch culture of Vero cells[7]. In this case, both glucose and glutamine have been estimated using software sensors based on metabolic activity. To estimate these concentrations, the dissolved-oxygen concentration, the stirrer speed, information from the mass-flow controllers and the base utilization were used as input parameters for the software sensors. All these parameters can easily be measured on-line, whereas both glucose and glutamine are very difficult to determine on-line. As can be seen, the glucose control is started after 50 hours of cultivation. In this case, the glucose is controlled using a peristaltic pump. Since the flow rate of such a pump is not very accurate, a small deviation occurs between measured and estimated glucose concentration. This example clearly shows the strength of software sensor applications in an MMI-based environment. Provided the knowledge about software sensors is available, it will take only a few days of programming to incorporate them into the MMI-software. Hence, without investments in analytical equipment, it is possible to extend the optimization possibilities significantly.

Looking at the amount of publications about software sensors, it is clear that a lot of research is being done in this field. The most important fields of interest are biomass determination, nutrient consumption, production of ammonium and lactate, and product formation. However, in addition to these common applications, software sensors are used for detection of contaminants in bioreactors[4] and for the automation of the *sake* brewing process[35]. Especially if software sensors are used for the optimization of complete production processes, this requires a software platform that is completely adjusted to the individual process steps. MMI-based

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systems then become an essential tool in the optimization process[22,46].

Next to software sensors, stat-systems can be implemented. These stats respond to changes in physical parameters like the oxygen uptake rate (DO-stat) or the acid production rate (pH-stat). If the value of such a parameter falls outside certain predefined limits, the feed rate is adjusted. This feed either consists of complete cultivation medium, or of one or more specific nutrients. Depending on the amount of knowledge about the physiology of the organism that is incorporated in the control system, stats can be developed that are either based on simple assumptions, or on physiological responses due to the complex interactions in the metabolic pathways. Especially stats for fed-batch systems rely on physiological knowledge to prevent waste of substrate and thereby the production of waste products that can be harmful to the organism. With this combination, a fed-batch cultivation with an optimal biomass or product yield is achieved. Several examples of stats are mentioned in literature. A DO-stat is used to control the acetic acid excretion by *Escherichia coli*[21,39]. By suppressing the acetic acid excretion the yield of recombinant human interleukin-2 was increased[39]. An example of a pH-stat is the ethanol production by a pH-mediated fermentation of *Zymomonas mobilis*[17]. When glucose is exhausted, the organism begins to metabolize amino acids. By changing its metabolism, the concentration of ammonia and as a consequence the pH increases. The increase of the pH-signal was converted in an increase of the feed rate of fresh medium. A more complex stat is used in the cultivation of *Alteromonas putrefaciens*[15]. In this stat, the yield of biomass on substrate was imposed on the culture. Fresh substrate was added when based on this yield and the biomass profile, all nutrients were theoretically spent. This stat resulted in an increase in biomass by a factor 1.5 and a reduction of the nutrient feed by a half.

In the development of both stat systems and software sensors and in the control of fermentation conditions, reliable sensor reading is a prerequisite. This

may seem trivial but up to now, only sensors which measure physical parameters like temperature, gas or liquid flows and pressure function highly dependable during the complete course of a fermentation. Basic chemical sensors like pH and DO sensors whose employment is widespread and which have been used for many years for the production of biologicals have to be treated with great care to obtain reliable values[29]. Problems with sensor reading can occur at several stages of the processing of the raw signal. The characteristics of the sensor (e.g. sensitivity to the parameter to be measured, sensitivity to other environmental conditions, signal to noise ratio, linearity), the electrical processing of the signal (wiring, electrical connections, grounding, AD/DA-conversion), the calibration, validation and maintenance of the sensor play an important part in obtaining sound data. The situation is even more troublesome with sensors which measure biologically relevant parameters such as biomass, nutrient and product concentration. These sensors are still very delicate (sensitive towards fouling and influenced by other chemical components) and not yet suited for exploitation in an industrial environment.

Besides the use of sophisticated software sensors and stats, historical data of previous production runs can be used to detect deviations in the characteristics of the current production process. This is possible since for each production run a complete batch record exists, containing culture conditions, operator actions and trends of all sensor signals. These data are accessible at any time during the production process. If compared to the historical data a deviation occurs, any predefined action can be triggered. Other deviations of the process characteristics such as a bacterial or a fungal infection can be detected in a very early stage too. This will save both time and production costs. Finally, it is possible to apply on-line statistics to the process signals. This can be used to detect failures in the measurement equipment[45] or deviations in the process characteristics. Especially

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in combination with historical data, on-line statistical analysis offers an important tool in the optimization of production processes.

CONCLUSIONS

The current trend for the production of biologicals is directed towards the automation of the complete production plant. This means that all operations and conditions inside the production facility are controlled by a Manufacturing Execution System (MES). Next to implementation of current good manufacturing practice (cGMP), an MES aids in increasing the reproducibility and consistency of the production process. To achieve this, standard operating procedures (SOPs) are implemented in the operator interface of the measurement and control systems for the different production steps. This interface is either the MES itself, or a Man-Machine Interface that is linked to an MES or functions stand alone. The incorporation of SOPs on the process floor ensures identical process conditions during all production runs. This is beneficial for both the production department and the research department. The production department benefits since the production process is executed in a very reproducible way, thereby obtaining consistent production results. This is essential for obtaining a production license. On the other hand, the research department is able to obtain large quantities of easily accessible process data that are obtained from identical processes. This enables a thorough analysis of the complete production process. This issue is often neglected but very important. Since all process steps interdepend, analysis and optimization of a production process should include the complete process and not just isolated steps like a fermentation or purification step.

A recipe-driven control system such as an MES or an MMI-based system that

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follows ISA S88 standards for batch processes is an important tool to accomplish the above. Next to highly consistent production runs, it offers additional features that will be useful in the optimization of the production process. The most important control features are process-dependent control, the use of software sensors, stats and predictive models, the application of historical data and on-line statistical techniques for trend analysis and detection of instrumentation failures.

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SUMMARY

In this thesis software sensors are introduced that predict the biomass activity and the concentrations of glucose, glutamine, lactic acid, and ammonium on line. The software sensors for biomass activity, glucose and lactic acid can be applied for any type of animal cell that is grown in a bioreactor system. The glutamine and ammonium software sensors are determined experimentally by correlating them to the acid-production rate. Therefore, they can only be used for Vero cells.

In the development of these software sensors, much attention is paid to the verification of their basic elements, i.e. the oxygen uptake rate (OUR) and the acid production rate (APR). For the estimation of the OUR, information about the course of the oxygen solubility and the oxygen transfer coefficient during cultivation is considered very important, since fluctuations in one of these parameters will introduce a systematic error in the OUR. Experiments have shown that the oxygen solubility remains approximately constant throughout the cultivation. However, the oxygen transfer coefficient can vary significantly during cultivation. Because of

this, a method for on-line determination of the oxygen transfer coefficient is developed. Application of this method results in an estimation of the OUR that is more reliable and contains less noise than the determination of the OUR using an off-gas mass spectrometer.

The lactic-acid production rate (LPR) is estimated from the APR, which in turn is determined from the amount of base utilized to control the pH at a fixed level. The estimated LPR provides reliable results, until nutrient limitation occurs. After that, the predicted LPR is higher than the measured LPR. Since at the end of the cultivation the lactic-acid concentration starts to drop, the cells are likely to take up lactic acid as an energy source. This uptake is supposed to be at least partially responsible for the discrepancy between estimated and measured LPR. However, since the estimation of the LPR is accurate up to the moment that nutrient limitation occurs and since the aim of control is to prevent nutrient limitation, the estimated LPR can still be used for control purposes.

After evaluation of the two basic elements of the software sensors, the sensors themselves are tested. Two software sensors use both the OUR and the LPR. These sensors, the glucose sensor and the biomass activity sensor, defined as the total energy requirement of the cultivated cells, both provide reliable signals. It is shown that the maximum error in the biomass activity signal, caused by the error in the LPR, is 4.6%. This error is small enough to be neglected. Also the estimation of the glucose concentration is accurate throughout the culture. The overall error in the estimation of the glucose uptake rate is found to be 6%.

The software sensors for lactic acid, glutamine and ammonium are solely based on the APR. Since the APR only provides reliable values until nutrient limitation occurs, these software sensors can only be used up to that point too. However, in the range at which the software sensors are valid, they are accurate.

Applying the software sensors, the effect of controlled addition of glucose

and glutamine is studied. For hybridoma cells a significant reduction of the lactic-acid production rate is described in literature when the glucose concentration is controlled at a fixed low level. Contradictory to this, the specific lactic-acid production rate for Vero cells is found not to change when glucose is controlled at different levels from 10 down to 0.25 $mol \cdot m^{-3}$. In fact, no significant changes in the cultivation conditions are observed at all. Reducing the glutamine concentration from 4 down to 0.5 $mol \cdot m^{-3}$ yields in a significant reduction (38%) of the specific ammonium production rate. This reduction is corrected for the ammonium that is formed during the spontaneous decomposition of glutamine. Lowering the glutamine concentration in the culture also results in a reduced decomposition of glutamine. The total reduction in ammonium formation caused by these two effects is 62%. Although a significant reduction of the ammonium formation is achieved, no effect is observed on the growth rate and the maximum cell density of Vero cells.

Analysis of the cultivation medium using off-line HPLC has shown that after approximately 100 hours of cultivation, two amino acids are depleted. To find out whether or not these amino acids, methionine and serine, are responsible for the limitations in growth rate and cell density, they are added sequentially at the end of a culture. At that point the biomass activity is already dropping. Adding first methionine results in a stabilization of the biomass activity, whereas first addition of serine does not seem to have any effect. Further addition of the second amino acid instantaneously results in an increase of the biomass activity, the growth rate and the cell density in both cases. This indicates that even serine, which is a non-essential amino acid, is essential to maintain cell growth. At the moment serine is depleted, the cells have to make serine themselves, which causes the growth rate to drop. When methionine, an essential amino acid, is depleted the cell growth stops completely.

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In an attempt to increase the maximum cell density, additional amounts of both amino acids are added to the cultivation medium prior to the start of the culture. Unexpectedly, serine again is depleted after 100 hours of cultivation, in spite of an initial serine concentration of 0.4 instead of 0.1 $mol \cdot m^{-3}$. The biomass activity drops instantaneously at the moment serine is depleted. After the serine concentration in the cultivation medium is restored, the biomass activity only partially recovers. Despite the short serine limitation, the cell density doubles compared to the reference culture that is executed without glucose and glutamine control and without supplementing limiting amino acids.

If this kind of control systems are to be implemented into production systems, some kind of plant automation is required. This can vary from computer-controlled measurement- and control systems up to fully automized manufacturing execution systems (MES) that control all parts of the production process. These processes can vary from stock management and generation of reports, to man-machine interfaces that operate the measurement- and control systems. An MES becomes important when the production facility has to produce according to governmental regulations such as current good manufacturing practice (cGMP). The system ensures that all parts of the production process are executed according to these rules. Additional features are process-dependent control, the use of software sensors and predictive models, the use of historical data and on-line statistical techniques for trend analysis and detection of instrumentation failures.

SAMENVATTING

In dit proefschrift worden schatters gepresenteerd die in staat zijn om tijdens een dierlijke-celkweek de biomassa activiteit, gedefinieerd als de totale energiebehoefte van de cellen in de bioreactor, en de glucose-, glutamine-, lactaat- en ammonium-concentratie te voorspellen. De schatters voor biomassa activiteit, glucose en lactaat zijn toepasbaar voor elk type dierlijke cel dat in een bioreactor wordt gekweekt. De glutamine en de ammonium software sensor zijn experimenteel bepaald door de glutamineconsumptiesnelheid en de ammoniumproduktiesnelheid te correleren aan de zuurproduktiesnelheid. Daarom zijn deze alleen maar geschikt voor Vero cellen.

Er is tijdens de ontwikkeling van de schatters veel aandacht besteed aan de verificatie van de twee basiselementen, te weten de zuurstofopnamesnelheid (OUR) en de zuurproduktiesnelheid (APR). Om de OUR goed te kunnen schatten, is het erg belangrijk dat er informatie beschikbaar is over het verloop van de oplosbaarheid van zuurstof en over het verloop van de zuurstofoverdrachtscoëfficiënt tijdens een

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kweek. Een verandering in één van beide waarden introduceert namelijk een systematische fout in de OUR. Experimenteel is vastgesteld dat alleen de zuurstofoverdrachtscoëfficiënt significant kan veranderen. De oplosbaarheid van zuurstof in het medium blijft nagenoeg constant gedurende de kweek. Om systematische fouten in de OUR te voorkomen is er een methode ontwikkeld om de zuurstofoverdrachtscoëfficiënt tijdens de kweek te kunnen bepalen. Dit heeft geresulteerd in een schatting van de OUR die betrouwbaarder is en minder ruis bevat dan de bepaling van de OUR met behulp van een massaspectrometer.

De lactaatproduktiesnelheid (LPR) is geschat uit de APR. Deze wordt bepaald uit de hoeveelheid loog die nodig is om de pH op een constante waarde te regelen. Zolang er geen nutriëntlimitatie optreedt is de schatting van de LPR goed. Na dat punt is de voorspelde LPR hoger dan de werkelijke. Omdat aan het einde van een celkweek de lactaatconcentratie daalt, is het waarschijnlijk dat de cellen lactaat gebruiken als energiebron. Deze lactaatopname kan in ieder geval een deel van het verschil tussen gemeten en geschatte LPR verklaren. Aangezien de schatting van de LPR goed is verlopen tot het moment dat nutriëntlimitatie is opgetreden en omdat het doel van regeling het voorkomen van dit soort limitaties is, kan de geschatte LPR toch goed worden gebruikt voor dit soort regelingen.

Na evaluatie van de twee basiselementen van de schatters, zijn de schatters zelf getest. Twee typen schatters maken gebruik van zowel de OUR als de LPR. Dit zijn de biomassa activiteit en de glucose sensor. Beide sensoren blijken betrouwbare resultaten te produceren. De maximale fout in de biomassa activiteit die is opgetreden als gevolg van de fout in de LPR is 4.6%. Deze fout is zo klein dat ze verwaarloosd mag worden. Ook de glucose sensor is in staat gebleken om gedurende de gehele kweek de glucose concentratie nauwkeurig te voorspellen. De totale fout in de geschatte glucoseopnamesnelheid is 6%.

De schatters voor lactaat, glutamine en ammonium zijn afgeleid van de APR.

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Omdat deze alleen maar betrouwbare waarden geeft totdat er een nutriëntlimitatie optreedt, kunnen de hieruit afgeleide schatters alleen maar gebruikt worden tot dat punt. In het gebied waar de schatters gebruikt mogen worden functioneren ze goed.

Met behulp van de ontwikkelde schatters is het effect van geregelde toevoeging van glucose en glutamine bepaald. Uit de literatuur is bekend dat bij hybridoma cellen een verlaging van de glucose concentratie ook een verlaging van de lactaat produktie tot gevolg heeft. Echter, bij Vero cellen verandert de specifieke lactaatproduktiesnelheid niet op het moment dat de glucoseconcentratie op een vaste waarde is geregeld tussen 10 en 0.25 mol·m^{-3}. In feite wordt geen enkel significant verschil in de kweekcondities waargenomen. Verlaging van de glutamineconcentratie van 4 naar 0.5 mol·m^{-3} heeft tot gevolg dat de specifieke ammoniumproduktiesnelheid met 38% vermindert. Deze waarde is gecorrigeerd voor ammonium dat vrijkomt bij de spontane ontleding van glutamine. Verlaging van de glutamine concentratie heeft ook tot gevolg dat de totale hoeveelheid glutamine die ontleedt afneemt. De totale afname in ammonium veroorzaakt door beide effecten loopt hierdoor op tot 62%. Hoewel de geproduceerde hoeveelheid ammonium dus drastisch is afgenomen, heeft dit geen effect op de groeisnelheid en de maximale celdichtheid gehad.

Na analyse van het kweekmedium met een HPLC is gebleken dat na ca. 100 uur kweken twee aminozuren compleet zijn verbruikt. Om na te gaan of beide aminozuren, methionine en serine, verantwoordelijk zijn voor de limitatie in de groeisnelheid en in de maximale celdichtheid, zijn deze aan het einde van een celkweek na elkaar toegevoegd. Op dat moment is de biomassa activiteit al aan het afnemen. Na toevoeging van methionine stabiliseert de biomassa activiteit. Toevoeging van alleen serine lijkt geen enkel effect te hebben. Toevoeging van het tweede aminozuur heeft in beide gevallen tot gevolg dat zowel de biomassa

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activiteit als de celdichtheid momentaan toenemen. Dit resultaat geeft aan dat zelfs serine, wat een niet-essentieel aminozuur is, essentieel is om de groeisnelheid op peil te houden. Als serine op is, moeten de cellen zelf serine maken, waardoor de groeisnelheid daalt. Als methionine (een essentieel aminozuur) op is, stopt de celgroei compleet.

In een poging om de maximale celdichtheid te verhogen, zijn aan het begin van een kweek extra hoeveelheden methionine en serine aan het medium toegevoegd. Ondanks een serineconcentratie van 0.4 in plaats van 0.1 $mol \cdot m^{-3}$, is na 100 uur alle serine weer verbruikt. Op dat moment daalt de ook biomassa activiteit weer. Na toevoeging van serine herstelt de biomassa activiteit zich slechts gedeeltelijk. Ondanks dat, verdubbelt de maximale celdichtheid vergeleken met de referentiekweek die is uitgevoerd zonder glucose- en glutamineregeling en waaraan geen extra aminozuren zijn toegevoegd.

Als dit type regelingen ingebouwd moet worden in produktiesystemen, vereist dit een bepaalde vorm van automatisering. Dit kan variëren van een computergestuurd meet- en regelsysteem tot een volledig geautomatiseerd produktiesysteem (MES) dat alle onderdelen van het produktieproces controleert. Bij deze processen kan gedacht worden aan magazijnbeheer en het genereren van rapporten tot aan zogenaamde mens-machine interfaces die gebruikt worden om meet- en regelsystemen aan te sturen. Vooral wanneer geproduceerd moet worden volgens bepaalde overheidsregels zoals 'current good manufacturing practice' (cGMP), wordt het gebruik van een MES belangrijk. Zo'n systeem zorgt ervoor dat alle onderdelen van het produktieproces worden uitgevoerd volgens de voorgeschreven regels. Verdere mogelijkheden van een MES zijn procesafhankelijke regelingen, het gebruik van schatters en voorspellende modellen, het gebruik van historische data en on-line statistische technieken voor trendanalyse en detectie van storingen in de apparatuur.

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CURRICULUM VITAE

Richard Christiaan Dorresteijn werd op 9 september 1965 geboren in Dieren. In 1981 behaalde hij zijn MAVO diploma aan de St. Bernhard MAVO te Dieren. Hierna begon hij aan de opleiding voor Middelbaar Laboratorium Onderwijs aan de OLAN te Arnhem. Tijdens deze opleiding werd stage gelopen bij de Keuringsdienst van Waren te Zutphen en bij de Hollandse Metallurgische Industrie Billiton BV te Arnhem. Nadat in 1985 het MLO werd voltooid, begon hij aan de HTS-SVL in Enschede aan de opleiding HTS chemische technologie. Voor deze opleiding werden stages gelopen bij het RIVM te Bilthoven en bij de ENKA te Arnhem. Ook de afstudeeropdracht werd uitgevoerd bij het RIVM. Het HTS-CT diploma werd in 1989 behaald met als specialisatie produktkunde. Vanaf september 1989 tot en met december 1993 was de auteur werkzaam als Assistent In Opleiding bij de sectie Proceskunde van de Landbouwuniversiteit te Wageningen. Het onderzoek zelf werd echter uitgevoerd op het RIVM. Vanaf 1994 kwam hij uiteindelijk officieel in dienst van het RIVM, waarna hij in augustus 1996 overstapte naar de proces-automatisering door te gaan werken bij Compex te Capelle aan de IJssel.