Stellingen

1. De aannome dat de reactiekinetiek van in een gel geïmobiliseerde gisten gelijk is aan die van groeiende, gesuspendeerde cellen en het op basis hiervan bepalen van de effectieve diffusiecoefficient in een gel is onjuist.


2. Een microsensor is een goed hulpmiddel voor de experimentele bepaling van de kinetiek van in gel geïmobiliseerde biokatalysatoren.

   Dit proefschrift.

3. Bij de gevoeligheid- en foutenanalyse van de verschillende methoden ter bepaling van een effectieve diffusiecoefficient door Itamunoala ontbreekt een parametergevoeligheidsanalyse voor de afzonderlijke methoden.


4. Voor het zinvol vergelijken van het effect van immobiliseren op het gedrag van enzymen of cellen moet vermeld worden om welke immobilisatietechniek het gaat.


5. Een glucose-microsensor gebaseerd op geïmobiliseerde glucose-oxidase heeft zeer beperkte toepassingsmogelijkheden in geïmobiliseerde systeem vanwege de noodzaak tot aanwezigheid van zuurstof.


6. Bij het bepalen van de kinetiek van een conversie-reactie in sediment door middel van een langdurig batch-experiment moet rekening worden gehouden met groei en in oplossing gaan van geïmobiliseerde cellen.

7. Een goede interdisciplinaire samenwerking op wetenschappelijk gebied stimuleert tot hogere output.

8. Wie ernst maakt met het milieu zorgt ervoor dat de mensen lopen, fietsen of thuis blijven maar de overheid doet het omgekeerde.

9. Het idee van Popper, dat het testen van een theorie bestaat uit een poging haar te falsificeren, is geen gemeengoed in de academische wereld.

10. Het is met name voor studentes belangrijk zich te realiseren dat universitaire bluf een veel voorkomend verschijnsel is.

11. De gemiddelde levensduur van de mens kan verlengd worden door het verhogen van de leeftijd waarop voortplanting plaatsvindt.

Stellingen behorende bij het proefschrift “*Diffusion coupled with bioconversion in immobilized systems*” van C.M. Hooijmans.

Delft, 16 september 1990.
DIFFUSION COUPLED WITH BIOCONVERSION IN IMMobilIZED SYSTEMS

USE OF AN OXYGEN MICROSENSOR
Diffusion coupled with bioconversion in immobilized systems

Use of an oxygen microsensor

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus, prof. drs. P.A. Schenck, in het openbaar te verdedigen ten overstaan van een commissie aangewezen door het College van Dekanen op 30 oktober 1990 te 14.00 uur.

door

Christine Maria Hooijmans

geboren te 's Gravenhage
landbouwkundig ingenieur
Dit proefschrift is goedgekeurd
door de promotor
prof. ir. K. Ch. A. M. Luyben
Contents

1 Introduction 1
1.1 Immobilization and bioconversions 1
1.2 Immobilization techniques and materials 3
1.3 Microsensors 4
1.4 Aim and scope of this thesis 4
1.5 Bibliography 5

2 Oxygen profiles in biocatalyst particles 9
2.1 Introduction 9
2.1.1 Oxygen sensors 10
2.1.2 Oxygen microsensors 11
2.2 Materials and Methods 13
2.2.1 Construction of the microsensors 13
2.2.2 Testing of the oxygen microsensors 13
2.2.3 Biocatalyst particle 15
2.2.4 Microsensor measuring equipment 15
2.3 Results 17
2.4 Acknowledgment 20
2.5 Bibliography 20

3 Intrinsic kinetic parameters determined from oxygen profiles 23
3.1 Introduction 24
3.2 Theoretical background 24
3.2.1 Free enzyme: kinetics 24
3.2.2 Marquardt's algorithm for least squares estimation of non-linear parameters 26
3.2.3 Immobilized enzyme: kinetics and diffusion 26
3.2.4 Calculation of the overall effectiveness factor 28
3.3 Materials and methods 29
3.3.1 Enzyme 29
3.3.2 L-lactic acid 29
3.3.3 Immobilization procedure 30
3.3.4 Biological Oxygen Monitoring system 31
3.3.5 Oxygen microsensor 31
5.3.4 Experimental procedure 67
5.4 Results 67
5.4.1 Mass transfer only by diffusion 67
5.4.2 Mass transfer under flow conditions 69
5.5 Conclusion 72
5.6 Nomenclature 73
5.7 Bibliography 73

6 Oxygen profiles in immobilized E. coli 75
6.1 Introduction 75
6.2 Materials and Methods 76
6.2.1 Bacterial strain and nutrient medium 76
6.2.2 Free cell growth 77
6.2.3 Gel-immobilized cell growth 77
6.2.4 Determination of cell concentration 78
6.2.5 Oxygen microsensors 78
6.2.6 Microsensor measurements of oxygen concentration profiles 78
6.3 Oxygen profile model 79
6.4 Results and Discussion 81
6.4.1 Oxygen profiles 81
6.4.2 Reaction-diffusion model 84
6.4.3 Maximum specific growth rate determination for the immobilized cells 86
6.4.4 Oxygen penetration depth 87
6.5 Conclusions 89
6.6 Acknowledgments 89
6.7 Nomenclature 89
6.8 Bibliography 90

7 Metabolically structured model for T. pantotropha 93
7.1 Introduction 93
7.2 Materials and methods 95
7.3 Metabolically structured modelling 95
7.4 Aerobic nitrification/denitrification of NH₃ and HNO₃ 96
7.4.1 The internal reaction rates 96
7.4.2 Linear equation between observable conversion rates 100
7.4.3 Measurements aerobic experiments 103
7.4.4 Results of fitting of the aerobic experiments 103
7.5 Anaerobic denitrification of HNO₃ 105
7.5.1 The internal reaction rates 105
7.5.2 Linear equation between observable conversion rates 106
7.5.3 Measurements anaerobic experiments 107
7.5.4 Results of fitting of the anaerobic experiments 107
7.6 Evaluation of the results 108
7.7 Conclusions 111
7.8 Acknowledgements 111
7.9 Nomenclature 112
7.10 Bibliography 112

8 Growth and coupled nitrification/denitrification by immobilized T. pantotropha 115
8.1 Introduction 116
8.1.1 General introduction 116
8.1.2 Linear relation between net conversion rates 117
8.1.3 Introduction of kinetic equations 118
8.1.4 Oxygen profiles as a result of oxygen limitation 119
8.1.5 Oxygen profiles as a result of acetate limitation 120
8.2 Materials and methods 121
8.2.1 Organism 121
8.2.2 Continuous cultures (suspended cells) 121
8.2.3 Continuous culture reactors (immobilized cells) 122
8.2.4 Immobilization procedure 122
8.2.5 Oxygen microsensor 122
8.2.6 Microsensor measuring system 122
8.2.7 Analytical techniques 123
8.2.8 Diffusion and mass transfer coefficients 123
8.3 Results and discussion 124
8.3.1 Steady state situation 124
8.3.2 Specific growth rate of immobilized cells 125
8.3.3 Biomass concentration in the biocatalyst particle 127
8.3.4 Influence of acetate limitation on oxygen profiles 130
8.4 Conclusion 132
8.5 Acknowledgements 133
8.6 Nomenclature 134
8.7 Bibliography 134

A Calculation of the oxygen consumption rate from an internal oxygen profile 137

B Calculation of the influence of acetate on the metabolism of T. pantotropha 139

Summary 143
Samenvatting 147
Dankwoord 151
Curriculum vitae 153
Chapter 1

Introduction

Biotechnology can be defined as the industrial application of biological organisms, systems or processes. The scientific disciplines such as microbiology, biochemical engineering and biochemistry form the basis of biotechnology. Subjects which are of interest to biotechnology are for example recombinant DNA technology, fermentation, bioreactor design, downstream processing and biocatalysis. The work presented in this thesis deals with biocatalysis. An oxygen microsensor was used in the study of the effect of immobilization on bioconversions.

1.1 Immobilization and bioconversions

Immobilization is a biotechnological technique mainly used for the retention of enzymes or cells in a reactor system. The term bioconversion is frequently used for the conversion of a particular substrate into a desired product by these enzymes or cells (biocatalysts). Numerous immobilization methods have been described in the literature [1–4] and will be discussed in more detail in the following section. Examples are attachment to a solid surface or entrapment within a porous membrane.

The advantages of immobilization can be many, the most obvious are listed below:

- Continuous use or re-use of the biocatalyst is made possible.
- During continuous operation high dilution rates can be used without wash-out of the biocatalyst.
- Toxic metabolites or inhibitant products can be removed continuously.
- There is almost no contamination of the product by enzymes or cells.

There are also drawbacks associated with immobilization, such as diffusion limitation of substrates or products, or alteration of the activity of the catalyst. However, it depends on the kinetics and type of alteration if these aspects are really disadvantageous. The biocatalyst properties can be
altered favourably by immobilization. Most of the research on immobilization started in the late sixties [5] and has generally focussed on artificially binding enzymes or cells to or in an immobilization material. The early publications described the immobilization methods that could be used for binding enzymes or cells, but the emphasis of later papers has gradually moved towards the kinetics of the reactions carried out by the biocatalysts, and practical applications.

There are at least four areas in which immobilized enzymes or cells are used, i.e. industrial, environmental, analytical and therapeutic [6]. For the purposes of this thesis, only the industrial and environmental applications will be considered.

In the environmental applications, waste-water treatment is the most outstanding. Active sludge processes and trickling filters have been used for decades [7]. The fluidized bed reactor and anaerobic sludge blanket reactor making use of acidogenic and methanogenic bacteria attached to sand or forming granular sludge are being used since the seventies [8, 9]. Immobilization by entrapment is beginning to appear in modern, biotechnological wastewater treatment systems. There are some processes working on lab-scale, such as the entrapment of biomass in porous support particles like polyurethane foam cubes [10].

With respect to the industrial applications, some already very old ones make use of naturally attached immobilized cells. An example of this type of process is the vinegar production invented by Pasteur in the mid 19th century, which used a biofilm of Acetobacter cells attached to the surface of wood chips in a trickling filter [11]. Examples of modern industrial applications include amino acid and antibiotic syntheses. In 1969, a continuous process for the production of several L-amino acids (L-methionine, L-valine, L-phenylalanine) using immobilized aminoacylase became the first industrial application of immobilized enzymes in the world [5]. In order to eliminate the necessity of extracting enzymes for these processes, attempts to directly immobilize intact microbial cells began in the early seventies [12]. In 1973, an industrial process for the continuous production of L-aspartic acid using immobilized Escherichia coli cells was launched [13]. The number of industrial processes utilizing a specific immobilized organism is still limited. Until 1989 nine different applications have been described in literature involving fourteen different companies [4, 14–16]. The process most frequently used is the isomerization of glucose to fructose by glucose isomerase, an enzyme present in different microorganisms. The processes utilizes simple one-step reactions without a cofactor requirement, using dead cells. Current research is tending to focus more on immobilized live cell systems. They may be employed for a wide variety of bioconversions, ranging from single enzyme reactions to conversions requiring complete metabolic pathways. Of course, for a successful exploitation of immobilized cells, the physical and chemical influence of im-
mobilization on their physiology should be known. Several researchers report that viable cells undergo changes when immobilized. The kinetic properties of *Saccharomyces cerevisiae* immobilized on cross-linked gelatin were found to be substantially different from those of the suspended cells [17]. Entrapment of *S. cerevisiae* in calcium-alginate beads altered the cell metabolism compared to that of suspended cells [18]. In κ-carrageenan gel immobilized recombinant *E. coli* cells showed a high plasmid stability [19]. Some more examples can be found in the review by Ghose [20]. The importance of elucidating the influence of immobilization on the physiology of immobilized cells was illustrated during the symposium ‘Physiology of Immobilized Cells’ in the Netherlands (Wageningen, December 1989). It will be one of the more important aspects for further development of immobilized cell technology.

1.2 Immobilization techniques and materials

The techniques for immobilizing a biocatalyst artificially can basically be divided into three categories: cross-linking, carrier binding and entrapment [12]. These techniques were first used for the immobilization of enzymes, but were later also applied to cells. For whole cells, physical entrapment in polymeric matrices is the most widely used technique, whereas single enzymes are most often bound to carriers, or cross-linked by bi- or multi-functional reagents such as glutaraldehyde or hexamethylene diisocyanate [21]. For the work presented in this thesis, almost exclusively the entrapment in agarose beads was used for immobilization. The reason for this was that all types of agarose can be penetrated by an oxygen microsensor without deformation or disruption of the bead. Agarose is a purified preparation of agar, a polysaccharide isolated from sea weed. It is basically composed of a repeating agarobiose unit consisting of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose [22]. The mechanism behind the gelation of agarose involves a shift from a random coil in solution to a double helix in the initial stage of gelation, and then to bundles of helices in the final stage [23]. It is a thermally reversible process, just like the gel formation of agar, gelatin and κ-carrageenan. κ-Carrageenan, however, requires cations such as K⁺, NH₄⁺ and Ca⁺⁺ or amines to form a firm gel. It appeared that gelatin and κ-carrageenan without cations ions were also suitable for microsensor measurement. For the other polysaccharides like alginate and chitosan the gelation principle is ionotropic. These gels deform when penetrating with an oxygen microsensor. Besides the aspect of easy penetration by a microsensor, there are other selection criteria that can be important such as temperature sensitivity of the biocatalyst, influence of growth media on the strength of the gel and physiologically inertness of the gel.
1.3 Microsensors

For most of the work presented in this thesis an oxygen microsensor was used. Microsensors can be described as sensors which have a tip diameter in the range of 1 to about 100 μm. Because of this small tip diameter, they can be used to measure concentration profiles in immobilized systems, giving high spatial resolution. The information obtained from such measurements can then be used to elucidate the behaviour of these systems. The experimental results can be used, for example, to develop and validate mathematical models. With these models, the behaviour of the biological system can be predicted for other situations. For the research presented in this thesis a Clark-type oxygen microsensor was used. This was constructed at Delft University of Technology according to the method described by Revsbech and Ward [24], and had a tip diameter of about 10 μm. In a Clark-type sensor the measuring electrode, the cathode, is assembled together with the reference electrode in one body. The tip of the sensor is sealed with a silicon membrane. The contact between the cathode and reference electrode is made by an electrolyte solution. The result is a sensor with a very stable signal and a short response time. It is relatively insensitive to poisoning by ions, can be used for measurements in gaseous environments, and has a life-time of several months. Until a few years ago, microsensors were only available for the measurement of oxygen, sulfide and pH [25]. Now, ion-selective micro-electrodes for the measurement of ammonium and nitrate are also available [26, 27]. A nitrous oxide microsensor [28] and a light microprobe [29] have recently been described. In addition, the use of small electrodes (tip diameter 250 μm) for the detection of hexacyanoferrate III have been used in the study of flow regimes in a packed bed reactor [30]. Until now, microsensors have mainly been used in microbial ecology [27, 31–35] rather than in the study of the behaviour of immobilized enzymes or cells. One recent publication shows the application of an oxygen microsensor for the determination of oxygen gradients in Ca-alginate beads [36].

1.4 Aim and scope of this thesis

The use of mathematical models describing aspects of the behaviour of real systems is an important tool for making scientific progress [37]. The basis of the work described in this thesis is formed by the combination of experimental techniques and mathematical models. Various systems have been investigated in order to discover and describe the behaviour of immobilized enzymes and metabolically active, growing microorganisms. Mathematical models were formulated to describe the behaviour of suspended enzymes and (growing) cells, and were validated using experimental results. The models then were extended to describe the behaviour of immobilized biocatalysts,
using an oxygen microsensor for the experimental validation. By combining the information obtained for the suspended and the immobilized systems, the influence of immobilization on the behaviour of such systems could be determined.

In Chapter 2, the use of an oxygen microsensor for measuring oxygen profiles in biocatalyst particles under steady state conditions is introduced. The particles were made of agarose and contained L-lactate 2-monoxygenase.

Chapter 3 deals with the determination of the Michaelis-Menten kinetics of immobilized L-lactate 2-monoxygenase, using profile measurements and mathematical models. The kinetic parameters of the immobilized model enzyme are compared with those for the suspended enzyme.

In Chapter 4, two different mathematical methods for the determination of Michaelis-Menten kinetics of an immobilized enzyme are compared, using data from batch experiments. The results are also compared with those obtained from steady state measurements using an oxygen microsensor.

The determination of external mass transfer for liquid flow around a spherical particle is described in Chapter 5. Measurements were made under different flow conditions and with various particle diameters. The results are compared with published correlations.

Chapter 6 deals with the determination of growth of *Escherichia coli* B(pTG201), immobilized in carrageenan gel slabs and spheres. The growth rates obtained are compared with the values measured in suspensions.

Chapter 7 presents a metabolically structured model based on intracellular reaction equations. This model was developed for the description of the behaviour of suspended continuous cultures of *Thiospaera pantotropha*, a species which is both a heterotrophic nitrifier and an aerobic denitrifier. This model forms the basis for Chapter 8 in which the structured model is extended with diffusion and reaction equations in order to describe the behaviour of immobilized *T. pantotropha*. The model description was combined with measured oxygen concentration profiles, resulting in several kinetic parameters of the immobilized cells. A comparison of these values with those obtained for the suspended cells was then used to gain more insight in the immobilization effects on the behaviour of *T. pantotropha*.

### 1.5 Bibliography


1. Introduction
Chapter 2

Determination of oxygen profiles in biocatalyst particles by means of a combined polarographic oxygen microsensor

When studying the effect of immobilization of enzymes or whole cells on the conversion of substrate, more information is gained if measurements of substrate inside the biocatalyst particle are possible. With the methods used until now only measurements outside the particle can be performed. In this article a method for measuring oxygen profiles in a biocatalyst particle under steady state conditions is described. The biocatalyst particle was made of agarose and contained the enzyme l-lactate 2-monooxygenase. This enzyme decarboxylates lactic acid to acetic acid in the presence of oxygen. The experiments were carried out in a flow chamber with the use of a micromanipulator and a stereomicroscope. The data were sampled by means of a computer. Four different profiles were measured using four different enzyme concentrations. The measured oxygen profiles were reproducible and the signal was very stable. It was also possible to measure the boundary layer around the particle. With the use of the oxygen microsensor measurements in a biocatalyst particle could be performed accurately, giving way for model validation.

2.1 Introduction

Because of an increasing use of immobilized biocatalysts (cells, enzymes) it is important to study the effects of immobilization on the activity of biocatalysts. Effects might be due to diffusion limitation of the substrate or

2. Oxygen profiles in biocatalyst particles

product within the particle. In the case of enzymes, the effects of immobilization also include possible alteration of the kinetic parameters due to conformational changes, and/or chemical denaturation by the reagents used for immobilization. As a result of these influences the activity of the immobilized biocatalyst differs from the activity of the free biocatalyst. Once the concentration profiles inside the biocatalyst particle (and knowing the diffusion limitation of the substrate or product) have been measured, the kinetic parameters of the immobilized enzyme can be calculated. Furthermore, when these parameters are known it is possible to predict the conversion capacity of the immobilized biocatalyst for other diameters of the particle or different enzyme concentrations.

In this chapter a method for determining the oxygen profile in a biocatalyst particle by means of a self-made oxygen microsensor is described, and some preliminary experimental results are shown. The particle consists of a gel containing an amount of homogeneously distributed oxygen reducing enzyme.

The use of microsensors in biotechnology is not new. Oxygen microsensors have been used for some years in research into the ecology of marine sediments [1–3], lake sediments [4], slimes and other slime systems trickling filter [5, 6] and mycelial pellets [7]. However, the application described here, in which a profile is measured inside a gel particle, is new.

2.1.1 Oxygen sensors

The principle of a polarographic (also called voltammetric) oxygen sensor is based on the diffusion of oxygen to the polarized cathode, resulting in an electrical signal. The cathode consists mostly of platinum with an Ag/AgCl electrode as reference. For the Clark-type sensor [8], the measuring electrode, the cathode, is assembled together with the reference electrode in one body. The contact between the cathode and the reference electrode is made by an electrolyte solution. The oxygen diffuses through a selective membrane and dissolves in the electrolyte solution. Noncombined sensors, consisting of a shielded cathode (in most cases protected by a membrane) and a separate reference electrode, are also used. The contact between these two electrodes is made by the measuring solution. Hence these electrodes can not be used to measure in air or other gaseous environments.

By means of an external polarization voltage, the cathode becomes negatively charged with respect to the reference electrode. The oxygen diffuses to the cathode and is reduced, resulting in a flow of electrons. For this current the following relation holds:

\[ I = nF\bar{A}J_0, \]  \hspace{1cm} (2.1)

in which:
\[ I = \text{electrical signal (A)} \]
\[ n = \text{number of electrons that is added to an oxygen molecule arriving at the cathode} \]
\[ F = \text{Faraday’s constant} = 9.6487 \cdot 10^4 \text{ C mol}^{-1} \]
\[ A = \text{surface of the cathode (m}^2\text{)} \]
\[ J_{O_2} = \text{flux of oxygen (mol m}^{-2}\text{s}^{-1}\text{)} \]

It is clear that the magnitude of the electrical signal depends on the flux of oxygen towards the cathode and the dimension of the surface of the cathode. The flux is influenced by the resistance caused by a stagnant layer in the measuring solution near the tip of the electrode, the resistance due to the membrane (type and thickness), and the resistance due to the electrolyte solution.

2.1.2 Oxygen microsensors

The advantage of microsensors is that they are very small and can be used for specific purposes like measuring in microenvironments without causing much disturbance. Other advantages, which are mostly inherent in the dimension of the electrode, include a fast response and the reduction of very small amounts of oxygen, making measurements in a stagnant medium possible.

The first (polarographic) oxygen microsensors with a sensing tip of a few microns was made in the 1960s. The initiative was taken in the physiological world to measure in tissue and blood vessels [9]. These electrodes (cathodes) consisted of a shielded platinum wire, of which the tip was in free contact with the solution. The reference electrode was separated from the measuring electrode. As a consequence this sensor had some bad properties. The response of the sensor was influenced by ions in the solution and poisoning of the electrode surface occurred relatively rapidly. Whalen [10] overcame these problems by covering the tip of the cathode with a membrane. His electrode did not contain a platinum wire, but consisted of a glass capillary with an alloy of a mixture of Wood’s metal and gold powder. This alloy adheres to glass very well. At the base of the recess the electrode had a layer of gold. The tip of the electrode was filled with a membrane made of collodion. The electrode had a tip with a diameter smaller than 5 \( \mu \text{m} \) and a separate Ag/AgCl electrode as a reference electrode. After testing the electrodes, Whalen reported that their response time was about 0.25 to 8 seconds and no stirring effect had been observed. The electrodes frequently broke during operation, so the long term stability could not be tested. This type of electrode is now commercially available from Diamond Electro-Tech (Ann Arbor, MI). Whalen, Huang and Sanders [10–12] used these microelectrodes for measuring oxygen tension in tissue and oxygen profiles in microbial slime films. They reported that the electrode showed a strongly fluctuating signal.
Baumgartl and Lubbers [13] made a slightly different sensor with a tip of 2 to 10 μm in diameter. Different from the electrode of Whalen is the glass shaft that was coated with a number of thin layers, the outermost consisting of silver. The silver layer served as a reference electrode and shielded the platinum wire inside the microsensor from electrical noise. The platinum cathode and reference electrode were situated behind the same membrane, made of collodion and polystyrene. Researchers of the group of Baumgartl and Lubbers used this microsensor for physiological research [14]. They reported that they had succeeded in optimizing this sensor resulting in a tip of 0.6 μm, the microcoaxial sensor. This very small electrode was required in order to determine the oxygen tension in cellular tissue which would be damaged by an electrode tip greater than 1 μm in diameter.

The problem with the microsensors as described above is that the membrane does not only allow oxygen to diffuse through. Other ions (Mg$^{2+}$, Ca$^{2+}$) will pass through as well and poison the electrode by direct precipitation of the formed hydroxides. A thicker membrane would also become less permeable for oxygen, which results in a higher response time. Consequently the signal of these sensors is not very stable when they are used in natural media, resulting in a slow drift and decreased sensitivity to oxygen after periods of low oxygen tension [4]. This problem was solved by Revsbech [15] by devel-

![Figure 2.1 Sensing tip of oxygen microsensor as developed by Revsbech [15]. It consists of electrolyte (1), anode (2), inner casing (3), outer casing (4), cathode (5) and silicon membrane (6).]
oping a combined sensor which is a small version of the conventional clark sensor [8]. This sensor (see figure 2.1) consists of a shielded platinum wire which has been gold plated (the cathode) attached to a glass capillary filled with a 1 m KCl electrolyte solution. The glass capillary is sealed with a membrane made of silicon rubber. This membrane allows only gases to pass. the tip of the electrode is a few µm above the membrane. In the electrolyte solution an Ag/AgCl electrode is immersed. The electrolyte serves as an electrical conductor but also as an electrical shielding of the cathode. Because of this, the signal of the sensor is very stable, while the other specifications, such as response time and possibility of measuring in a stagnant medium, are more or less the same as for the electrodes mentioned above. The major problem with this type of sensor is that it can be poisoned by hydrogen sulfide.

Revsbech et al. used this microsensor to determine photosynthetic activity in benthic microbial mats [4, 16] and oxygen microprofiles of trickling filter biofilm [17].

2.2 Materials and Methods

2.2.1 Construction of the microsensors

For the measurements in a biocatalyst particle as described in this chapter an oxygen microsensor constructed at our laboratory according to Revsbech and Ward [4, 15] was used.

Many phenomena associated with a working oxygen microsensor, such as the stirring effect or response time, depend on a number of factors. These are the thickness of the silicon membrane, the dimension of the cathode, and the dimension of the cathode relative to the glass shaft. This can be explained as follows. If the membrane is thick, the flux of oxygen to the cathode is reduced, and consequently the response time increases. The same effect is caused by a relatively big gap between the cathode and the membrane. If the cathode is much smaller than the glass shaft and the membrane does not form a great resistance for the oxygen to diffuse, oxygen can accumulate in the electrolyte and the sensor will not be able to discriminate between different oxygen concentrations.

2.2.2 Testing of the oxygen microsensors

The manufacturing of the microsensors resulted in a variety of oxygen sensors with different specifications, so every sensor was tested individually.

An advantage of the Clark-type oxygen microsensor is that the linearity of the signal can be tested in air. For this purpose three gas mixtures with different oxygen concentrations were used (100%, 40% and 20% oxygen). Nitrogen gas was used to determine the zero current.
Table 2.1 Working specifications of five oxygen microsensors.

<table>
<thead>
<tr>
<th>microsensor number</th>
<th>signal (nA) in N₂</th>
<th>Air</th>
<th>H₂O unstirred</th>
<th>H₂O stirred</th>
<th>response time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.06</td>
<td>2.4</td>
<td>2.1</td>
<td>2.2</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>2.4</td>
<td>1.7</td>
<td>1.8</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>3</td>
<td>0.02</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>0.01·10⁻³</td>
<td>0.75·10⁻²</td>
<td>0.75·10⁻²</td>
<td>0.75·10⁻²</td>
<td>&lt; 3</td>
</tr>
<tr>
<td>5</td>
<td>0.01·10⁻²</td>
<td>0.14</td>
<td>0.14</td>
<td>0.14</td>
<td>&lt; 3</td>
</tr>
</tbody>
</table>

Table 2.2 Manufacturing characteristics of five oxygen microsensors.

<table>
<thead>
<tr>
<th>microsensor number</th>
<th>Diameter (µm)</th>
<th>Cathode</th>
<th>Shaft</th>
<th>Membrane</th>
<th>Gap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>10</td>
<td>40</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>10</td>
<td>15</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>&lt; 5</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>&lt; 5</td>
<td>10</td>
<td>5</td>
<td></td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

The response time was measured with nitrogen gas and air. The time was registered for the sensor signal to reach 90% of the signal in air after setting the zero point in nitrogen.

The signal in water deviated by a few percent from that in air. The stirring effect was determined by registering the signal in water with and without stirring. The water was stirred at such a speed that a further increase did not influence the signal. The difference between the two sets of signals should not be above 5 percent. When the difference was greater the sensor was rejected. Table 2.1 gives an example of some of the working specifications of five sensors made at this laboratory, including the electrode that was used for the profile measurements described in this chapter (microsensor number 1). The signal of all the sensors appeared to be linear. Table 2.2 gives the manufacturing characteristics of the same five sensors.

If the sensors were well sealed, no leakages of electrolyte occurred and storage for months was possible. A response of three seconds was the minimal time that could be achieved, this limitation being imposed by the self-made amplifier.
2.2.3 Biocatalyst particle

The biocatalyst particle was made of agarose (gel type 7, Sigma), which contained the enzyme L-lactate-oxygen 2-oxidoreductase, EC 1.13.12.4 from *Mycobacterium smegmatis* provided by Sigma. The type of agarose used has a gelling temperature of 30°C at a concentration of 2% (w/w). Agarose appeared to be a good gel to use for the microsensor measurements because it was not too rigid and did not deform during penetration.

The enzyme used for immobilization catalyses the following decarboxylation reaction:

\[
\text{enzyme} \\
\text{CH}_3\text{CHOHCOOH} + \text{O}_2 \xrightarrow[]{} \text{CH}_3\text{COOH} + \text{CO}_2 + \text{H}_2\text{O} \\
\text{lactic acid} \quad \text{acetic acid}
\]

The immobilization was carried out at 39°C. The experiments were performed in a phosphate buffer, pH 6.2, at 37°C. The L-lactic acid (Sigma) was added in excess to make sure that only oxygen was the limiting substrate.

To obtain different profiles in the particle different amounts of enzyme were immobilized. The concentrations were 0.05, 0.10, 0.25 and 0.50 units per millilitre gel, respectively. For the experiments spherical particles with a diameter of 4 mm were used.

2.2.4 Microsensor measuring equipment

The current of the sensor was amplified by means of a home-made amplifier. The amplifier was connected to a recorder (Kipp & Zonen BD40) and a computer (HP A600), see figure 2.2.

The microsensor was put in a holder. This holder was connected to a motor drive and a micromanipulator (Oriel Model 18011 Encoder Mike controller). It was possible to move the sensor by hand, either stepwise or continually. The distance and speed of movement of the microsensor were regulated by the manipulator. During the experiments the penetration velocity of the sensor was $3 \cdot 10^{-6}$ or $5 \cdot 10^{-6}$ m s$^{-1}$.

For the profile measurements, a flow chamber of perspex was used (see figure 2.3). It consisted of an inner and outer chamber. The volume of the inner chamber was 50 ml, the volume of the outer chamber was 150 ml. The flow chamber was illuminated with an Euremex cold-light illuminator EK-1 and the walls of the inner and outer chamber were plan-parallel so that the process could be followed with a stereomicroscope (Olympus S071).

For each measurement a biocatalyst particle was selected and set in a holder in the inner chamber. The microsensor was positioned by hand right above the particle. The sensor was located so that the tip was at the surface of the particle, corresponding with a distance of 0 μm on the display of the manipulator. A stereomicroscope was used to determine the surface of the
Figure 2.2 Schematic description of the measuring equipment.

Figure 2.3 Perspex flow chamber. The scheme includes an oxygen microsensor (1), flow of liquid in (2) and out (3), outer (4) and inner (5) flow chamber, biocatalyst particle (6), and a holder (7).
particle. The microsensor was then adjusted to a position 300 \( \mu \text{m} \) above the particle to take into account a possible boundary layer around the particle. At the beginning of a measurement the data acquisition and the manipulator were started simultaneously. The data were taken at time intervals of 0.5 s. The signal coming from the sensor was also registered on the recorder in order to follow the process.

Phosphate buffer containing an excess amount of L-lactate was pumped into the outer chamber (the flow was 5 ml s\(^{-1}\)). It flowed over into the inner chamber, parallel to the microsensor, to avoid movement of the sensor. The reason for the recirculation of the medium is to assure a constant oxygen concentration in the liquid phase and a constant temperature. The medium was saturated with air and at a temperature of 37°C.

2.3 Results

The profiles resulting from the measurements are given in the figures 2.4, 2.5 and 2.6. In these figures the normalized oxygen concentration (i.e. the concentration in the particle divided by the concentration in the liquid phase) is shown as a function of the normalized radius (i.e. the radius of the particle minus the location of the electrode divided by the radius of the particle). On the horizontal axis the normalized distance zero corresponds to the center of the particle. It can be seen that the profiles are very smooth. The data for

![Figure 2.4 Oxygen profiles measured in one single particle.](image)
Figure 2.5 Oxygen profiles measured in two different particles having the same enzyme concentration.

Figure 2.6 Oxygen profiles measured in particles with increasing enzyme concentration. 0.05 units ml\(^{-1}\) (---); 0.10 units ml\(^{-1}\) (----); 0.25 units ml\(^{-1}\) (-----); 0.50 units ml\(^{-1}\) (-----).
the figures were not smoothed artificially, the noise level of the signal coming from the sensor was very low. There are slight signal disturbances at the outside of the particle due to the movement of the water.

Because it was possible to use one particle several times (no deformation of the particle took place) the reproducibility of the measurements could be tested. The result can be seen in figure 2.4. These two profiles were determined in a single particle, at two different spots only a few μm apart. As can be seen the profiles match well.

The difference between the profiles of two different particles with the same enzyme concentration was also tested. These profiles were also much alike (figure 2.5).

The profiles shown in figure 2.6 were measured in particles with increasing enzyme concentration. The effect of the internal diffusion limitation is very clear. Diffusion limitation becomes increasingly important as the enzyme loading increases. Using the highest enzyme loading, part of the biocatalyst is not used for the conversion of the substrate.

Because of the high liquid turbulence around the particle the external diffusion layer was relatively small. As can be seen for instance in figure 2.4 the layer was only about 50 μm thick (the diameter of the particle was 4 mm).

For model validation it is important to make sure that the profiles in the particles are symmetrical. One way to check this is to find out if the concentration gradient in the center of the particle becomes zero. This is only an accurate method if the concentration of the substrate in the particle does not become zero. Another way to check the symmetry is to continue the measurement once the center of the particle is reached. It appeared that the profiles were not symmetrical (profiles are not shown). The external mass transfer at the top of the particle was higher than at the bottom because

Figure 2.7 Laminar flow around the particle (1) creates a stagnant zone (2). A plate (3) under the particle creates turbulence and increases the external mass transfer.
of a stagnant zone under the particle. To break the flow and cause more turbulence under the particle a plate placed under this proved to be sufficient. (see figure 2.7). The profiles shown in this chapter were measured after the adaptation of the flow chamber had taken place.

2.4 Acknowledgment

The authors wish to thank Schott Nederland B.V., Tiel, for their gift of different technical glass tubes.

2.5 Bibliography

2. Oxygen profiles in biocatalyst particles
Chapter 3

Use of an oxygen microsensor for the determination of intrinsic kinetic parameters of an immobilized oxygen reducing enzyme*

An oxygen microsensor was used to measure internal oxygen profiles in biocatalyst particles of different diameter and activity. The particles were made of agarose gel and contained an oxygen reducing enzyme, L-lactate monooxygenase. The kinetics of the enzyme could be well described by the Michaelis-Menten equation. From the internal substrate concentration profile the intrinsic kinetic parameters were determined by means of fitting a simulated model to the measurements, using Marquardt’s algorithm. The intrinsic kinetic parameters found following this procedure appeared to be independent of particle radius or enzyme loading used, proving the method to be reliable. These parameters were also compared with the kinetic parameters of the free enzyme which were determined in a biological oxygen monitoring system. The intrinsic kinetic parameters showed a decrease with a factor 2.3 for the $V_m$ value and with a factor 2.7 for the $K_m$ value compared to the parameters for the free enzyme. From this the conclusion can be drawn that the immobilization as such or the carrier material not only can have an effect on the maximum intrinsic conversion rate ($V_m$) but also on the affinity of the enzyme ($K_m$) for oxygen.

3. Intrinsic kinetic parameters determined from oxygen profiles

3.1 Introduction

There is much interest in the possibilities to use immobilized enzymes as industrial catalysts [1]. A large variety of methods exist for the immobilization of enzymes like for example entrapment in or attachment to solid supports. There are, however, only a very limited number of industrial applications of immobilized enzymes. The production of high-fructose syrups from corn starch by means of glucose isomerase is the most important one from an economic point of view [2]. Other enzymes used as industrial biocatalysts are: L-amino-acylase, penicillinamylase, L-aspartase and lactase. The reactions catalysed are all single-step, without the requirement of a cofactor [3].

For the application of immobilized enzymes it is important to know what effects immobilization has on the enzyme. The possible change of overall enzyme activity due to immobilization can be caused by a variety of reasons, including chemical denaturation by the reagents used for immobilization, conformational changes of immobilized enzyme molecules, hindering of the rotational mobility of the substrate (rotational masking [4]), and diffusion limitation of the substrate within the immobilized particle and in the laminar layer surrounding it.

Due to several of the reasons mentioned the kinetic parameters of the immobilized enzyme (the so-called intrinsic kinetic parameters), can be different from the kinetic parameters of the enzyme in free solution (free kinetic parameters). Diffusion limitation does not influence these parameters but only the observed overall enzyme activity. When knowing the intrinsic parameters and the effective diffusion coefficient, insight is gained on the effects of the immobilization on the enzyme. Furthermore, it is possible to predict the conversion capacity of the immobilized biocatalyst for different situations, such as different particle diameters or different enzyme concentrations.

In this chapter a method is introduced, by which the intrinsic kinetic parameters of an immobilized oxygen reducing enzyme, L-lactate monooxygenase, can be determined. The method is based on the measurement of the oxygen concentration profile, making use of an oxygen microsensor. The reaction kinetics have been described by the Michaelis-Menten equation. The kinetic parameters of the free enzyme in solution were also measured, making a comparison with the intrinsic parameters possible.

3.2 Theoretical background

3.2.1 Free enzyme: kinetics

The enzyme catalysed reaction used as a model system in our experiments involves two substrates. When one substrate will be kept in excess (at a concentration high enough to obtain zero order kinetics and relatively small
conversion), it is safe to assume that the kinetics of substrate conversion occur according to Michaelis-Menten kinetics whose well known steady-state velocity equation is nonlinear in terms of $K_m$ and linear in terms of $V_m$. For a batch reactor the substrate conversion rate can be described as follows:

$$\frac{dS}{dt} = \frac{E V_m S}{K_m + S}$$

(3.1)

in which $S$ is the rate limiting substrate concentration (mol m$^{-3}$), $E$ the enzyme concentration (kg m$^{-3}$) and $t$ the time (s).

The maximum velocity $V_m$ (mol kg$^{-1}$ s$^{-1}$) is the result of the conversion of both substrates, the Michaelis-Menten constant $K_m$ (mol m$^{-3}$) holds for the rate-limiting substrate.

Basically one can distinguish two approaches to determine the $K_m$ and $V_m$, the kinetic parameters of the enzyme. The first one is the initial velocity approach as often followed by enzymologists [5]. A problem with this method is the difficulty to estimate the value of the initial velocity because most of the reaction curves are not straight at the beginning of the reaction due to start-up phenomena. When determining the kinetic parameters from these experiments the non-linear Michaelis-Menten equation is transferred into a linear one such that linear regression can be applied. One of the most frequently used transformations is the double-reciprocal plot, the so-called Lineweaver-Burke plot. However, this method is not the most accurate one to determine $V_m$ and $K_m$ because of the unequal weighting of the data points [6]. A statistically better graphical method is the direct-linear plot of Cornish-Bowden and Eisenthal [7].

The initial velocity method is used for the following reasons: the use of simple mathematical techniques; to avoid product inhibition during the experiment so Michaelis-Menten kinetics can be applied; and simply out of tradition.

Another method to determine the kinetic parameters is to monitor continuously the progress of the reaction by measuring product formation and/or substrate disappearance. This approach is followed in our study. The advantage is that the entire curve results from just one experiment. The dependent variable is the substrate concentration, so the rate equation (3.1) should be integrated numerically. After integration, non-linear regression must be used to fit the model to the experiments. This requires more cumbersome mathematical handling and may be one of the important reasons why this method is less often applied. In this chapter, we used the technique as follows. The substrate concentration as a function of time is fitted using a fourth-order Runge Kutta method and the Michaelis-Menten equation (3.1). The sum of squares of the difference between the observed and calculated substrate concentration is minimized. In using this method the following assumption has implicitly been made: the random error is presumed to be normally distributed with
constant standard deviation (independent of the substrate concentration) and independent of previous errors. The concentration at zero time, which forms a boundary condition for the first-order differential equation, is not known. To solve this problem the concentration at zero time is used as a parameter for estimation, as well as $K_m$ and $V_m$.

3.2.2 Marquardt's algorithm for least squares estimation of non-linear parameters

Marquardt's algorithm [8] was chosen as a non-linear regression technique. This numerical optimization method performs an optimum interpolation between the Taylor series method and the gradient method, based upon the maximum neighbourhood in which the truncated Taylor series gives an adequate representation of the non-linear model. It is one of the best non-linear regression techniques with respect to ability and speed of convergence [6]. The method makes use of derivatives in the parameters to determine the direction of the search towards the desired minimum. Our model consists of a first-order differential equation (3.1) that must be solved numerically. This means that for each parameter, the substrate concentration should be determined via a fourth-order Runge Kutta integration routine. This demands a lot of calculation, but profit of time can be gained against derivative-free methods because the algorithm converges in 6 to 10 steps when the initial values are well chosen. In that case the sum of squares changes less than 0.001 % around the minimum.

3.2.3 Immobilized enzyme: kinetics and diffusion

As a model system an oxygen consuming enzyme immobilized in a carrier material was used [9]. The immobilization resulted in spherical biocatalyst particles. Figure 3.1 gives a schematic representation of the concentration gradient due to diffusion limitation and external mass transfer resistance of the rate limiting substrate. For the model describing transport and reaction of the limiting substrate the following assumptions have been made:

- The kinetics of the enzyme can be well described by the Michaelis-Menten equation.
- The enzyme is homogeneously distributed in the particle.
- There is no interaction between the substrates and/or products.
- There is no interaction between the substrates and the carrier material.
- Mass transport of substrates can be described by Fick's law.
- The effective diffusion coefficient for substrate is independent of the substrate concentration.
3.2 Theoretical background

Figure 3.1 Schematic representation of a biocatalyst particle and the substrate gradient.

Taking these assumptions into account the concentration of the substrate as a function of the radius of the particle can be described by the following differential equation:

\[
\frac{\partial S}{\partial t} = D_e \left[ \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial S}{\partial r} \right) \right] - \frac{E V_m S}{K_m + S}
\]  

(3.2)

in which \( D_e \) is the effective diffusion coefficient (m²s⁻¹) and \( r \) the radial distance (m).

For steady state conditions this equation reduces to:

\[
\frac{d^2 S}{dr^2} + \frac{2}{r} \frac{dS}{dr} = \frac{1}{D_e} \frac{E V_m S}{K_m + S}
\]  

(3.3)

There are two boundary conditions needed to solve equation (3.3). The ones normally used are as follows. For a spherical geometry it follows that in the centre of the particle there is no net transport of substrate, so the concentration gradient is zero (the substrate profile in the particle must be symmetrical). Furthermore the transport towards the biocatalyst particle equals the transport into the particle. The gradient in the boundary layer is assumed to be linear (film theory).
\[ r = 0, \quad \frac{dS}{dr} = 0 \quad (3.4a) \]

\[ r = r_p, \quad \frac{dS}{dr} = \frac{k_l}{D_e} (S_l - S_i) = \frac{D}{\delta D_e} (S_l - S_i) \quad (3.4b) \]

in which \( r_p \) is the particle radius (m), \( k_l \) the external mass transfer coefficient (m s\(^{-1}\)), \( \delta \) the thickness of the boundary layer and the subscript \( l \) stands for liquid and \( i \) for interface.

In comparison to other researchers we used a microsensor to measure the substrate concentration profile in the particle, so information about the boundary conditions (3.4) of the differential equation was gained that is normally unknown: the substrate concentration in the centre and at the surface of the particle. From the known boundary conditions, two were selected to solve the differential equation. These were the substrate concentration (measured) and concentration gradient (to be zero), both in the centre of the particle. In the calculation procedure the concentration in the centre of the particle was used also as a parameter to avoid fixing the profile on one point.

In solving the differential equation we used the same integration and optimization techniques as for the free kinetic measurements. The procedure is as follows. The substrate concentration in the particle is calculated with starting parameters using a fourth-order Runge Kutta integration routine. Comparing this profile with the measured profile results in the adjustment of three parameters: the intrinsic kinetic parameters \( V_m \) and \( K_m \), and the concentration in the centre of the particle. So for describing the profile the concentration gradient in the centre of the particle in combination with the parameters is enough. The sum of squares of the differences between the calculated and measured substrate concentration is minimized using Marquardt’s algorithm.

The radius of the particle, the substrate concentration in the liquid phase and the effective diffusion coefficient of the substrate have been determined independently.

3.2.4 Calculation of the overall effectiveness factor

When the intrinsic kinetic parameters and the first derivative at the surface of the particle have been determined, the overall effectiveness factor can be calculated. This factor, which forms a measure for the degree of diffusion limitation and external transport resistance, is defined as the ratio of the observed reaction rate and the reaction rate that would occur at liquid phase substrate concentration. The equation for the effectiveness factor \( \eta \) is as follows:
\[ \eta = \left( \frac{4\pi r_p^2 D_e}{r=r_p} \frac{dS}{dr} \bigg|_{r=r_p} \right) \frac{E V_m S_l}{K_m + S_l} \]  

(3.5)

3.3 Materials and methods

3.3.1 Enzyme

The enzyme used for the experiments was L-lactate 2-monoxygenase, EC 1.13.12.4, from *Mycobacterium smegmatis* obtained from Sigma (package of 5 mg protein corresponding to 100 units). This enzyme was chosen as a model enzyme for reasons of simplicity of the reaction. It is a flavoprotein with FMN as prosthetic group. The substrate itself serves as the electron donor. The decarboxylation reaction is as follows:

\[
\text{enzyme} \quad \text{CH}_3\text{CHOHCOOH} + \text{O}_2 \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 + \text{H}_2\text{O} \quad \text{lactic acid} \quad \text{acetic acid}
\]

An enzyme solution was made by dissolving 5 mg of protein, corresponding with a certain amount of the enzyme, in 20 ml sterile millipore filtered water. It was stored at 0°C for several days. Before this solution was used for the immobilization, the Michaelis-Menten kinetics of the free enzyme were determined. Before each experiment meant to determine the free kinetic parameters of the enzyme, the enzyme solution was vibrated in an ultrasonical bath to make sure that a representative sample was taken.

From the determination of the free kinetic parameters of the enzyme it appeared that the enzyme adsorbed on the finn-pipettes of polypropene which were used for the addition of the enzyme to the buffer solution. This effect was overcome by flushing the pipettes by the enzyme solution. The enzyme did not adsorb on the other materials used, such as glass, perspex or teflon.

No significant deactivation of the free enzyme appeared during the experiments. This was tested by performing several consecutive experiments with the free enzyme. For the immobilized enzyme no difference in profiles between two duplo experiments was observed, within measuring accuracy, thus no significant deactivation during the measurements occurred.

3.3.2 L-lactic acid

Because it was necessary that only oxygen was the rate-limiting substrate some experiments were performed with different L-lactate concentrations. The L-lactate concentration above which the oxygen conversion did not increase was 2.5 kg m\(^{-3}\). Due to this high L-lactate concentration the oxygen
3. Intrinsic kinetic parameters determined from oxygen profiles

solubility decreased to 88% of its standard solubility in water. This was determined by the Winkler titration method [10].

Furthermore, the oxygen profile in the particle must be in equilibrium with the oxygen concentration in the bulk, so it was important to determine the time necessary for reaching a steady state situation in the particle. This time was determined by putting the particles in the medium and measuring oxygen profiles after increasing time. The effective diffusion coefficient of L-lactate was also calculated \((1.4 \cdot 10^{-6} \text{ m}^2 \text{s}^{-1}, \ [11])\) to determine the stabilization time for the situation that the diffusion process is carried out for 90% [12]. From the first method it appeared that 15 minutes was enough to achieve a steady-state situation for a particle of 6 mm. The calculation resulted in a stabilization time of 42 minutes at 37°C. So before the start of each measurement the particles were stored in the medium for at least 30 minutes at 4°C, and for 15 minutes at 37°C. This time was also long enough for the stabilization of the oxygen profile because oxygen was already present in the particle.

3.3.3 Immobilization procedure

For the immobilization of the enzyme agarose was used (gel type 7, Sigma). This type of agarose has a gelling temperature of 30°C at a concentration of 2% (w/w). Agarose appeared to be a very good gel to use for microsensor measurements because it was not rigid and it was not deformed when penetrated by the microsensor.

An agarose solution (4 ml) and some of the dilute enzyme solution (varying from 0.4 to 0.04 ml) were mixed at 39°C (at 30°C the agarose solution was too viscous) during 30 seconds in a 20 ml tube on a whirl-mixer to form a 5% (w/w) agarose-enzyme mixture. This mixing method was tested beforehand by adding a few drops of a brilliant red suspension to the agarose solution. Test-tubes or other tubes with a round bottom did not suffice, only tubes with a flat surface gave good degree of mixing.

The agarose-enzyme mixture was injected into a mould. The temperature of the equipment used was kept at 39°C with an infra-red lamp. This mould was made of perspex and contained spherical holes with a diameter of 4, 5, and 6 mm. After injection the mould was placed at 0°C for half an hour. Hereafter the particles were put in the polyethyleneimine (1% (v/v), pH 8.5) and in the glutaraldehyde (0.25% (v/v), pH 8.5). These chemicals were used to prevent enzyme leakage as observed with previous experiments not using the chemicals. The mechanistic background of the chemical reaction was as follows. Polyethyleneimine diffused only a few micrometers into the particle. After putting the particles in glutaraldehyde it reacted with the amine compounds of the polyethyleneimine and formed a network impermeable to the enzyme. (This procedure is also described by Birnbaum [13]). The treatment with these chemicals did not hinder the penetration of the particles with the
3.3 Materials and methods

microsensor, probably because the matrix itself was not changed. The particles were washed with tap water to remove remnants of glutaraldehyde and stored in a 1,2 propanediol solution (25% (v/v), pH 7). Measurements were performed within a few days.

The immobilization procedure resulted in perfect round spheres.

3.3.4 Biological Oxygen Monitoring system

A Biological Oxygen Monitoring (BOM) system was used to determine the kinetic parameters of the enzyme in solution. This equipment consisted of a reaction vessel, thermostated bath, polarographic oxygen sensor, amplifier, recorder and a data acquisition connection to a computer.

The reaction vessel was made of glass with a volume variable between 10 and 40 ml. The content of the vessel was being stirred at a constant high speed by means of a magnetic stirrer. The reaction vessel was placed in a vessel of perspex that was continuously flushed by water of 37°C. The reaction vessel was closed free from air bubbles with a tightly fitting stopper. The stopper was equipped with a Clark-type polarographic oxygen sensor (Orbisphere, System 4714). The signal from the sensor went via the amplifier to a recorder (Kipp & Zonen BD40) and a computer (HP A690).

Before each measurement the vessel was cleaned with alcohol and filled with a sterile phosphate buffer, and a few ml of a 20% (w/w) lactate solution (L-lactic acid No L-2250, Sigma) resulting in a L-lactate concentration of \(0.44 \times 10^3\) mol m\(^{-3}\) and a pH of 6.0 (optimal pH according to Makovos and Liu [14]). The oxygen concentration at the beginning of an experiment varied between 0.3 and 0.7 mol m\(^{-3}\). The temperature was 37°C. The amount of enzyme added resulted in an average protein concentration of \(2.5 \times 10^{-3}\) kg m\(^{-3}\). The total reaction volume was about 20 ml. The reaction vessel was closed with the stopper and the decrease of the oxygen concentration as function of time was registered.

3.3.5 Oxygen microsensor

The oxygen microsensor used was a combined one, constructed at our laboratory according to Revsbech and Ward [15]. The specimen used for the experiments described in this article had a sensing tip of about 15 μm. The signal coming from the sensor was in water of 37°C: 1 nA, the zero current was \(5 \times 10^{-2}\) nA. The signal was very stable and no measurable drift was found during the experiments.

3.3.6 Microsensor measuring system

The microsensor equipment is described by Hooijmans et al. [9]. A part of the equipment is formed by a perspex flow chamber. The design of the flow chamber guarantees a constant oxygen concentration and temperature in the
bulk of the liquid, and an equal mass transfer resistance around the particle. The flow chamber consists of an inner and outer chamber. The volume of the inner chamber, in which the particle was set, was 50 ml. The flow chamber was illuminated with a cold-light illuminator (Euromex EK-1), so that the particle could be seen through a stereomicroscope (Olympus S071) that was placed before the flow chamber. The surface of the particle could be determined accurately with the use of the microscope, the resolution being about 10 μm.

The microsensor itself was put in a holder above the flow chamber and was positioned a few micrometers above the particle. The holder was connected to a motor drive and a micromanipulator (Oriel Model 18011 Encoder Mike Controller). The signal coming from the sensor was sent through a self-made amplifier to a recorder (Kipp & Zonen BD40) and a computer (HP A600).

For the determination of the intrinsic kinetic parameters particles with a diameter of 4, 5 and 6 mm were used. For each diameter four different oxygen profiles were obtained by using four different enzyme concentrations in the particles.

The optimal enzyme content per particle to obtain substantially different profiles was calculated by means of simulation of the profiles with the kinetic parameters \( V_m \) and \( K_m \) of the free enzyme, determined in the BOM. For this simulation it was also necessary to know the external mass transfer existing in the flow chamber, as well as the effective diffusion coefficient of oxygen in agarose. The mass transfer coefficient was calculated from the results of earlier experiments [9]. From these experiments the thickness of the boundary layer could be determined and consequently the mass transfer coefficient by using equation (3.4b). The average thickness of the boundary layer was 50 μm, the corresponding mass transfer coefficient was \( 4.4 \times 10^{-5} \text{ m s}^{-1} \). In 5% agarose the diffusion coefficient of oxygen is about 20% lower than the value in water [16]. The diffusion coefficient of oxygen in water at 37°C is according to [17] about \( 2.9 \times 10^{-9} \text{ m}^2 \text{s}^{-1} \), so the effective diffusion coefficient used was \( 2.3 \times 10^{-9} \text{ m}^2 \text{s}^{-1} \).

The result of the simulation was that the optimal enzyme concentration was: 0.025, 0.005, 0.0125 and 0.025 kg protein per m³ agarose-solution.

### 3.4 Results and discussion

#### 3.4.1 Kinetics of the free enzyme

Three experiments were performed in the BOM to determine the free kinetic parameters. Figure 3.2 shows an example of the results. The reaction kinetics were determined from the slope of the curve using the non-linear numerical optimization procedure.

As can be seen from figure 3.2 the slope is initially constant indicating zero order reaction, thus for most of the experiment the oxygen concentration was
far higher than the value of $K_m$. From a very low $K_m$ value follows that it was necessary to assure there was no significant leaking of air. Furthermore it was important to start with an oxygen concentration high enough to determine the $V_m$ accurately. The equipment fulfilled these demands.

The average value of the numerical fits for the maximal conversion rate, $V_m$, was $3.60 \cdot 10^{-1}$ mol kg$^{-1}$ s$^{-1}$ with a standard deviation of $0.20 \cdot 10^{-1}$ mol kg$^{-1}$ s$^{-1}$. The $K_m$ value was $0.49 \cdot 10^{-1}$ mol m$^{-3}$ with a standard deviation of $0.05 \cdot 10^{-1}$ mol m$^{-3}$.

According to Lockridge [18] phosphate is, like other anions such as sulphate and chloride, an inhibitor of the lactate-oxidase reaction, competitive with lactate with a $K_i$ of $1.0 - 1.6 \cdot 10^{-2}$ mol m$^{-3}$. Nevertheless phosphate was used as a buffer solution for the experiments. This means that the reaction constant $K_m$ is made up of $K_m(1 + C_i/K_i)$ actually. During the experiments L-lactate was added in excess and did not diminish measurably. When knowing $K_i$, $K_m$ could be calculated. This resulted in a real $K_m$ of $1.8 \cdot 10^{-3}$ mol m$^{-3}$. However, in the following we will only use the overall reaction constant. The interest is not in the real $K_m$, but in the effect of immobilization.

### 3.4.2 Kinetics of the immobilized enzyme

Different diameters ($4 \cdot 10^{-3}$, $5 \cdot 10^{-3}$ and $6 \cdot 10^{-3}$ m) and different enzyme concentrations (0.025, 0.005, 0.0125 and 0.025 kg m$^{-3}$ gel) were used. For each particle the profile was measured at two places varying only a few micrometers, so the total number of measurements was 24. Afterwards it appeared that the particles with a diameter of $5 \cdot 10^{-3}$ m and a protein concentration of
0.0125 and 0.0025 kg m\(^{-3}\) gel were deformed, so the results were not reliable and therefore have not been used.

Figures 3.3 to 3.5 give examples of measured profiles together with the calculated profiles. For the calculation of the line outside the particle the film theory was applied, assuming the mass transfer resistance to be located in a stagnant boundary layer with a certain thickness through which the mass transfer takes place. Because the difference between the oxygen diffusion coefficient in gel and water is only 20\%, the small bend in the curve at the surface of the particle is hard to see. Fitting a data set takes about 10 to 30 seconds CPU-time on the HP Agoo computer. As can be seen the measured and calculated profiles match very good. This was the case with all the profiles, mostly resulting in a very low value of the standard deviation in the kinetic parameters. The average value for the kinetic parameters when all the measurements were taken into account was for \(V_m\) \(1.58 \cdot 10^{-1}\) mol kg\(^{-1}\) s\(^{-1}\) with a standard deviation of \(0.41 \cdot 10^{-1}\) mol kg\(^{-1}\) s\(^{-1}\) and for \(K_m\) \(0.18 \cdot 10^{-1}\) mol m\(^{-3}\) with a standard deviation of \(0.12 \cdot 10^{-1}\) mol m\(^{-3}\). When only one profile per particle was taken into account it resulted in the same kinetic parameters, for \(V_m\) \(1.57 \cdot 10^{-1}\) mol kg\(^{-1}\) s\(^{-1}\) with a standard deviation of \(0.44 \cdot 10^{-1}\) mol kg\(^{-1}\) s\(^{-1}\), for \(K_m\) \(0.18 \cdot 10^{-1}\) mol m\(^{-3}\) with a standard deviation of \(0.13 \cdot 10^{-1}\) mol m\(^{-3}\). This result means that there was hardly any difference between the profiles measured in one particle at two places varying a few micrometers.

![Figure 3.3 Influence of enzyme concentration on oxygen profile. \(d_p = 4\) mm, \(S = 0.2\) mol m\(^{-3}\), measured data for \(E\) is resp. 0.0025 (\(*\)), 0.005 (\(+\)), 0.0125 (\(\circ\)) and 0.025 kg m\(^{-3}\) (\(#\)), and fitted curve.](image-url)
3.4 Results and discussion

Figure 3.4 Influence of enzyme concentration on oxygen profile. \( d_p = 5 \text{ mm}, \ S = 0.2 \ \text{mol m}^{-3} \), measured data for \( E = 0.0025 \ (\ast) \) and \( 0.005 \ \text{kg m}^{-3} \ (\ast\ast) \), and fitted curve.

Figure 3.5 Influence of enzyme concentration on oxygen profile. \( d_p = 6 \text{ mm}, \ S = 0.2 \ \text{mol m}^{-3} \), measured data for \( E \) resp. \( 0.0025 \ (\ast) \), \( 0.005 \ (\ast\ast) \), \( 0.0125 \ (\circ) \) and \( 0.025 \ \text{kg m}^{-3} \ (\#) \), and fitted curve.
Table 3.1 The intrinsic kinetic parameters determined from experiments with varying enzyme concentrations in the particles.

<table>
<thead>
<tr>
<th>number of measurements</th>
<th>prot.conc. ( \cdot 10^{-2} ) (kg m(^{-3})gel)</th>
<th>( V_m \cdot 10^{-1} ) (mol kg(^{-1})s(^{-1}))</th>
<th>( K_m \cdot 10^{-1} ) (mol m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.25</td>
<td>1.60 ± 0.19</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>2.02 ± 0.17</td>
<td>0.28 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
<td>1.52 ± 0.13</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>0.94 ± 0.07</td>
<td>0.09 ± 0.03</td>
</tr>
</tbody>
</table>

The standard deviation in the value of \( V_m \) can be explained by the addition of the enzyme. The enzyme solution was probably not vibrated enough before a sample was taken, resulting in a deviation of the concentration that was intended. This is illustrated by the results presented in table 3.1. Table 3.1 gives an overview of the average value of the intrinsic parameters for the different enzyme concentrations. As can be seen there was a variation between the average values of the \( V_m \). When looking at one enzyme concentration it is clear that the standard variation is much lower than when taking all the profiles into account.

There appeared to be a kind of optimal profile and consequently protein concentration to determine \( K_m \) accurately. This can be made clear as follows. When the parameter value is low compared to the average oxygen concentration in the particle, as is the case for the protein concentration of 0.0025 kg m\(^{-3}\) gel (see the flat profiles in figure 3.3 the \( K_m \) value is about 0.1), the \( K_m \) is difficult to determine. Fitting the data from the flat profiles result in a \( K_m \) with a high standard deviation (results not shown), indicating that the profile is insensitive for the value of \( K_m \). On the other hand, when the profile is very steep, as is the case for the protein concentration of 0.025 kg m\(^{-3}\) gel (see figure 3.3 and 3.5) the standard deviation for \( K_m \) is also high. The influence of diffusion limitation is too large for an accurate determination of the \( K_m \). The profile is then almost completely determined by the maximal conversion rate and the diffusion limitation. The optimal protein concentration for the determination of \( K_m \) was 0.0125 kg m\(^{-3}\) gel. The results given in table 3.1 support this theory. The standard deviation is highest for the protein concentration of 0.0025 kg m\(^{-3}\) gel and decreases till the protein concentration is 0.0125 kg m\(^{-3}\) gel. The standard deviation is higher again for the protein concentration of 0.025 kg m\(^{-3}\) gel.

Table 3.2 gives the intrinsic parameters as a function of the diameter of the particle. The effects mentioned disappear when the results obtained with different enzyme concentrations are mixed. There is no difference to be seen between the average values of \( V_m \) and \( K_m \) per diameter, which is in accor-
3.4 Results and discussion

Table 3.2 The intrinsic kinetic parameters determined from experiments with varying particle diameter.

<table>
<thead>
<tr>
<th>diameter $\cdot 10^{-3}$ (m)</th>
<th>number of measurements</th>
<th>$V_m \cdot 10^{-1}$ (mol kg$^{-1}$a$^{-1}$)</th>
<th>$K_m \cdot 10^{-1}$ (mol m$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8</td>
<td>1.49 $\pm$ 0.37</td>
<td>0.16 $\pm$ 0.09</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1.68 $\pm$ 0.37</td>
<td>0.14 $\pm$ 0.16</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>1.61 $\pm$ 0.49</td>
<td>0.22 $\pm$ 0.12</td>
</tr>
</tbody>
</table>

The data are calculated as the mean values that resulted from experiments with $E = 0.0025$, 0.0050, 0.0125 and 0.025 kg m$^{-3}$.

dance with what would be expected. Although the standard deviation for the intrinsic kinetic parameters is rather high they appear to be, in contradiction to the apparent parameters, independent of the particle diameter or enzyme loading used.

In earlier measurements there was no significant difference to be seen between profiles measured in different particles having the same diameter and protein concentration [9]. This again was tested for one protein concentration to make sure that the enzyme was really homogeneously distributed in the protein-agarose solution. The results can be seen in figure 3.6. The variation in concentration was about 3%. The values of the intrinsic parameters were resp. $1.39 \cdot 10^{-1}$ and $1.43 \cdot 10^{-1}$ mol kg$^{-1}$ s$^{-1}$ for $V_m$ and 0.32 $\cdot 10^{-1}$ and

Figure 3.6 Duplo measurement. $d_p = 4$ mm, $S = 0.2$ mol m$^{-3}$, $E = 0.0125$ kg m$^{-3}$. 
Table 3.3 The overall effectiveness factor for the different enzyme concentrations in the particles.

<table>
<thead>
<tr>
<th>prot.conc. ( \cdot 10^{-3} ) (kg m(^{-3})gel)</th>
<th>diameter ( \cdot 10^{-3} ) (m)</th>
<th>overall effectiveness factor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>0.25</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>6</td>
<td>90</td>
</tr>
<tr>
<td>0.50</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>0.50</td>
<td>5</td>
<td>78</td>
</tr>
<tr>
<td>0.50</td>
<td>6</td>
<td>68</td>
</tr>
<tr>
<td>1.25</td>
<td>4</td>
<td>75</td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>1.25</td>
<td>6</td>
<td>51</td>
</tr>
<tr>
<td>2.50</td>
<td>4</td>
<td>67</td>
</tr>
<tr>
<td>2.50</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>2.05</td>
<td>6</td>
<td>51</td>
</tr>
</tbody>
</table>

0.26 \( \cdot 10^{-1} \) mol m\(^{-3}\) for \( K_m \).

Table 3.3 gives the overall effectiveness factor calculated for the different enzyme concentrations in the biocatalyst particles. For a steep oxygen profile the overall effectiveness factor is still 50%, because the effectiveness factor is determined by the volume of the biocatalyst and not by the radius.

When the results of the microsensor measurements are compared with the results of the BOM, the free kinetic parameters with a \( V_m \) of 3.60 \( \cdot 10^{-1} \) mol kg\(^{-1} \) s\(^{-1} \) with a standard deviation of 0.20 \( \cdot 10^{-1} \) mol kg\(^{-1} \) s\(^{-1} \), and a \( K_m \) of 0.49 \( \cdot 10^{-1} \) mol m\(^{-3} \) with a standard deviation of 0.05 \( \cdot 10^{-1} \) mol m\(^{-3} \) the following conclusions can be drawn: The value of the \( V_m \) decreased with a factor 2.3, the value of \( K_m \) decreased with a factor 2.7. When only the protein concentration of 0.0125 kg m\(^{-3} \) gel is taken into account for the intrinsic \( K_m \), the \( K_m \) value decreased with a factor 2.0. The kinetic parameters are actually changed due to the immobilization procedure or effect of the carrier material.

It is interesting to note that the affinity of the enzyme for oxygen increased. A good explanation for this phenomenon is missing. The decrease in \( V_m \) can be caused by denaturation of the enzyme during immobilization.

### 3.5 Conclusions

The oxygen microsensor appeared to be a new and accurate tool for the determination of the intrinsic kinetic parameters of an immobilized enzyme, that catalyses a reaction involving oxygen. The measured internal oxygen profiles showed a good reproducibility, the variation in concentration was about 3%.
A model describing mass transfer and reaction (Michaelis-Menten kinetics) was used to fit the experimental profiles. Because the concentration profile in the particle was measured the concentration at the surface and by that the external mass transfer resistance was accurately known, in comparison to other methods found in literature.

Although the standard deviation for the intrinsic kinetic parameters was rather high it seemed they were, in contradiction to the apparent parameters, independent of the particle diameter and enzyme loading used, proving the method to be reliable. The average value found for the intrinsic kinetic parameters was for \( V_m \): \( 1.58 \cdot 10^{-1} \text{ mol kg}^{-1} \text{ s}^{-1} \) with a standard deviation of \( 0.41 \cdot 10^{-1} \text{ mol kg}^{-1} \text{ s}^{-1} \), and for \( K_m \): \( 0.18 \cdot 10^{-1} \text{ mol m}^{-3} \) with a standard deviation of \( 0.12 \cdot 10^{-1} \text{ mol m}^{-3} \).

There appeared to be an optimal profile and consequently enzyme concentration for the determination of \( K_m \). The resulting \( K_m \) value for the optimal protein concentration was \( 0.25 \cdot 10^{-1} \text{ mol m}^{-3} \) with a standard deviation of \( 0.05 \cdot 10^{-1} \text{ mol m}^{-3} \).

The free kinetic parameters were different from the intrinsic kinetic parameters, having a \( V_m \) of \( 3.60 \cdot 10^{-1} \text{ mol kg}^{-1} \text{ s}^{-1} \) with a standard deviation of \( 0.20 \cdot 10^{-1} \text{ mol kg}^{-1} \text{ s}^{-1} \), and a \( K_m \) of \( 0.49 \cdot 10^{-1} \text{ mol m}^{-3} \) with a standard deviation of \( 0.05 \cdot 10^{-1} \text{ mol m}^{-3} \).

Comparing the intrinsic kinetic parameter with the free kinetic parameters it can be seen that the value of \( V_m \) decreased with a factor 2.3, the \( K_m \) value decreased with a factor 2.7. When only the optimal protein concentration is taken into account for the intrinsic \( K_m \), the \( K_m \) decreased with a factor 2.0. The kinetic parameters change as a consequence of the immobilization procedure or the effects the carrier material might have on the enzyme. The decrease in \( V_m \) can be explained by denaturation of the enzyme during immobilization, a good explanation for the decrease in \( K_m \) is missing.

The results obtained from the experiments have shown that an oxygen microsensor can be applied to elucidate the behaviour of immobilized, oxygen consuming enzymes or cells.

### 3.6 Acknowledgements

The authors wish to thank H.J. Vos for carefully reading this manuscript, and J.W. Schilperoort for his assistance in implementing Marquardt's algorithm.
3.7 Nomenclature

- $D$: diffusion coefficient (m$^2$s$^{-1}$)
- $D_e$: effective diffusion coefficient (m$^2$s$^{-1}$)
- $d_p$: particle diameter (m$^3$)
- $E$: enzyme concentration (kg m$^{-3}$)
- $k_l$: external mass transfer coefficient (m$s^{-1}$)
- $K_m$: Michaelis-Menten constant for the rate limiting substrate (mol m$^{-3}$)
- $r$: radial distance (m)
- $r_p$: radius of the particle (m)
- $S$: rate limiting substrate concentration (mol m$^{-3}$)
- $t$: time (s)
- $V_m$: maximal substrate conversion rate (mol kg$^{-1}$s$^{-1}$)

Greek symbols

- $\delta$: thickness of boundary layer (m)
- $\eta$: effectiveness factor (-)

Subscripts

- $i$: at the surface
- $l$: in the liquid
- $o$: in the centre

3.8 Bibliography


3. Intrinsic kinetic parameters determined from oxygen profiles
Chapter 4

Comparison of two experimental methods for the determination of Michaelis-Menten kinetics of an immobilized enzyme

For the application of immobilized enzymes, the influence of immobilization on the activity of the enzyme should be known. This influence can be obtained by determining the intrinsic kinetic parameters of the immobilized enzyme, and by comparing them with the kinetic parameters of the suspended enzyme. This chapter deals with the determination of the intrinsic kinetic parameters of an agarose-gel bead immobilized oxygen consuming enzyme: L-lactate 2-monoxygenase. The reaction rate of the enzyme can be described by Michaelis-Menten kinetics. Batch conversion experiments using a biological oxygen monitor, as well as steady state profile measurements within the biocatalyst particles using an oxygen microsensor were performed.

Two different mathematical methods were used for the batch conversion experiments, both assuming a pseudo-steady state situation with respect to the shape of the profile inside the bead. One of the methods used an approximate relation for the effectiveness factor for Michaelis-Menten kinetics which interpolates between the analytical solutions for zero- and first-order kinetics. The other mathematical method was based on a numerical solution and combined a mass balance over the reactor with a mass balance over the bead. The main difference in the application of the two methods is the computer calculation time; the completely numerical calculation procedure was about 20 times slower than the other calculation procedure.

The intrinsic kinetic parameters resulting from both experimental methods were compared with each other to check the reliability of the methods. There was no significant difference in the intrinsic kinetic parameters obtained from the two experimental methods. By comparison of the kinetic parameters for
the suspended enzyme with the intrinsic kinetic parameters for the immobilized enzyme, it appeared that immobilization caused a decrease in the value of $V_m$ by a factor two, but there was no significant difference in the values obtained for $K_m$.

4.1 Introduction

For the description of the diffusion-limited reaction rate of an immobilized enzyme, the intrinsic kinetic parameters are important parameters. They are, in contrast to the apparent kinetic parameters, independent of carrier dimension or enzyme loading. Moreover, the kinetic parameters for the immobilized enzyme may be different from those of the suspended enzyme because of, among others, interactions with the carrier or deactivation due to immobilization.

The conversion of substrate by an immobilized enzyme is described by a differential equation combining diffusion and reaction. The steady state mass balance equation for a single rate limiting substrate in a spherical particle assuming Michaelis-Menten kinetics is given by:

$$D_e \left[ \frac{1}{r^2} \frac{d}{dr} \left( r^2 \frac{dS}{dr} \right) \right] = \frac{EV_m S}{K_m + S}$$  \hspace{1cm} (4.1)

The boundary conditions used to solve this equation are as follows. At the centre of the particle there is no net transport of substrate, so the concentration gradient is zero. Furthermore the transport towards the particle equals the radial transport in the particle.

$$r = 0, \quad \frac{dS}{dr} = 0 \hspace{1cm} (4.2a)$$

$$r = r_p, \quad \frac{dS}{dr} = \frac{k_l}{D_e} (S_l - S_i) \hspace{1cm} (4.2b)$$

To determine the kinetic parameters $V_m$ and $K_m$ of the immobilized enzyme, the differential equation (4.1) must be solved. This is not possible analytically for Michaelis-Menten kinetics. In literature various techniques have been described to determine intrinsic kinetic parameters. Some researchers have used the kinetic parameters of the suspended enzyme as an approximation of the intrinsic kinetic parameters [1]. Engasser [2] assumed that the conversion rate will reach the maximum value when measured at very high substrate concentrations. Narisham [3] made use of the fact that, at very high and very low substrate concentrations, Michaelis-Menten kinetics can be approximated by zero- and first-order kinetics, respectively. For zero- and first-order kinetics an analytical solution is available.
Using a numerical calculation procedure, the intrinsic kinetic parameters can be determined from a single batch experiment using enzyme immobilized in beads [4]. During the experiment, the bulk substrate concentration is monitored as function of time. The batch experiment is then simulated using the kinetic parameters of the suspended enzyme as first estimation for the parameters to be determined. The intrinsic kinetic parameters can be obtained by adjusting the estimated parameters such that the simulation will fit the experiments [5]. A disadvantage of this method is the relatively long computer time needed.

In this chapter, a new method for the calculation of the intrinsic kinetic parameters from a single batch experiment is introduced. The method makes use of the generalized Thiele modulus as defined by Aris [6] and Bischoff [7]. An expression for the effectiveness factor which interpolates between the analytical solutions for zero- and first-order cases, as was suggested by Kobayashi [8], provides the basis for the iterative procedure. An advantage of this method is the short computer calculation time. Application of the method is illustrated using oxygen conversion data obtained from batch experiments in a biological oxygen monitor. The biocatalyst beads were made of agarose-gel containing the enzyme L-lactate 2-monoxygenase. For comparison the data have also been processed using the numerical calculation method mentioned above. Furthermore, another experimental approach [9], which involves steady state measurements carried out with an oxygen microsensor, was used. The combination of the results obtained from both types of experimental methods using the same biocatalysts beads completes the analysis. This procedure is schematically represented in figure 4.1.

4.2 Modelling

4.2.1 Kinetics of the suspended enzyme

The enzyme-catalysed model reaction in our experiments involves two substrates. When one substrate is kept in excess, the Michaelis-Menten equation for a single substrate can be applied. The kinetic parameters for the suspended enzyme were determined using the experimental data resulting from measuring the substrate conversion in batch as function of time. After numerical integration of the Michaelis-Menten rate equation, non-linear regression was applied in order to fit the model to the experiments, resulting in values for $K_m$ and $V_m$. A more explicit description was given by Hooijmans [10].

4.2.2 Kinetics of the immobilized enzyme

The intrinsic kinetic parameters of the immobilized enzyme can be determined from batch experiments and steady state profile measurements using
Figure 4.1 Schematic representation of the dynamic and steady state method for the determination of intrinsic kinetic parameters of an immobilized, oxygen consuming enzyme.
an oxygen microsensor. These methods will be explained in the following sections.

### 4.2.3 Batch experiments assuming pseudo-steady state

**Completely numerical approach**

Combination of the steady state mass balance equation (4.1) with a mass balance over the reactor results in a description of the substrate decrease in the liquid phase as a function of time. For this, pseudo-steady state with respect to the internal profile is assumed, so every measured substrate concentration in the liquid corresponds to a certain concentration profile inside the particle. Simulation of the measured curve can be obtained using the kinetic parameters of the suspended enzyme as starting values. By adjustment of the parameters such, that the simulation fits the measured substrate decrease in the liquid, the intrinsic kinetic parameters are obtained. This method is relative computer-time consuming.

**Alternative mathematical approach**

The alternative mathematical approach makes use of the relation between the effectiveness factor and the generalized Thiele modulus and an approximate expression for the effectiveness factor for Michaelis-Menten kinetics which interpolates between the zero- and first-order kinetics, and can be explained as follows. The measured curve describing the substrate decrease in the liquid is fitted with Michaelis-Menten kinetics neglecting the influence of diffusion limitation, using numerical integration of the Michaelis-Menten rate equation and non-linear regression. The resulting values are the so-called apparent $K_m$ and $V_m$. Next, from the substrate concentration measured as function of time, the experimental conversion rates for each measured concentration are obtained. The rest of the procedure can best be explained on the basis of the equations needed.

The decrease of substrate concentration in the batch is the product of maximum conversion rate and overall effectiveness factor. This is expressed by the following relation:

$$\frac{dS_i}{dt} = v(S_i) = \eta_{ov} \left( \frac{E V_m S_i}{K_m + S_i} \right) \quad (4.3)$$

When the overall effectiveness factor is known the values for the intrinsic $K_m$ and $V_m$ can be obtained. The overall effectiveness factor is the product of the internal and external effectiveness factors:

$$\eta_{ov} = \eta_{in} \eta_{ex} \quad (4.4)$$
For Michaelis-Menten kinetics the following equation gives the relation for the external effectiveness factor, which accounts for the decrease in reaction rate due to external mass transfer resistance:

\[ \eta_{ee,MM} = \frac{S_i/(K_m + S_i)}{S_i/(K_m + S_i)} \]  \hspace{1cm} (4.5)

The internal effectiveness factor is the actual reaction rate relative to the reaction rate that would be observed when no internal diffusion limitation would occur:

\[ \eta_{in,MM} = \frac{dS_i/dt}{(EV_mS_i)/(K_m + S_i)} \]  \hspace{1cm} (4.6)

It is possible to give an approximate relation for the internal effectiveness factor for Michaelis-Menten kinetics which is valid for all geometries, by interpolating between the analytical solutions available for first- and zero-order reactions, the two extremes of the Michaelis-Menten kinetics [8, 11].

\[ \eta_{in,MM} = \Theta \eta_{in,0} + (1 - \Theta) \eta_{in,1} \]  \hspace{1cm} (4.7)

\( \Theta \) is a weighting factor with a value between 0 and 1 and fulfills the following asymptotic values:

\[ S \gg K_m, \quad \Theta \rightarrow 1, \quad \eta_{MM} \rightarrow \eta_0 \]  \hspace{1cm} (4.8a)

\[ S \ll K_m, \quad \Theta \rightarrow 0, \quad \eta_{MM} \rightarrow \eta_1 \]  \hspace{1cm} (4.8b)

Kobayashi [8] gave an approximate expression for \( \Theta \) that was further optimized, yielding the following correlation for spherical geometry:

\[ 0 \leq \Phi_{MM} \leq \frac{1}{\sqrt{3}}, \quad \Theta = \Theta_1 - \sqrt{3} (\Theta_1 - \Theta_2) \Phi_{MM} \]  \hspace{1cm} (4.9a)

\[ \Phi_{MM} > \frac{1}{\sqrt{3}}, \quad \Theta = \Theta_2 + \frac{1}{0.7 + \Phi_{MM} - \sqrt{3}} \Theta_2 (\Phi_{MM} - \sqrt{3}) \]  \hspace{1cm} (4.9b)

in which:

\[ \Theta_1 = \frac{S_i}{S_i + 1.4K_m} \]  \hspace{1cm} (4.10)

\[ \Theta_2 = \frac{S_i}{S_i + 2.9K_m^{0.87}} \]  \hspace{1cm} (4.11)

These equations resulted in a deviation of the internal effectiveness factor of maximal 1% to the exact numerical solution. In the above equations \( \Phi_{MM} \)
is the generalized Thiele modulus for Michaelis-Menten kinetics. The general-ized Thiele modulus [6] is the ratio of diffusion and reaction time and is given by the following equation:

$$\Phi = \frac{V_p}{A_p \sqrt{2}} \left( \int_{S_{eq}}^{S_i} D_e R(s) dS \right)^{-\frac{1}{2}}$$  \hspace{1cm} (4.12)

$S_{eq}$, the equilibrium concentration that would be attained at the centre of an infinite bead [12], is zero if irreversible kinetics are used. For zero- and first-order reaction kinetics analytical integration of equation (4.12) results in:

$$\Phi_0 = \frac{r_p}{3} \left( \frac{V_m}{2D_e S_i} \right)^{\frac{1}{2}}$$  \hspace{1cm} (4.13)

$$\Phi_1 = \frac{r_p}{3} \left( \frac{V_m}{D_e K_m} \right)^{\frac{1}{2}}$$  \hspace{1cm} (4.14)

The relationships between the Thiele moduli and the effectiveness factors in the limiting zero- and first-order cases are as follows. For zero-order:

$$\Phi_0 \leq \frac{1}{\sqrt{3}}, \quad \eta_0 = 1$$  \hspace{1cm} (4.15a)

$$\Phi_0 > \frac{1}{\sqrt{3}} \frac{1}{\Phi_0^2} = \left[ 9 - 9 (1 - \eta_0)^{\frac{3}{2}} - 6 \eta_0 \right]$$  \hspace{1cm} (4.15b)

and for first-order:

$$\eta_1 = \frac{1}{3\Phi_1^2} \left[ 3\Phi_1 \coth(3\Phi_1) - 1 \right]$$  \hspace{1cm} (4.16)

Substituting the Michaelis-Menten rate equation in equation (4.12) and replace it by the experimental conversion rate resulting from the measured curve, and the effectiveness factor for Michaelis-Menten kinetics, $\eta_{in,MM}$ [13], results in:

$$\Phi_{MM} = \frac{r_p}{3} \left( \frac{V_{exp}/\eta_{in,MM}}{2D_e (K_m + S_i) \left( 1 + \frac{K_m}{S_i} \ln \left( \frac{K_m}{S_i} - S_i \right) \right) \right)^{\frac{1}{2}}$$  \hspace{1cm} (4.17)

$S_i$ is found experimentally if the mass transfer process is described by equation (4.18).

$$k_1 a (S_i - S_i) = \frac{dS_i}{dt}$$  \hspace{1cm} (4.18)

A value for the external mass transfer coefficient ($k_1$) is obtained by using an empirical correlation from literature [14]. The values for internal effectiveness factors ($\eta_{in,MM}$) for each measured substrate concentration in
the liquid, can now be determined as follows. Starting from an initial estimate for \( \eta_{in,MM} = 1 \), the Thiele modulus (\( \Phi_{MM} \)) for a certain surface concentration is calculated using the estimated value for \( \eta_{in,MM} \), the value for \( K_m \) (which results from fitting the substrate curve) and the experimental conversion rate (equation (4.17)). Values for \( \eta_0 \) and \( \eta_1 \) are calculated using the estimated \( K_m, V_m \) and the surface concentrations, \( S_i \), see equations (4.13)-(4.16). Then the weighting factors, \( \Theta \), are calculated using \( \Phi_{MM}, S_i \) and \( K_m \) (see equation (4.9)). The internal effectiveness factors (\( \eta_{in,MM} \)) are calculated using \( \eta_0, \eta_1 \) and \( \Theta \) (equation (4.7)), and subsequently compared with the present estimated internal effectiveness factors. This procedure is repeated until convergence is obtained. The values for the external effectiveness factors, \( \eta_{ex} \), are calculated using equation (4.5), the value for \( K_m \) which results from the fit, and the surface concentration for each time period. With the values for the internal and external effectiveness factors, the overall effectiveness factors can be calculated for each measured concentration and used to correct the conversion rates for that situation, in which diffusion would be infinitely fast, resulting in a new substrate concentration curve. This new curve, being steeper than the previous one, is fitted again assuming Michaelis-Menten kinetics, and the whole procedure is repeated until convergence is obtained, i.e., until \( K_m \) and \( V_m \) do not change any more within certain tolerance (sum of squares less than 0.001% around the minimum). The curve obtained in this way is no longer influenced by diffusion, and is being described by the intrinsic kinetic parameters.

4.2.4 Steady state situation

For the description of the substrate concentration within the biocatalyst beads under steady state conditions, only a mass balance over the biocatalyst as a function of the location (equation (4.1)) is necessary. Instead of boundary condition (4.2b), another boundary condition can be used to solve the equation (4.1), namely:

\[
\tau = r_p, \quad S = S_i \quad (4.19)
\]

This boundary condition can be selected when a microsensor is used because the concentration at the surface of the bead can be measured. This in contrast to the other experimental method used. The numerical fit procedure is simpler to explain as that for the pseudo-steady state approach. After numerical integration of equation (4.1), the sum of squares of the differences between the calculated and measured substrate concentration profile inside the bead is minimized, directly resulting in values for \( V_m \) and \( K_m \).
4.3 Materials and methods

4.3.1 Enzyme

The enzyme used for the experiments was L-lactate 2-monoxygenase, EC 1.13.12.4., from *Mycobacterium smegmatis* obtained from Sigma. This enzyme has been used before as a model system [10]. The decarboxylation reaction involves L-lactate and oxygen. It appeared that at a L-lactate concentration of 2.5 kg m\(^{-3}\), oxygen was the sole rate-limiting substrate, so Michaelis-Menten kinetics could be applied.

4.3.2 Biological Oxygen Monitor

A Biological Oxygen Monitor (BOM) was used to determine the free kinetic and intrinsic kinetic parameters of the enzyme, by measuring the decrease of oxygen in a buffer. The BOM consisted of a thermostatically controlled glass vessel (37° C) which could be closed with a tightly fitting stopper containing a Clark-type oxygen sensor. A personal computer (IBM-PS/2) was used for data acquisition. The data file created by the data acquisition program was transferred to a HP 9000 computer which was used for calculations and data fitting. For the determination of the kinetics of the suspended enzyme, enzyme was added to the buffer solution (pH 6.2) resulting in a final protein concentration of 1.2 – 2.0 \( \cdot 10^{-3} \) kg m\(^{-3}\).

4.3.3 Microsensor

The microsensor measurements were carried out using a Clark-type oxygen microsensor which was constructed according to the method described by Revsbech and Ward [15]. Three electrodes were used. For the measurements performed with hexane-made beads, the diameter of the tip of the sensor was 20 \( \mu \)m, the standard current in air-saturated water (37° C) 0.25 nA and the zero-oxygen reading was 0.04 nA. For the measurements carried out with oil-made beads, two sensors were used, the diameters of the electrode tip were 5 \( \mu \)m and 20 \( \mu \)m, the standard currents in air-saturated water (37° C) were 7.5 pA and 0.27 nA, and the zero-oxygen readings were 0.01 pA and 0.03 nA.

4.3.4 Microsensor measuring system

The equipment used was described in detail by Hooijmans et al. [9]. A biocatalyst particle was placed in a flow chamber and an air-saturated buffer solution (pH 6.2, 37° C, oxygen concentration is 0.21 mol m\(^{-3}\)) containing L-lactate (2.5 kg m\(^{-3}\)) was continuously circulated (4 ml s\(^{-1}\)) in order to provide a constant environment for the biocatalyst. Measurements were started after stabilization of the sample and positioning the microsensor 300 \( \mu \)m above the surface. The holder, in which the microsensor was mounted, was connected
to a motor drive and a micromanipulator in order to regulate the distance and speed (5 \( \mu \text{m s}^{-1} \)) of the sensor movement. After amplification the signal from the sensor was recorded by a personal computer (IBM-PS/2). The data file created by the data acquisition program was transferred to a HP A900 computer which was used for calculations and data fitting.

4.3.5 Criteria for immobilization

Because the intrinsic kinetic parameters were determined using the biocatalysts under steady state (profile measurement) as well as pseudo-steady state (BOM) conditions, some criteria for the optimal enzyme loading and particle diameter were formulated. For the profile measurement, the minimum diameter of the beads was 2 mm. This limitation was imposed by the bead holder in the flow chamber and moreover, the smaller the bead the greater the influence of the microsensor. Furthermore, the immobilization material should be easily penetrated by the microsensor. It was found that agarose fulfills this requirement. For the BOM measurements, the experiment should be short enough to limit the influence of oxygen leakage into the reaction vessel at very low oxygen concentrations. A first-order deactivation constant of \( 6 \cdot 10^{-7} \text{s}^{-1} \) was found for the free enzyme (number of data points equals 5), resulting in a half life of 321 h. No significant deactivation for the immobilized enzyme was found during three consecutive experiments. At a low oxygen concentration (0.005 mol m\(^{-3}\)), 1 \( \cdot \) 10\(^{-5} \) mol m\(^{-2} \) s\(^{-1} \) oxygen leaked into the reaction vessel, 15 times lower than the oxygen conversion at that particular concentration. If the conversion experiment does not last longer than 1 hour, the influence of oxygen leakage is negligible.

4.3.6 Immobilization procedure

Agarose (gel type 7, Sigma) and a dilute enzyme suspension were mixed at 37°C for 30 s on a whirlmixer. The final mixture had an enzyme concentration of 0.0125 kg m\(^{-3}\) suspension, and the agarose concentration was 5% (w/w). A thermostatically controlled (39°C) perspex vessel was filled with the mixture. The mixture in the vessel was the forced (by air pressure) through a nozzle made of perspex and teflon. By blowing wet air along the teflon point, the diameter of the drops could be regulated. The drops fell into a tube filled with either hexane or olive oil at a temperature of 0°C, giving spherical beads in the apolar phase because agarose, a polysaccharide, is polar. Bead which had formed in oil were then washed with hexane. Because of its low evaporation temperature, the remaining traces of hexane could easily be removed by evaporation. The beads were then put into polyethyleneimine (1% v/v, pH 8.5) and then into glutaraldehyde (0.25% v/v, pH 8.5), each for 5 minutes, in order to prevent enzyme leakage. Finally, the beads were washed with tap water and stored in a buffer solution (pH 7). Measurements were carried out within a few days.
4.3.7 Calculation procedure

The same mathematical techniques were used in the numerical calculations to determine the kinetic parameters of the suspended and immobilized enzymes. In all cases, numerical integration was applied using the fourth-order Runge Kutta integration method, and as non-linear regression technique Marquardt's compromise algorithm [16] was employed to fit the calculated data to the measurements. From the data sets, about 100 data points were used in the fit procedure.

In the calculations, values for the diffusion and mass transfer coefficients were needed. Although necessary for the BOM measurements, a value for the mass transfer coefficient was not necessary for the profile measurements because the external mass transfer resistance was determined by measuring the surface concentration with the microsensor. A value for the external mass transfer coefficient was calculated using an empirical relation from the literature [14] \( (7 \cdot 10^{-5} \text{ m s}^{-1}) \). As a comparison, a value of \( 5 \cdot 10^{-5} \text{ m s}^{-1} \) was measured for the flow chamber under the conditions used [17]. The effective diffusion coefficient for oxygen in 5% agarose 37°C is \( 2.3 \cdot 10^{-9} \text{ m}^2\text{s}^{-1} \) [18]. The effective diffusion coefficient for L-lactate needed to calculate the stabilization time for the beads, is \( 1.4 \cdot 10^{-9} \text{ m}^2\text{s}^{-1} \) [10]. This results in a stabilization time of 10 minutes for a bead of 3 mm for the situation that the diffusion process is carried out for 90%.

4.4 Results

4.4.1 Comparison of the methods by means of simulations

The mathematical calculation procedures assuming pseudo-steady state were compared with each other by simulation as follows. With the completely numerical method a concentration-time curve was simulated using certain values for \( V_m \) and \( K_m \). This curve was fit with the alternative mathematical method. This resulted in exactly the same values for the intrinsic kinetic parameters and proves the procedure to be principally correct.

A comparison between the steady state and pseudo-steady state method using simulations was also made. Using the standard numerical method, a concentration profile in a bead was simulated. This profile was fitted with the steady state program. Again this resulted in the same values for the kinetic parameters.

4.4.2 Kinetics of the suspended enzyme

The kinetic parameters for the Michaelis-Menten equation of the suspended enzyme were determined in six-fold. The average value found for the maximum conversion rate \( V_m \) is \( 0.48 \pm 0.05 \text{ mol kg}^{-1}\text{s}^{-1} \), and for \( K_m \) is \( 0.07 \pm \)
0.01 mol m\(^{-3}\). Fitting the equation for one data set took about 10 seconds CPU time on the HP A900 computer.

### 4.4.3 Kinetics of the immobilized enzyme

The experiments were performed in duplicate using two different batches of immobilized enzyme. The diameter of the beads varied between 2 and 3 mm. By means of sieving, several fractions were obtained. The beads used for the first experiment were made in hexane. The relative error in the diameter of the sieved fractions was about 2% because the particles were slightly elliptical. For the second experiment, beads made in olive oil were used. These beads were almost completely spherical with a relative error in their diameter of the sieved fractions of about 0.7%.

### 4.4.4 Results of the pseudo-steady state experiments

The experimental data of the BOM conversion experiments were processed using the two mathematical procedures, the 'fully numerical approach' (FNA) and the 'alternative mathematical approach' (AMA).

As described in section 4.2, procedure (FNA) makes use of a mass balance over the biocatalyst as a function of the location, combined with a mass balance describing the substrate decrease in the liquid phase as a function of time. The result can be seen in figure 4.2, which shows a measured curve and the fitted substrate concentration. The experiment lasted about 50 minutes. Fitting the data set took about 30 minutes CPU time on the HP A900 computer, starting from accurate initial values for the parameters.

The first step in procedure AMA is the calculation of the apparent kinetic parameters from the measured curve, assuming the curve can be described simply using the Michaelis-Menten equation. By using the calculated overall effectiveness factor, the measured rate of decrease of substrate in the liquid phase was adjusted, finally resulting in a curve that would have been measured if the effectiveness factor had been 1. An example of such a measured curve including the fitted curve, and the resulting pseudo-measured curve for \(\eta_{ow} = 1\) including fitted curve, can be seen in figure 4.3. Fitting the data set took about 90 seconds on the HP A900 computer. The experiment was the same as that shown in figure 4.2. In both figures it can be seen that the fits are quite accurate. However, procedure AMA is much faster than procedure FNA. The average CPU time using procedure FNA was about 30 minutes to 2 hours, using procedure AMA about 1 to 5 minutes.

**Hexane-made biocatalyst particles**

Two fractions of the biocatalysts made in hexane were used in duplicate experiments. Table 4.1 contains the results of fitting the experimental results with the two mathematical approaches. For both intrinsic kinetic parameters
Figure 4.2 Measured substrate concentration (•) and fitted curve for a dynamic measurement using the FNA procedure. \( d_p = 2.6 \) mm, \( S_I = 0.46 \) mol m\(^{-3}\).

Figure 4.3 Measured substrate concentration (○), fitted with Michaelis-Menten kinetics, and the corrected concentration (•) including the fitted curve for a dynamic measurement using the AMA procedure. \( d_p = 2.6 \) mm, \( S_I = 0.46 \) mol m\(^{-3}\).
Table 4.1 Intrinsic kinetic parameters resulting from the hexane-made beads, using dynamic measurements.

<table>
<thead>
<tr>
<th>measurement number</th>
<th>( d_p ) (mm)</th>
<th>number of particles</th>
<th>( V_m ) (mol kg(^{-1}) s(^{-1}))</th>
<th>( K_m ) (mol m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMA</td>
<td>PNA</td>
</tr>
<tr>
<td>1a</td>
<td>2.8</td>
<td>135</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>1b</td>
<td>2.8</td>
<td>135</td>
<td>0.34</td>
<td>0.33</td>
</tr>
<tr>
<td>2a</td>
<td>2.6</td>
<td>246</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>2b</td>
<td>2.6</td>
<td>246</td>
<td>0.29</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Two batches of beads were used, for each batch duplicate experiments were performed. The data were fit with the AMA-procedure and with the PNA-procedure.

Table 4.2 Intrinsic kinetic parameters resulting from the oil-made beads, using dynamic measurements.

<table>
<thead>
<tr>
<th>measurement number</th>
<th>( d_p ) (mm)</th>
<th>number of particles</th>
<th>( V_m ) (mol kg(^{-1}) s(^{-1}))</th>
<th>( K_m ) (mol m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMA</td>
<td>PNA</td>
</tr>
<tr>
<td>1</td>
<td>2.1</td>
<td>220</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>281</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>2.9</td>
<td>102</td>
<td>0.34</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Three batches of beads were used. The data were fit with the AMA-procedure and with the PNA-procedure.

there was a rather large variation in the results. The average overall value for \( V_m \) was 0.26 ± 0.08 mol kg\(^{-1}\) s\(^{-1}\) and for \( K_m \) of 0.07 ± 0.04 mol m\(^{-3}\). Furthermore, it can be seen that both methods give approximately the same result.

Oil-made biocatalyst particles

Three fractions of the biocatalyst beads made in oil were used. The results are shown in table 4.2. These results are better than those of the hexane-made biocatalysts. The average value for \( V_m \) was 0.31 ± 0.05 mol kg\(^{-1}\) s\(^{-1}\), for \( K_m \) 0.07 ± 0.02 mol m\(^{-3}\). This may be due to the fact that, as already mentioned, these beads were more spherical formed than the hexane-made beads. Both methods gave exactly the same result here.
4.4 Results

![Graph showing normalized oxygen concentration vs. normalized radius.]

Figure 4.4 Measured substrate concentration (o) including the fitted curve for a steady state profile measurement. The dashed line marks the surface of the bead. \( d_p = 3.0 \text{ mm}, S_I = 0.2 \text{ mol m}^{-3} \).

4.4.5 Results of the steady state experiments

The results of the profile measurements were processed using the steady state numerical fit procedure. An example of a measured profile, including the fitted curve, can be found in figure 4.4. The fit looks accurately. Fitting of the data set took about 20 seconds CPU time on the HP A900 computer.

Hexane-made biocatalyst particles

For the determination of the intrinsic kinetic parameters, 2 different beads were used. With each bead, duplicate microsensor profile measurements were performed, at slightly different locations (a few \( \mu \text{m} \) apart) on the bead. The results are given in table 4.3. The duplicate results from one bead agree well, but the agreement between the results for the two different beads was not as good. The overall average value for \( V_m \) is \( 0.21 \pm 0.08 \text{ mol kg}^{-1} \text{s}^{-1} \) and for \( K_m 0.04 \pm 0.02 \text{ mol m}^{-3} \).

Oil-made biocatalyst particles

Again, duplicate measurements with two separate beads were performed at slightly different locations on each of the beads. The results are given in table 4.4. Again the duplicate results from the individual beads agree well, but comparing the outcome for the different diameters, the results are somewhat
4. Intrinsic kinetics determined by two experimental methods

Table 4.3 Intrinsic kinetic parameters resulting from the hexane-made beads, using steady state measurements.

<table>
<thead>
<tr>
<th>measurement number</th>
<th>( d_p ) (mm)</th>
<th>( V_m ) (mol kg(^{-1}) s(^{-1}))</th>
<th>( K_m ) (mol m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>2.8</td>
<td>0.15</td>
<td>0.03</td>
</tr>
<tr>
<td>1b</td>
<td>2.8</td>
<td>0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>2a</td>
<td>3.0</td>
<td>0.30</td>
<td>0.06</td>
</tr>
<tr>
<td>2b</td>
<td>3.0</td>
<td>0.26</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Two different beads were used, for each bead duplicate experiments were performed.

Table 4.4 Intrinsic kinetic parameters resulting from the oil-made beads, using steady state measurements.

<table>
<thead>
<tr>
<th>measurement number</th>
<th>( d_p ) (mm)</th>
<th>( V_m ) (mol kg(^{-1}) s(^{-1}))</th>
<th>( K_m ) (mol m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>3.3</td>
<td>0.28</td>
<td>0.05</td>
</tr>
<tr>
<td>1b</td>
<td>3.3</td>
<td>0.31</td>
<td>0.05</td>
</tr>
<tr>
<td>2a</td>
<td>3.0</td>
<td>0.19</td>
<td>0.02</td>
</tr>
<tr>
<td>2b</td>
<td>3.0</td>
<td>0.21</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Two different beads were used, for each bead duplicate experiments were performed.

different. The overall average value for \( V_m \) is 0.25 ± 0.06 mol kg\(^{-1}\) s\(^{-1}\) and for \( K_m \) 0.04 ± 0.02 mol m\(^{-3}\).

4.4.6 Effectiveness factor

With the numerical calculation procedures, it is possible to calculate the overall effectiveness factor (\( \eta_{ov} \)). For the dynamic situation, \( \eta_{ov} \) changes continuously with the substrate concentration in the liquid phase. To compare the \( \eta_{ov} \) resulting from the dynamic batch experiments with the \( \eta_{ov} \) determined from the profile measurements, it is necessary to select the specific hov which was calculated for the same substrate concentration used for the profile measurements, being 0.2 mol m\(^{-3}\). The result is given in table 4.5. The value for \( \eta_{ov} \) was the same for both approaches, it seems that \( \eta_{ov} \) is a less sensitive parameter than the intrinsic kinetic parameters.
Table 4.5 Overall effectiveness factor resulting from the dynamic as well as the steady state measurements, for an oxygen concentration of 0.2 mol m\(^{-3}\).

<table>
<thead>
<tr>
<th>type of bead</th>
<th>type of measurement</th>
<th>(\eta_{ov})</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexane-made</td>
<td>pseudo-steady state</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>hexane-made</td>
<td>steady state</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>oil-made</td>
<td>pseudo-steady state</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>oil-made</td>
<td>steady state</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

4.4.7 Discussion and conclusion

The two mathematical methods used to process the data from the dynamic experiments gave similar results for the intrinsic kinetic parameters. However, the computer calculation time for alternative method, making use of an expression for \(\eta_{in,MM}\) which interpolates between the analytical solutions for zero- and first-order cases, was about 20 times shorter as for the fully numerical approach.

Averaging the results from all the experiments, values for the intrinsic kinetic parameters for the dynamic batch measurements were \(V_m = 0.28 ± 0.07\) mol kg\(^{-1}\) s\(^{-1}\) and \(K_m = 0.07 ± 0.03\) mol m\(^{-3}\). For the profile measurements the values were \(V_m = 0.23 ± 0.07\) mol kg\(^{-1}\) s\(^{-1}\) and \(K_m = 0.04 ± 0.02\) mol m\(^{-3}\). The calculated value for the overall effectiveness factor, \(\eta_{ov}\), was virtually the same for identical liquid bulk concentrations and diameters in the two types of experiments. It can be concluded that the results of the two experimental methods do not differ significantly, although the standard deviation is rather large. However, the average value for the \(K_m\) resulting from the profile measurements was somewhat lower than that obtained with the BOM. In a previous section was shown that when using simulated data, the dynamic and steady state approach give the same result, so differences in the results can only be caused by the experiments. Experimental deviation between the two approaches can be caused by errors that are different for each system. One of these errors might lay in the value chosen for the mass transfer coefficient \(k_l\). This parameter was only necessary in the dynamic approach, because the external mass transfer limitation could be determined during the profile measurements by measuring the concentration on the surface of the bead. In the range of \(k_l\) applicable for the BOM measuring system, a variation in the parameter value of 20% will affect the \(K_m\) more (about 10%) than the \(V_m\) (about 1%). This is one possible explanation for the difference found between the average values for \(K_m\) from the two systems. Also deviations from the spherical form will effect the parameter values when a sphere is assumed in the model. The magnitude of this effect of course depends on the particle di-
ameter. It may therefore have a greater impact on the profile measurements than on the BOM measurements.

Comparing the kinetic parameters of the suspended enzyme ($V_m = 0.48 \pm 0.05 \text{ mol kg}^{-1} \text{s}^{-1}$ and $K_m = 0.07 \pm 0.01 \text{ mol m}^{-3}$) with the results presented above for the intrinsic kinetic parameters, it can be concluded that immobilization in this case causes a reduction in $V_m$ by a factor of about 2, but the $K_m$ does not show a significant change. It is also clear that the alternative method for calculating the intrinsic kinetic parameters for enzymes obeying Michaelis-Menten kinetics is much faster and gives essentially similar results as the fully numerical method.

4.5 Nomenclature

- $a$: specific surface bead (m$^{-1}$)
- $A_p$: surface bead (m$^{-2}$)
- $D_e$: effective diffusion coefficient (m$^2$ s$^{-1}$)
- $d_p$: particle diameter (m)
- $E$: enzyme concentration (kg m$^{-3}$)
- $k_i$: external mass transfer coefficient (m s$^{-1}$)
- $K_m$: Michaelis-Menten constant for the rate limiting substrate (mol m$^{-3}$)
- $r$: radial distance (m)
- $R$: reaction rate (mol m$^{-3}$ s$^{-1}$)
- $r_p$: radius of the bead (m)
- $S$: rate limiting substrate concentration (mol m$^{-3}$)
- $t$: time (s)
- $V_m$: maximal substrate consumption rate (mol kg$^{-1}$ s$^{-1}$)
- $V_p$: volume bead (m$^{-3}$)

Greek symbols

- $\eta$: effectiveness factor (-)
- $\Theta$: weighting factor (-)
- $\Phi$: generalized Thiele modulus (-)

Subscripts

- $0$: zero-order
- $1$: first-order
- $MM$: Michaelis-Menten
- $i$: at the surface
- $l$: in the liquid
4.6 Bibliography

4. Intrinsic kinetics determined by two experimental methods
Chapter 5

Experimental determination of mass transfer boundary layer around a spherical biocatalyst particle *

An oxygen microsensor was used to determine the thickness of the boundary layer outside a spherical biocatalyst particle in liquid flow. Different particle diameters and fluid velocities were used. The results were compared with correlations available in the literature using the superficial liquid velocity. The measured thickness of the boundary layer appeared to be smaller than the thickness calculated with the literature correlations. The reason for this disagreement could be that the local liquid velocity existing in the measuring equipment was higher than the superficial velocity used in the calculations. No experimental distinction could be made between the results obtained with different particle diameters. The diameter dependence is small at high Reynolds numbers, while at low Reynolds numbers the measuring error was too large due to vortices. However, it is clear that care must be taken when using correlations from the literature to predict external mass transfer resistance.

5.1 Introduction

This chapter describes boundary layer measurements performed with an oxygen microsensor around a spherical biocatalyst particle made of gel containing an oxygen consuming enzyme. The aim was to see if the approximate literature relations describing mass transfer for flow around a spherical particle could be used for the situation of a fixed sphere in a flow chamber. This

flow chamber is part of a piece of equipment that is being used for measuring internal oxygen profiles in biocatalysts [1], and will be presented in section 5.3.3.

An immobilized biocatalyst is constructed by the immobilization of enzymes or cells in a gel or other carrier material. Due to this immobilization a concentration profile of the substrate consumed inside the particle will result. Apart from the effective diffusion coefficient and the intrinsic kinetics, the external mass transfer resistance will influence the exact profile in the particle. This resistance is the result of a boundary layer around the particle with a thickness dependent on the velocity or corresponding Reynolds number of the liquid flowing around the particle. If there is no flow, transport is only by diffusion.

5.2 Literature Relations

Various relations can be found in the literature to calculate the thickness of the boundary layer for different conditions. They all assume the mass transfer resistance to be located in a stagnant boundary layer with a certain thickness through which mass transfer takes place (film theory). The mass transfer coefficient is then defined by the following relation:

$$k_t = \frac{D}{\delta} = \frac{Sh D}{d_p}$$  \hspace{1cm} (5.1)

If mass transfer is only by diffusion the thickness of the assumed boundary layer is equal to the radius of the particle (the Sherwood number equals 2 for this situation). When there is liquid flow, the thickness of the boundary layer will decrease. The relations describing the mass transfer for flow around a sphere can have an empirical or theoretical background.

A theoretical analysis, checked by experiment, is that of Brian and Hales [2]. The equation resulting from their analysis is well known and widely accepted [3]. The equation is valid for $10^3 < Re < 10^5$ and is as follows:

$$k_t = \frac{D}{\delta} = \left[ 2.0 + 0.57 Re^{1/2} \left( \frac{\nu}{D} \right)^{1/3} \right] \frac{D}{d_p}$$  \hspace{1cm} (5.2)

Ranz & Marshall [4] came up with an empirical relation for $Re < 10$:

$$k_t = \frac{D}{\delta} = \left[ 4.0 + 1.21 \left( \frac{d_p U}{D} \right)^{2/3} \right]^{1/2} \frac{D}{d_p}$$  \hspace{1cm} (5.3)

The intersection of these equations is at $Re$ is 30.

Recently, Brauer [5] showed an empirical equation that corresponded with data obtained by numerical solution of the differential equations describing
the velocity field around a spherical particle. The advantage of this relation is the broader range of application, being valid for $0 < Re < 3 \cdot 10^5$. The relation is as follows:

$$k_l = \frac{D}{\delta} = \left[2 + f_k \frac{(Re \, Sc)^{1.7}}{1 + (Re \, Sc)^{1.2}}\right] \frac{D}{d_p} \tag{5.4}$$

in which:

$$f_k = \frac{0.66}{\left[1 + (0.84 \, Sc^{1/6})^3\right]^{1/3}} \tag{5.5}$$

Oxygen microsensors have been used before to determine external mass transfer. Kasche et al. [6] measured the thickness of the diffusion layer outside immobilized biocatalysts. The diameter of the particles they used was 0.6 mm. The thickness of the layer decreased with increasing flow velocity. Rietheus et al. [7] investigated mass transfer with a microsensor more quantitatively. They were interested in the bubble diameter for optimal mass transfer and determined the mass transfer from gas bubbles into liquid gassed with nitrogen. They found that for their system Sherwood numbers were lower than the values obtained from theory, and that for a small bubble diameter (1 mm) the mass transfer was distinctively lower than for a higher bubble diameter (4.9 mm). They came to the conclusion that it was better to use larger bubble diameters in a bubble column, which differs from general experience.

5.3 Methods and materials

5.3.1 Oxygen microsensor

The polarographic oxygen microsensor used was constructed at our laboratory according to Revsbech and Ward [8]. It was a combined sensor, which means that the cathode as well as the reference electrode are combined in one sensor. The diameter of the sensing tip of the microsensor used for these experiments was about 10 μm. The response of the sensor was linearly dependent on the oxygen concentration. The sensor showed no measurable stirring effect. The signal of the sensor was very stable, and did not drift could be detected during the experiments. In water of 20°C the current was 0.46 nA, the zero current was 0.05 nA. The specifications of the sensor did not alter with frequent use over a period of one month.

5.3.2 Immobilized biocatalyst

The biocatalyst particle was made of 5% agarose (gel type 7, Sigma), which contained the enzyme L-lactate 2-monooxygenase, EC 1.13.12.4 from My-
5. Mass transfer boundary layer around a biocatalyst bead

Figure 5.1 Perspex flow chamber. The scheme includes an oxygen microsensor (1), flow of liquid in (2) and out (3), outer (4) and inner (5) flow chamber, biocatalyst particle (6) and a holder (7).

cobacterium smegmatis provided by Sigma. These biocatalyst particles were also used for measuring internal oxygen profiles [1]. For the external mass transfer measurements relatively high concentrations of the enzyme were used to guarantee a very steep profile inside the particle and consequently a distinct boundary layer.

5.3.3 Experimental configuration

A perspex flow chamber consisting of an inner and outer chamber was used (see figure 5.1). The volume of the inner chamber, in which the particle was mounted, was 50 ml. The length of the walls was 30 mm. The volume of the outer chamber was 150 ml. A silicon tube with little holes in it was placed on the bottom of the outer chamber. The medium was pumped through this holes, ascending in the outer chamber and flowing over into the inner chamber, parallel to the sensor. The reason to create such a flow was to avoid too much turbulence around the microsensor that approached the particle from above. The flow chamber was square with parallel walls and was illuminated with a cold-light illuminator (Euromex EK-1), so that the particle could be seen through a stereomicroscope (Olympus S071) that was placed in front of the flow chamber. The surface of the particle could be determined quite accurately with the use of the microscope, the resolution being about 10 μm.

The sensor itself was put in a holder above the flow chamber. The holder was connected to a motor drive and a micromanipulator (Oriel Model 18011 Encoder Mike controller). The signal coming from the sensor was amplified
and monitored by use of a recorder (Kipp & zonen BD40) and a personal computer (IBM-PS/2).

5.3.4 Experimental procedure

For the mass transfer measurements the sensor was initially placed several hundred micrometers above the particle and moved step-wise by means of the manipulator. Steps of 10 μm were performed and an average of 30 readings per step was taken.

Particles of diameter 2.9, 3.9, 5.0 and 5.8 mm were used. For the liquid velocity six different superficial velocities were taken, namely $4.9 \cdot 10^{-4}$, $9.6 \cdot 10^{-4}$, $1.9 \cdot 10^{-3}$, $2.8 \cdot 10^{-3}$, $3.9 \cdot 10^{-3}$, $5.0 \cdot 10^{-3}$ and $6.2 \cdot 10^{-3}$ m s$^{-1}$. The velocities were determined by dividing the liquid flow by the area of the inner chamber.

The medium used was a phosphate buffer with excess of L-lactate and an oxygen concentration of 0.25 mmoll$^{-1}$, at a temperature of 20°C. The diffusion coefficient used was $2.0 \cdot 10^{-9}$ m$^2$s$^{-1}$ [9].

The boundary layer in the absence of flow was measured before a boundary layer measurement under flow conditions was performed. This measurement was carried out to see if the equipment was suitable for the boundary layer measurements. According to the film theory, the mass transfer resistance for this situation is located in a layer with a thickness equal to the radius of the particle. The biocatalyst particle was embedded in a 1% agarose gel to guarantee that there was no convection of any kind. Due to the difference in agarose concentration the particle (5% agarose) was still visible. The diffusion layer was measured by continuously moving the sensor with a velocity of 5 μms$^{-1}$.

5.4 Results

5.4.1 Mass transfer only by diffusion

The measurements to determine the diffusion layer were performed in duplicate with a particle having a diameter of 5.8 mm. Figure 5.2 gives the result of one of these measurements. The diffusion layer was calculated by means of a regression line through the first five points outside the particle, the distance between the points was 30 μm. The resulting boundary layer was 2.9 mm, for the duplicate it was 2.6 mm. This result is in good agreement with the theory. The slight deviation could be due to measuring errors, such as the determination of the location of the surface of the particle, the resolution of the sensor itself or the small number of data points available for regression.
Figure 5.2 Oxygen concentration as function of the distance relative to the particle surface as a result of mass transfer by diffusion only. The dotted line marks the surface ($d_p = 5.8$ mm).

Figure 5.3 Oxygen concentration (measured data (*)) as function of the distance relative to the particle surface under flow conditions. The dotted line marks the surface, the dashed line marks the external boundary layer ($d_p = 3.9$ mm, $U = 3.9 \cdot 10^{-3}$ m s$^{-1}$).
5.4.2 Mass transfer under flow conditions

An example of a boundary layer measurement under flow conditions can be seen in figure 5.3. For this measurement the diameter of the particle was 3.9 mm, the superficial velocity was $3.9 \times 10^{-3}$ m s$^{-1}$. The data points in the figure are averages over 30 readings. The measurement started at 100 µm above the particle and was completed at 60 µm inside the particle. The dotted line marks the surface of the sphere. The boundary layer was calculated by means of a regression line through the first five points outside the particle.

The thickness of the boundary layers for two different particle diameters under varying flow conditions is given in the figures 5.4 and 5.5. Each data point is the average of two measurements. The figures depict the measured boundary layer as a function of the Reynolds number. The Reynolds numbers were calculated by means of the superficial fluid velocity. Data calculated from relations in the literature (equations (5.2)-(5.5)) are also given in figures 5.4 and 5.5. From the results in table 5.1 an impression of the

![Figure 5.4 Thickness of boundary layer vs. the Reynolds number for a particle diameter of 2.9 mm. Measured data (o) and calculated curvatures, --, eqn. (5.3); ---, eqn. (5.2); ----, eqns. (5.4) and (5.5);](image)
Table 5.1 Thickness of the boundary layer (including standard deviation) for different $Re$. $d_p = 5.8$ mm.

<table>
<thead>
<tr>
<th>$\log Re$</th>
<th>$\delta$ (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>114 ± 34</td>
</tr>
<tr>
<td>1.2</td>
<td>69 ± 8</td>
</tr>
<tr>
<td>1.4</td>
<td>58 ± 8</td>
</tr>
</tbody>
</table>

Experimental error can be obtained. The data are averages of four experiments. The particle diameter was 5.8 mm and three different fluid velocities were used. The results of these measurements make clear that the standard deviation is rather large, and is especially large at low fluid velocities. This was also found for the duplicate measurements (results not shown). The explanation is that at low fluid velocities large vortices occurred, resulting in a

Figure 5.5 Thickness of boundary layer vs. the Reynolds number for a particle diameter of 5.8 mm. Measured data (□) and calculated curvatures, ---, eqn. (5.3); ——, eqn. (5.2); ----, eqns. (5.4) and (5.5);
large measuring error (see below).

It is clear that there is a deviation between the experimental results and the given correlations. The measured boundary layer is much smaller than what would be expected. The curvature, however, looks more or less the same. It seems as if the Reynolds number is larger than that calculated from the superficial velocity. This can be caused by turbulence or other unforeseen movement in the flow. Due to the design of the flow chamber some deviation could be expected. An other reason for the deviation might be the location at which the microsensor pierced the particle. The sensor measured the boundary layer right on top of the particle. This point where the streamlines hit the particle is perhaps the region with the smallest boundary layer. The two hypotheses mentioned were tested separately.

Small amounts of brilliant red dye suspension were used to investigate the deviation from a laminar flow pattern. This procedure showed that vortices occurred due to the relatively small gap through which the water had to stream out. The square shape of the flow chamber introduced some extra turbulence. So although using the superficial velocity for the calculation of $Re$ is not strictly correct it is difficult to translate the flow pattern into a more realistic velocity.

The influence of the location on the thickness of the boundary layer appeared to be less important. This is explained by the fact that, although there was a deviation from the laminar flow pattern, the oxygen concentration was the same at various positions. This was checked by measuring the surface concentration on top, beside and under the particle. For a velocity smaller and equal to $3.9 \cdot 10^{-3}$ m s$^{-1}$ the surface concentration was the same everywhere, above this velocity a slightly thinner boundary layer was found on the top of the particle.

From figures 5.4 and 5.5 it can be seen that our measurements were performed in the intermediate region of two correlations [2, 4]. The approximate relation of Brauer [5] describes this region best and seems more applicable than the other relations [2, 4].

The results of the measurements performed with the different particle diameters are given by figure 5.6. In this figure the approximate relation based on ref. [5] is included also. One would expect a different curve for each particle size measured. However, with the limited accuracy of the measurements under low flow conditions it is not possible to discriminate between the results achieved. For higher flow velocities the accuracy is better, but there is hardly any difference in the thickness of the boundary layer because of the high Reynolds number. For a reasonable description of the average result it was calculated that the velocity to be used in the equation must be four times higher than the superficial velocity determined.
5. Mass transfer boundary layer around a biocatalyst bead

Figure 5.6 Thickness of boundary layer vs. the Reynolds number for a particle diameter of 2.9 mm (○); 3.9 mm (△); 5.0 mm (●); 5.8 mm (■). The curvatures were calculated with the correlation of ref. [5] for a particle diameter of 2.9 mm (---); 3.9 mm (---); 5.0 mm (---); 5.8 mm (—).

5.5 Conclusion

Direct measurement of the oxygen gradient outside a particle with an oxygen microsensor is a good method for determining the mass transfer resistance. However, the measuring results are not in full agreement with correlations available in literature. Relations (5.4) and (5.5), given by Brauer [5], agree best with the measured data.

The boundary layer layer thickness is calculated from the correlations using the superficial liquid velocity. The measurements can be brought in agreement with the correlations only if it is assumed that the local experimental velocity is some four times higher than the measured superficial velocity.

The theoretical relationship with the particle diameter can not be seen from the measurements. At high Reynolds numbers the dependence on the diameter is low and becomes higher at low Reynolds numbers. Due to the
increasing measuring error in the data at low fluid velocities no experimental
distinction can be made between the results obtained with different diam-
eters.

In general, using literature correlations for the predicting of external mass
transfer resistance should be used with care if these correlations are obtained
under other conditions than they are used for.

5.6 Nomenclature

\( d_p \) \hspace{1cm} \text{diameter particle (m)}

\( D \) \hspace{1cm} \text{diffusion coefficient (m}^2\text{s}^{-1})

\( k_t \) \hspace{1cm} \text{external mass transfer coefficient (m} s^{-1})

\( U \) \hspace{1cm} \text{superficial velocity (m} s^{-1})

\( Re \) \hspace{1cm} \frac{U d_p}{\nu} = \text{Reynolds number (–)}

\( Sc \) \hspace{1cm} \frac{\nu}{D} = \text{Schmidt number (–)}

\( Sh \) \hspace{1cm} \frac{k_t d_p}{D} = \text{Sherwood number (–)}

Greek symbols

\( \delta \) \hspace{1cm} \text{boundary layer (m)}

\( \nu \) \hspace{1cm} \text{kinematic viscosity (m}^2\text{s}^{-1})

5.7 Bibliography

1990, 12, 178.


neering (H. Brauer, H.J. Rehm and G Reed, Eds.) Weinheim, New York
1985, 77.


5. Mass transfer boundary layer around a biocatalyst bead
Chapter 6

Measurement of Oxygen Concentration Gradients in Gel-immobilized Recombinant *Escherichia coli*

In this study, an oxygen microsensor was used to measure oxygen concentration profiles in carrageenan gel particles containing growing, immobilized *Escherichia coli* B(pTG201). Profiles, which were measured at intervals during continuous culture of gel slabs and beads, became increasingly steep with time. The oxygen penetration depth in the gel decreased with time, eventually reaching a steady state value of approximately 100 μm for both gel beads and slabs. A reaction-diffusion model employing zero-order cell growth kinetics was found to provide an excellent fit to the experimental concentration data. Growth rates estimated from profiles obtained during the first few hours of culture were 0.24 h⁻¹ (gel slabs) and 0.18 h⁻¹ (beads), compared to a value of 0.30 h⁻¹ measured in free-cell suspensions at 25°C.

6.1 Introduction

The physical immobilization of growing bacterial cells has been shown to produce selected benefits such as increased volumetric productivity and ease of culture. In addition, recent work has demonstrated that immobilized recombinant bacteria exhibit a significantly lower rate of plasmid loss, thus suggesting various applications of immobilized cells for the commercial use of recombinant bacteria [14, 17]. Plasmid stability in both free and immobilized cells has been shown to be affected by many factors, including the level

---

of plasmid gene expression [1, 18] and the particular growth environment. For example, nutrient limitations such as glucose, phosphate and magnesium have been shown to reduce the stability of plasmids [6, 10], while plasmid stability was found to be increased under anaerobic growth conditions (dissolved oxygen concentration of 0%) relative to conditions with a dissolved oxygen concentration of 50% and 100% [9].

For assessing the importance of such factors as oxygen concentration on plasmid stability in gel-immobilized cells it is necessary to measure spatial variations in oxygen concentration within the gel. In practice, a gel-immobilized cell system is heterogeneous in nature, containing nutrient and product concentration gradients which can be severe, depending on the balance between reaction and diffusion in the gel matrix. For example, it is reasonable to assume that carrageenan gel particles containing *Escherichia coli* might contain significant anaerobic regions, even when submerged in supernatant medium at 100% dissolved oxygen concentration.

One method of measuring spatial variations in a system such as gel-immobilized *E. coli* is the use of an oxygen-sensitive microsensor. Past workers have employed microsensors to measure oxygen profiles in mycelial pellets [10, 21] and in bacterial colonies on an agar surface [20]. Recently, the use of a combined oxygen microsensor to measure oxygen profiles inside Ca-alginate beads and agarose gel particles has been described [7, 8]. The object of this work was to experimentally measure time-dependent oxygen concentration profiles in carrageenan gel-immobilized recombinant cells using a combined oxygen microsensor, and to interpret the results using a reaction-diffusion mathematical model of cell growth within the gel.

### 6.2 Materials and Methods

#### 6.2.1 Bacterial strain and nutrient medium

The bacterial strain used in this work was *E. coli* B containing plasmid pTG201. Plasmid pTG201 is derived from pBR322 and codes for the enzyme 2,3-catechol dioxygenase from *Pseudomonas putida* as well as for β-lactamase. The strain was conserved by mixing an overnight culture, containing 100 μg ml⁻¹ ampicillin, with sterile 30% (w/v) glycerol (1:1) and freezing aliquots at –80°C. Nutrient medium for all experiments was M9 minimal medium, pH 7.5, containing (in g l⁻¹): Na₂HPO₄, 6.0; KH₂PO₄, 3.0; NH₄Cl, 1.0; NaCl, 0.5; MgSO₄·7H₂O, 0.25; CaCl₂·2H₂O, 0.015; and glucose, 2.0. When used for gel-immobilized experiments, 0.1 M KCl was added to increase mechanical stability of the carrageenan gel. Petri plates were prepared with LB agar, containing (in g l⁻¹): bactopeptone, 10.0; yeast extract, 5.0; NaCl, 10.0; and agar, 15.0.
6.2.2 Free cell growth

The growth of a dense *E. coli* aggregate at 37°C with delivery of nutrients solely by diffusion can produce extremely steep gradients in nutrient concentrations, with a significant fraction of the aggregate starved for the limiting nutrient [12]. In order to reduce somewhat the cellular consumption of oxygen, and thus to reduce diffusional limitation of cell growth in the gel beads, and in order to facilitate measurement of oxygen gradients resulting from reaction-diffusion balances, the growth temperature for immobilized and free-cell experiments was selected to be 25°C. Pre-inoculation cultures were prepared by inoculating a flask containing 50 ml of M9 medium, containing 100 µg ml⁻¹ ampicillin, with a thawed aliquot of cells. The flask was placed in a rotary shaker at 37°C and 140 rpm overnight. Free-cell batch cultures were also used to measure anaerobic and aerobic growth rates and glucose yield coefficients. For these, a 1% (v/v) inoculum was added to a water-jacketed, stirred beaker at 25°C. Sparging gas, either nitrogen or oxygen, was filtered with a 0.2 µm filter. Cell growth was monitored by optical density at 660 nm, and cell dry mass was determined by filtering samples of the cell broth onto pre-rinsed, dried, and tared 0.2 µm filters (type GS, Millipore) followed by drying at 80°C and re-weighing.

6.2.3 Gel-immobilized cell growth

Cells were immobilized in both κ-carrageenan gel slabs, in the form of short cylinders, and gel beads. Moulds for the gel cylinders (1.31 cm inner diameter, 2.0 cm tall) were constructed from polypropylene tubing glued to glass plates, and autoclaved for 15 min at 121°C. The gel-cell mixture was formed by mixing 4.0 ml of an overnight aerobic pre-culture with 40 ml of sterile 2.2% (w/v) κ-carrageenan (E407, CECA, France) at 42°C, yielding a final carrageenan concentration of 2.0%. Following gentle swirling of the flask for 1-2 minutes, approximately 16 ml of the mixture was used to fill 8 sterile moulds and allowed to harden by cooling to room temperature (approximately 15 min). The cylinders were then submerged in 0.3 M KCl for 1 hour, then transferred to the reactor. Gel beads were formed by mixing 1.0 ml of pre-culture with 20 ml 2.2% κ-carrageenan, again giving a final gel concentration of 2.0%. A total of 2.5 ml of this mixture was then added dropwise with a sterile syringe and needle to a solution of 0.3 M KCl with stirring at room temperature, forming beads with an average diameter of 2.5 mm (initial experiment) or 2.8 mm (duplicate experiment). After an additional 15 min in the KCl solution, the beads were transferred to the reactor.

Continuous cultures were conducted in magnetically-stirred, water-jacketed reactors at 25°C, in a total volume of 170 ml (gel cylinders) or 130 ml (gel beads). Dilutions rates were maintained at 1 h⁻¹ for both types of experiments. Pure oxygen gas was sparged into the reactors at 1.5 to 1.8 vvm; this
flow rate was sufficient to ensure a dissolved oxygen concentration of 100% in the supernatant nutrient medium. Experiments were performed in duplicate.

6.2.4 Determination of cell concentration
Initial viable cell concentrations were determined by dilution plating. Several beads or sections from a gel cylinder were placed in individual, tared sterile tubes, and the actual volume of the sample was calculated from the gel weight and the measured density of 2% carrageenan gel (0.9919 g cm⁻³). The samples were dissolved by addition of 4.0 ml of 10 g l⁻¹ sodium citrate accompanied by heating to 37°C and vortexing. Successive 10-fold dilutions of the resulting solutions were made with 64 mol m⁻³ phosphate buffer, pH 7.5, as diluent, and 50-200 μl aliquots of the appropriate dilution were spread on LB agar plates. The viable cell concentration was calculated from the number of colonies appearing on the plates.

6.2.5 Oxygen microsensors
Experiments were carried out with two microsensors which were constructed according to Revsbech and Ward [15]. The first sensor exhibited a standard current of 0.39 nA (air-saturated water; 25°C). The zero current was 13% of the air-saturated signal. The second sensor produced a standard current of 0.29 nA with a zero current equal to 7% of the standard current. After stabilization for one hour, both sensors showed no measurable drift during the experimental measurements. The sensors also exhibited no significant stirring effects, and demonstrated a linear response to oxygen concentration.

6.2.6 Microsensor measurements of oxygen concentration profiles
The measuring equipment has previously been described [8]; a short summary is given here. The microsensor was placed in a holder, connected to a motor drive and micromanipulator (Oriel model 18017 Encoder Mike Controller) in order to regulate the distance and speed of sensor movement. The velocity used during measurements was 5 μm s⁻¹. The output signal from the sensor was amplified and recorded by a microcomputer (IBM-PS/2). The data file created by the data acceptance program was transferred to a HP A900, which was used for calculations and data fitting. The raw data were averages of 2 or 5 samples, taken at time intervals of 1 or 2 seconds, respectively, depending on the steepness of the measured gradients.

Measurement of the oxygen concentration profile within the gel beads and cylinders was performed at selected times during continuous cultures. For each measurement, a gel block or bead was removed from the reactor and placed in a flow chamber. Nutrient medium was continuously circulated in the flow chamber at 3.5 ml s⁻¹ and at a constant temperature of 25°C in order to provide a constant environment for the sample. The nutrient
medium used in the flow chamber was identical to that used in the continuous cultures, except that it did not contain any potassium. This change was necessary in order to relax the carrageenan gel: preliminary tests had shown that, in the presence of potassium, the gel was too firm to allow penetration of the microsensor tip, resulting in gel particle deformation and disturbance of the resulting signal due to a build-up of pressure at the sensor tip. The lack of potassium in the circulating medium is not expected to cause significant changes in cell metabolism during the relatively short time period (about 15 minutes) required for a profile measurement. The oxygen concentration in the circulating medium was regulated at 80% of saturation value by sparging pure oxygen into the medium reservoir. Measurements were begun after positioning the microsensor 300 μm above the sample surface.

6.3 Oxygen profile model

A model of oxygen diffusion and reaction within the cell/gel matrix was used to evaluate the experimental profiles. Although cell concentration and thus reaction rate are a function of both time and distance within the gel, a quasi-steady state approximation can be used at each sample time since the characteristic diffusion time (order of minutes) is smaller than the generation time for cell growth (order of hours for cell growth at 25°C). The governing equation for oxygen concentration within the gel at a given sample time is:

\[ D_e \frac{1}{r^n} \frac{d}{dr} \left( r^n \frac{dC_{ox}}{dr} \right) = R_{ox} \]  

(6.1)

Boundary conditions:

\[ r = r_i, \quad C_{ox} = C_{i,ox} \]  

(6.2a)

\[ r = r_d, \quad C_{ox} = 0, \quad \frac{dC_{ox}}{dr} = 0 \]  

(6.2b)

in which \( C_{ox} \) is the local oxygen concentration, \( R_{ox} \) is the specific volumetric consumption rate of oxygen, \( D_e \) is the effective diffusion coefficient of oxygen in the gel, and \( r_d \) is the distance at which the oxygen concentration reaches a minimum critical value, beyond which aerobic growth is not possible. For the gel cylinders, which are treated mathematically as slabs (diffusion perpendicular to the gel surface), \( n = 0 \), while for gel beads, \( n = 2 \). \( R_{ox} \) is obtained from the relation:

\[ R_{ox} = \frac{\mu X}{Y_{X/ox}} \]  

(6.3)
The primary assumption of this model is that the specific growth rate of *E. coli* is zero-order with respect to oxygen, and is thus given by the maximum specific growth rate $\mu_{max}$. This assumption is supported by the extremely small value of $K_m$ for oxygen ($2.3 \cdot 10^{-3}$ mol m$^{-3}$ [19]) relative to its bulk concentration (1.3 mol m$^{-3}$ in the reactor and 1.0 mol m$^{-3}$ in the measurement flow chamber).

With this assumption, equation (6.1) can be solved analytically with boundary conditions (6.2b) to obtain $C_{aox}$ in terms of $r_d$ (for a more thorough discussion, see [3] [but note typographical error in the concentration equation for a sphere in this paper]; and [4]. For the slab:

$$C_{aox}(r) = \frac{1}{2} \frac{R_{aox}}{D_e} \left[ (r^2 - r_d^2) - 2r_d(r - r_d) \right]$$  \hspace{1cm} (6.4)

and for the sphere:

$$C_{aox}(r) = \frac{1}{6} \frac{R_{aox}}{D_e} \left( r^2 + \frac{2r_d^3}{r} - 3r_d^2 \right)$$  \hspace{1cm} (6.5)

The location of $r_d$ can be determined by applying condition (6.2a), and the result can be expressed in terms of the penetration depth of oxygen in the gel, given by $d = r_i - r_d$. For the slab:

$$d = \sqrt{\frac{2C_{i,aox}D_e}{R_{aox}}}$$  \hspace{1cm} (6.6)

and for the sphere:

$$3d^2 - \frac{2d^3}{r_p} = \frac{6C_{i,aox}D_e}{R_{aox}}$$  \hspace{1cm} (6.7)

The above solutions, and in particular condition (6.2a), are applicable for our experimental system, in which the surface concentration of oxygen, $C_{i,aox}$, was measured directly with the microsensor. If $C_{i,aox}$ is not known and external mass transfer resistance is significant, then equation (6.2a) can be modified to take into account external mass transfer effects [3]. The following procedure was thus used to compare predicted profiles with experimental results. First, the combined parameter group $R_{aox}/D_e$ was estimated from the measured initial biomass concentration, the diffusion coefficient of oxygen in water at 25°C, and the growth rate and yield coefficient values measured in free-cell experiments. This estimate was used to predict the initial oxygen profile in the gel. The value of $R_{aox}/D_e$ was then adjusted to obtain a best fit between the predicted and actual profiles, using a least-squares estimation method [13].

An independent estimate of the immobilized maximum cell growth rate was also obtained using the fitted values of $R_{aox}/D_e$. In addition to the assumption of zero-order growth, it was assumed that for the first few hours of
immobilized-cell growth the parameters \( D_e \), \( Y_{X/\text{oz}} \), \( \mu_{\text{max}} \) and the maintenance coefficient were constant (that is, independent of biomass concentration). Therefore, the observed growth rate is given by:

\[
\ln \left( \frac{R_{\text{oz}}/D_e}{R_{\text{oz}}/D_e}_o \right) = \mu_{\text{max}} t
\]

(6.8)

where \( t \) is the time and subscript 'o' indicates the initial value of \( R_{\text{oz}}/D_e \).

6.4 Results and Discussion

6.4.1 Oxygen profiles

Four experiments were performed, two each with gel slabs (cylinders) and gel beads. Oxygen concentration profiles were measured every few hours during continuous culture, until a steady state profile was obtained. For each individual measurement, the profile was measured at least twice, either using a fresh sample in the case of the gel spheres, or using another surface location in the case of the gel blocks. Results of such duplicate measurements are presented in figures 6.1 and 6.2.

As can be seen, there was very little difference between duplicate measurements. For figure 6.1 the values found for \( R_{\text{oz}}/D_e \) are \( 49.2 \cdot 10^5 \) and \( 45.4 \cdot 10^5 \) mol m\(^{-2}\) and for figure 6.2 the values found for \( R_{\text{oz}}/D_e \) are \( 10.7 \cdot 10^5 \) and \( 10.3 \cdot 10^5 \) mol m\(^{-2}\). For the gel slab, there was an indication of oxygen, and

![Figure 6.1](image-url)

Figure 6.1 Duplicate measurements of oxygen concentration profiles in two different gel beads (\( \Delta t = 22.5 \) h). \( X_o = 0.04 \) kg m\(^{-3}\), \( d_p = 2.5 \) mm.
Figure 6.2 Duplicate measurements of oxygen concentration profiles using the same gel block but in two different locations near the centre of the cylinder surface ($\Delta t = 5$ h). Distance 0 corresponds with 1 cm, $X_o = 0.27$ kg m$^{-3}$.

hence nutrient medium, leakage between the wall of the sample container and the gel cylinder itself. This can be seen in figure 6.3 as a less steep profile close to the slab edge, compared to the measurement made near the centre of the gel block. The profiles shown were obtained at 10 hours. After 23 hours (the next measurement), there was no longer any observable difference between the centre and edges of the gel. Thus it appears that leakage of nutrient medium between the walls of the mould and the gel block itself may be significant in the earlier hours of the experiment. This complication has also been noted in the measurement of a biomass gradient in gel cylinders [2]. It is important to note that, in this work, all profiles used for parameter fitting were obtained from the centre of the blocks, so that leakage should have a minimal effect on the numerical results.

Following attainment of steady state oxygen concentration profiles in the gel slabs, growth of cells on the gel surface was observed, resulting in large surface colonies. The surface colonies were found to be approximately 160 $\mu$m in depth. As illustrated by figure 6.4, the oxygen profile in a surface colony appeared to be less steep than within the gel. This is likely the result of at least two differences between a surface colony and the gel-entrapped cells. Firstly, oxygen transport in the gel occurs by diffusion only, while transport within the colony probably includes contributions from convection, due to
Figure 6.3 Separate measurements of oxygen profiles using the same gel slab but in two widely-spaced locations ($\Delta t = 10$ h). Distance 0 corresponds with 1 cm, $X_o = 0.04$ kg m$^{-3}$; (*) near the centre of the gel block; (o) approximately 1 mm from the edge of the gel block.

Figure 6.4 Effect of surface colony growth on the oxygen profile measured in a gel slab ($\Delta t = 49$ h). Distance 0 corresponds to 1 mm. $X_o = 0.04$ kg m$^{-3}$. (*) within and below a colony; (o) bare spot located immediately adjacent to a surface colony.
Table 6.1 Best-fit values of the parameter $R_{ox}/D_e$ for the oxygen profiles depicted in figures 6.5 and 6.6.

<table>
<thead>
<tr>
<th>$t$ (h)</th>
<th>$R_{ox}/D_e \cdot 10^{-8}$ (mol m$^{-8}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sphere</td>
</tr>
<tr>
<td>0</td>
<td>1.41 [1.06]</td>
</tr>
<tr>
<td>4.2</td>
<td>2.40 [2.66]</td>
</tr>
<tr>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>7.8</td>
<td>5.21 [6.17]</td>
</tr>
<tr>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>11.0</td>
<td>8.25 [8.25]</td>
</tr>
<tr>
<td>13.5</td>
<td>-</td>
</tr>
<tr>
<td>14.3</td>
<td>15.3 [17.9]</td>
</tr>
<tr>
<td>49.0</td>
<td>348 [178]</td>
</tr>
</tbody>
</table>

The values in brackets resulted from duplicate profile measurements using the same gel, performed within minutes of the first measurement.

vigorous movement of the nutrient medium in the flow chamber. Secondly, the cell density in the surface colony is expected to be lower than that in the gel: previous studies using electron microscopy of gel bead sections have shown extremely high densities, accompanied by shape distortion of the _E. coli_ cells, within gel cavities close to the surface, presumably caused by the mechanical pressure of the surrounding gel [5].

No growth of surface colonies was noted on the gel spheres. This was probably due to larger shear forces present in the gel bead fermentors, in contrast to the gel block experiments in which the blocks remained stationary on a platform within the reactor and were thus more protected from shear forces.

6.4.2 Reaction-diffusion model

As described above, the measured profiles were fitted with a model assuming growth to be zero-order in oxygen. Fitted and measured profiles are summarized in figures 6.5 and 6.6 for one example each of the gel bead and block experiments. The data and the fitted profiles show close agreement. Thus the zero-order model used to fit the data seems to be sufficiently accurate to describe the growth of _E. coli_ in the carrageenan gel.

Table 6.1 lists the values of $R_{ox}/D_e$ resulting from the fitted profiles. The variation in the value of this parameter was usually less than 20% for duplicate measurements, except for duplicate measurements at large times. For both the sphere and slab geometries, the difference between duplicate measurements at $t = 49.0$ h is about 50%. This variation is due to the extreme steepness of the oxygen concentration profiles at large times, with small errors
Figure 6.5 Measured oxygen concentration profiles in gel bead-immobilized *E. coli* and the fitted curve. $X_o = 0.01$ kg m$^{-3}$, $d_p = 2.8$ mm. Measurements at 0 h ($*$); 4.2 h (o); 7.8 h (+); 11.0 h (#); 14.3 h (⊕); and 28.0 h (●).

Figure 6.6 Measured oxygen concentration profiles in gel slabs-immobilized *E. coli* and the fitted curve. $X_o = 0.27$ kg m$^{-3}$. Measurements at 0 h (+); 5.0 h (●); 10.0 h (+); 13.5 h (#); and 49.0 h (o). Distance 0 corresponds with 1 cm.
Figure 6.7 Logarithmic value of the normalized parameter $R_{oa}/D_e$ as a function of time. Symbols represent experimental data, and the lines are calculated using linear regression. Gel beads, $X_o = 0.09$ kg m$^{-3}$, (Δ, ----); gel beads, $X_o = 0.01$ kg m$^{-3}$, (+, ---); gel slabs, $X_o = 0.04$ kg m$^{-3}$, (o, -- --); gel slabs, $X_o = 0.27$ kg m$^{-3}$, (+, ·····).

in the profile measurement causing large differences in the estimate of the parameter $R_{oa}/D_e$.

6.4.3 Maximum specific growth rate determination for the immobilized cells

The first few profiles following start of the continuous cultures were used to calculate the maximum specific growth rate in both the gel slabs and spheres, by applying equation (6.8). The logarithmic values of the normalized oxygen conversion rates are plotted in figure 6.7. The slope of the line, obtained by linear regression, gives the maximum specific growth rate, $\mu_{max}$.

For the two slab experiments, the values of $\mu_{max}$ were $0.24 \pm 0.03$ h$^{-1}$ ($n = 3$, first experiment) and $0.24 \pm 0.04$ h$^{-1}$ ($n = 5$, second experiment). For the gel bead experiments $\mu_{max}$ was $0.18 \pm 0.03$ h$^{-1}$ ($n = 3$, first experiment) and $0.18 \pm 0.01$ h$^{-1}$ ($n = 5$, second experiment). The growth rate measured in free-cell suspensions at 25°C was $\mu_{max} = 0.30$ h$^{-1}$, which is close to that measured in the gel slabs.

As can be seen in figure 6.7, values for larger $t$ do not deviate from the regression line. The data points obtained from measurements in the region of steady state oxygen profiles, however, deviated from a straight line (data
Table 6.2 Biomass concentrations calculated for the gel spheres from numerical fitting of the oxygen profiles for duplicate experiments.

<table>
<thead>
<tr>
<th>t (h)</th>
<th>X (kg m(^{-3}))</th>
<th>experiment 1</th>
<th>experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.06</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>-</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>0.35</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>-</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>11.0</td>
<td>-</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>14.3</td>
<td>-</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>22.5</td>
<td>5.65</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>28.0</td>
<td>-</td>
<td>(\geq X_{\text{max}})</td>
<td></td>
</tr>
<tr>
<td>28.3</td>
<td>(\geq X_{\text{max}})</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>47.8</td>
<td>(\geq X_{\text{max}})</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

not shown), supporting the assumption that cell growth within the gel was no longer exponential.

For each determined oxygen conversion rate, the corresponding biomass concentration was calculated using equation (6.3). For the measurements performed after about 28 hours, the calculated biomass concentration was higher than the approximate biomass concentration, \(X_{\text{max}}\).

The value of \(X_{\text{max}} = 28\text{kg m}^{-3}\) was chosen based on maximum cell concentrations near the gel surface measured in other experiments [2]. Estimates of \(D_e\) and \(Y_{X/ox}\) were used for the biomass calculation. A value of \(D_e = 1.4 \cdot 10^{-9} \text{ m}^2\text{s}^{-1}\), which is 60% of the value of \(D_e\) for oxygen in water, was used [11], while \(Y_{X/ox} = 42.6 \cdot 10^{-3} \text{ kg mol}^{-1}\) was taken from previously reported work [19]. Table 6.2 reports the biomass concentration for the gel spheres. Initial biomass concentrations obtained from dilution platings were \(X_0 = 0.09 \text{ kg m}^{-3}\) for experiment 1 and \(X_0 = 0.01 \text{ kg m}^{-3}\) for experiment 2. Table 6.3 reports the biomass concentrations for the gel cylinders. Initial biomass concentrations obtained from dilution platings were \(X_0 = 0.04 \text{ kg m}^{-3}\) for experiment 1 and \(X_0 = 0.27 \text{ kg m}^{-3}\) for experiment 2.

6.4.4 Oxygen penetration depth

The oxygen penetration depth was also calculated for each sample (see equations (6.6) and (6.7)). An advantage of the microsensor measurement technique is that \(C_{\text{t,ox}}\), the actual concentration of oxygen at the surface of the gel, is known. This is especially important for the steep profiles in which external oxygen transport resistance plays a significant role. The oxygen penetration depths for the two gel slab experiments, using two different initial biomass concentrations, are shown in figure 6.8 as a function of time. As can be seen,
Table 6.3 Biomass concentrations calculated for the gel cylinders from numerical fitting of the oxygen profiles for duplicate experiments.

<table>
<thead>
<tr>
<th>$t$ (h)</th>
<th>$X$ (kg m$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>experiment 1</td>
</tr>
<tr>
<td>0.0</td>
<td>0.02</td>
</tr>
<tr>
<td>5.0</td>
<td>–</td>
</tr>
<tr>
<td>10.0</td>
<td>–</td>
</tr>
<tr>
<td>10.2</td>
<td>0.32</td>
</tr>
<tr>
<td>13.5</td>
<td>–</td>
</tr>
<tr>
<td>22.7</td>
<td>8.61</td>
</tr>
<tr>
<td>29.8</td>
<td>$\geq X_{\text{max}}$</td>
</tr>
<tr>
<td>48.0</td>
<td>$\geq X_{\text{max}}$</td>
</tr>
<tr>
<td>49.0</td>
<td>$\geq X_{\text{max}}$</td>
</tr>
<tr>
<td>55.0</td>
<td>$\geq X_{\text{max}}$</td>
</tr>
</tbody>
</table>

Figure 6.8 Oxygen penetration depth in gel slabs as a function of time following the start of continuous culture. $X_0 = 0.04$ kg m$^{-3}$, $(\triangle, \cdots)$; $X_0 = 0.27$ kg m$^{-3}$, $(\circ, \cdots)$. 
the steady state values for both experiments were about the same, approximately 100 µm. A steady state value of \( d = 100 \mu m \) was also measured in the experiments performed with gel beads. In fact, since the steady state ratio of \( d/r_i \) is very small for the gel particles used, it is expected that the final penetration depth should be insensitive to the geometry of the gel.

6.5 Conclusions

In this work, we have used an oxygen microsensor to measure oxygen concentration profiles in carrageenan gels containing immobilized cells. The profiles measured were reliable, reproducible, and obtained relatively rapidly. Oxygen concentration profiles in both slab and sphere geometries became steeper with increasing culture time, eventually reaching a steady state value of 100 µm for both types of gel particles. A reaction-diffusion model using the assumption that cell growth is zero-order in the limiting nutrient oxygen was found to provide an excellent fit to the experimentally-obtained concentration profiles. Growth rates estimated from values of \( R_{ox}/D_e \) at early culture times were lower than the value obtained from free-cell experiments, and this difference was especially pronounced in the gel beads.

6.6 Acknowledgments

This work was supported in part by the North Atlantic Treaty Organization (NATO) under a grant awarded to C. Briasco in 1987. We also thank Transgene S.A., France, for donation of plasmid pTG201.

6.7 Nomenclature

\[
\begin{align*}
C_{ox} & \quad \text{oxygen concentration in the gel (mol m}^{-3}\text{)} \\
C_{i,ox} & \quad \text{oxygen concentration at the gel surface (mol m}^{-3}\text{)} \\
d & \quad \text{oxygen penetration depth (m)} \\
d_p & \quad \text{diameter of gel bead (m)} \\
D_e & \quad \text{effective diffusion coefficient of oxygen in the gel (m}^2\text{s}^{-1}\text{)} \\
K_m & \quad \text{Michaelis-Menten constant (mol m}^{-3}\text{)} \\
r_i & \quad \text{characteristic gel length: bead radius or cylinder (slab) height (m)} \\
r_d & \quad \text{distance at which oxygen concentration reaches zero (m)} \\
R_{ox} & \quad \text{specific volumetric consumption rate of oxygen (mol s}^{-1}\text{m}^{-3}\text{)}
\end{align*}
\]
6. Oxygen profiles in immobilized *E. coli*

\[ t \quad \text{time (s)} \]
\[ \Delta t \quad \text{residence time of gel beads and blocks in continuous culture at time of measurement (s)} \]
\[ X \quad \text{cell concentration (kg m}^{-3}) \]
\[ X_0 \quad \text{initial cell concentration (kg m}^{-3}) \]
\[ X_{\text{max}} \quad \text{maximum possible cell concentration in the gel (kg m}^{-3}) \]
\[ Y_{X/ox} \quad \text{cell yield coefficient on oxygen (kg mol}^{-1}) \]

**Greek symbols**

\[ \mu \quad \text{specific growth rate (s}^{-1}) \]
\[ \mu_{\text{max}} \quad \text{maximum specific growth rate (s}^{-1}) \]

6.8 Bibliography

6. Oxygen profiles in immobilized *E. coli*
Chapter 7

The use of a metabolically structured model in the study of growth, nitrification and denitrification by *Thiosphaera pantotropha*

*Thiosphaera pantotropha* is capable of aerobic heterotrophic nitrification and both aerobic and anaerobic denitrification. These phenomena have been studied in acetate-limited aerobic and anaerobic continuous cultures supplied with ammonia and nitrate. The internal reaction rates were defined, based on biochemical knowledge. The observable external conversion rates are related through a linear equation on the basis of the specified internal reaction rates. The linear equation is a Pirt relation extended for microbial systems with multiple electron donors (acetate and ammonia) and electron acceptors (oxygen and nitrate). The coefficients in this equation were estimated from the continuous culture measurements, and are composed of parameters involved in ATP production and consumption by the microorganism. It is shown that with realistic values for these parameters, the metabolically structured model describes the aerobic as well as the anaerobic experiments.

7.1 Introduction

In various types of waste water, nitrogen compounds such as ammonia and nitrate can be an environmental problem. These compounds are normally removed microbiologically in two steps, exploiting two different types of microorganisms. The first step is aerobic, and involves the oxidation of ammonia to nitrate by autotrophic bacteria which derive energy from the reaction (nitrification). In the second step, the nitrate is reduced anaerobically to

nitrogen gas, generally by heterotrophs or methylotrophs (denitrification). The emphasis of studies on nitrification has mainly been concentrated on the autotrophic organisms. However, many heterotrophs are also capable of nitrification [1, 2]. The importance of heterotrophic nitrification is still a matter of debate, especially as heterotrophic nitrification does not generate any energy. The specific nitrifying activity of the heterotrophs is said to be $10^3 - 10^4$ times lower than that of the autotrophs, and therefore heterotrophic nitrification is often considered to be of minor ecological significance [1, 3, 4]. However, this activity was measured by the accumulation of nitrite or nitrate. Many heterotrophic nitrifiers are able to denitrify aerobically [5] as well as anaerobically [6]. In this way, ammonia is directly converted to nitrogen gas and nitrite or nitrate will not accumulate. When making mass balances for continuous cultures, it was found that the nitrification activity (in terms of ammonia oxidized) of at least one heterotrophic nitrifier/aerobic denitrifier, *Thiospaera pantotropha*, is only $10 - 10^3$ times lower than the autotrophs [5]. While growing as heterothrophs (during which heterotrophic nitrification takes place) the growth rate of *T. pantotropha* is much higher than when it would grow autotrophically. It seems likely that, as other bacteria of this physiological type are studied, it will be found that most nitrification rates have been underestimated because of the simultaneous nitrite reduction. Thus, in view of the fact that heterotrophs generally outnumber autotrophs in the bacteria communities found in most waste water treatment systems, sediments and soils, heterotrophic nitrifying organisms might well be of greater significance than previously thought.

In waste water treatment, the nitrification step is often a bottleneck. The residence time in the nitrification reactor is mainly determined by the maximum growth rate of the autotrophs, which is relatively low. In view of their higher growth rates and their ability to convert ammonia to nitrogen gas in one step, heterotrophic nitrifiers/denitrifiers might be an attractive alternative for waste water treatment. Additionally, a pre-treatment step, where organic material is removed prior to autotrophic nitrification, would not be necessary if heterotrophs were used. However, before these organisms can successfully be applied, the heterotrophic nitrification/denitrification processes must be fully understood.

To evaluate the potential application of such microorganisms, mathematical modelling of their performance is advisable. These mathematical models can also be used, when extended, to predict the behaviour of immobilized organisms [7].

In this chapter growth and simultaneous nitrification and denitrification by homogeneous, continuous cultures of *T. pantotropha* is described mathematically. Hereby it is assumed that the aerobic nitrification/denitrification is due to two reactions, in which ammonia is first oxidized to nitrite, and subsequently reduced to nitrogen gas, and that the anaerobic denitrification
proceeds according to the 'conventional' reaction in which nitrate is reduced to nitrite and consequently to nitrogen gas.

7.2 Materials and methods

The model is based on the results of experiments using chemostat cultures of *Thiosphaera pantotropha* LMD 82.5. This organism was originally isolated from a sulfide-oxidizing, denitrifying industrial waste water treatment system in which it was one of the dominant mixotrophs [8]. The growth media and analytical techniques, together with the full experimental results, can be found in ref. [9]. All of the cultures were acetate limited and supplied with ammonia as nitrogen source (32 mol m$^{-3}$). Aerobic cultures were maintained at 80% air saturation, nitrate was supplied to some cultures, as mentioned in the text.

7.3 Metabolically structured modelling

On the basis of biochemical knowledge and stoichiometry, the reactions which play a role in the metabolism of a heterotrophic microorganism can be formulated and are called internal reaction rates. The rates of these reactions can be related to the substrate consumption and product formation rates (observable conversion rates), as described by Roels *et al.* [10–13] whose approach and notation is followed here. First, the principles and the assumptions for the metabolically structured model will be given and applied to aerobic nitriﬁcation/denitriﬁcation of ammonia and denitriﬁcation of nitrate. Second, the model will be applied to the anaerobic denitriﬁcation of nitrate. For ease of notation, NADH$_2$ instead of NADH + H$^+$ + e$^-$ will be used in the text and formulae.

The assumptions necessary for a metabolically structured model are as follows:

- **Biomass** is in pseudo-steady state with respect to all compounds being considered in the metabolism. Consequently, there is no net accumulation of ATP, NADH$_2$ and precursors.
- Compounds such as NADH$_2$ and ATP do not leave the cell in significant amounts. There is no net transport to the environment.
- For the compounds for which a pseudo-steady state hypothesis is justified, and which are not transported to the environment, generation during metabolism must match consumption. This can be applied to NADH$_2$, ATP and precursors. Their net conversion rate is therefore zero.
7. Metabolically structured model for *T. pantotropha*

- Stoichiometric coefficients used in the metabolic reactions related to energy conversion are independent of the experimental growth conditions used (type of substrate, high or low oxygen concentration etc.).

The reactions and the procedure to make a metabolically structured model for *T. pantotropha* are outlined below.

7.4 Aerobic nitrification/denitrification of NH₃ and HNO₃

7.4.1 The internal reaction rates

The behaviour of aerobic cultures which have either been supplied with ammonia alone or ammonia and nitrate, can be described as follows:

Biomass formation reaction

Two overall reactions are involved in biomass formation. The first reaction is anabolism, the synthesis of biomass. This synthesis follows a specified stoichiometry which only depends on the nature of the substrate used. The nitrogen source is assumed to be ammonia:

\[
(1 + \alpha_{11}) \text{CH}_2\text{O}_2 + 4\text{NH}_3 \xrightarrow{r_1} (\alpha_{12} + K) \text{ATP} + \text{CH}_4\text{O}_6\text{N}_1 + \alpha_{11}\text{CO}_2 + [b_2 (1 + \alpha_{11}) - 2\alpha_{11} - b_1] \text{H}_2\text{O} + [(0.5a_2 - b_2) (1 + \alpha_{11}) + b_1 - 0.5a_1 + 2\alpha_{11} + 1.5c_1] \text{NADH}_2
\]

(7.1)

In this reaction it is assumed that NADPH₂ is equivalent to NADH₂. The extra energy content of NADPH₂ is implicitly present in the amount of ATP necessary for biomass synthesis. The amount of ATP involved in the anabolism is divided in the amount necessary for the formation of biomass precursors (\(\alpha_{12}\)) and the amount necessary for the polymerization of precursors to biomass (\(K\)). This amount is independent of the type of substrate used. Moreover, as can be seen in equation (7.1) the amount of biomass and substrate are expressed in carbon-mol, being the amount containing one mol of the element carbon.

The second reaction involves ATP used in maintenance. The ATP requirement for maintenance is first-order in the amount of biomass:

\[
r_{\text{ATP}} = -m_{\text{ATP}}C_X = \frac{-m_{\text{ATP}}}{\mu}r_1
\]

(7.2)

The amount of ATP required for maintenance generally ranges from 0.005 to 0.05 mol/Cmol h [10].
The biomass composition is CH$_{1.9}$O$_{0.5}$N$_{0.25}$ [9], determined from element analysis. Equations (7.1) and (7.2) can be combined to give an equation for biomass formation with an ATP requirement composed of maintenance, synthesis of precursors and precursor polymerization yielding the following balance equation for the biomass formation rate $r_1$:

$$(1 + \alpha_{11})\text{CH}_3\text{O} + 0.25 \text{NH}_3 \rightarrow_{T_1} \left(\alpha_{12} + K + \frac{\text{mATP}}{\mu}\right) \text{ATP} + \text{CH}_1\text{O}_{0.5}\text{N}_{0.25} + \alpha_{11}\text{CO}_2 + (0.5 - \alpha_{11})\text{H}_2\text{O} + (2\alpha_{11} - 0.075) \text{NADH}_2$$

(7.3)

The amount of ATP required for the synthesis of biomass from acetate ($K + \alpha_{12}$) generally ranges from 2 to 3 mol/Cmol [14]. Furthermore it is assumed that in the assimilation reaction, about 0.3 mol carbon dioxide is produced per Cmol biomass [15], $\alpha_{11} = 0.3$.

Substrate catabolism reaction

Part of the substrate is catabolized to produce ATP and reducing equivalents:

$$\text{CH}_3\text{O}_2 \rightarrow_{T_2} \text{CO}_2 + \alpha_{21}\text{ATP} + a2\text{NADH}_2 + (b2 - 2)\text{H}_2\text{O}$$

(7.4)

For acetate this gives the following balance equation for the catabolism reaction rate $r_2$:

$$\text{CH}_3\text{O} + \text{H}_2\text{O} \rightarrow_{T_2} \text{CO}_2 + \alpha_{21}\text{ATP} + 2\text{NADH}_2$$

(7.5)

To find a value for $\alpha_{21}$, information is required about the dissimilation of acetate. Acetate is taken up by the cell by means of active transport and must be activated to form acetyl-CoA. Both processes theoretically require, in total, 2 ATP per mol acetate [16]. The citric acid (TCA) cycle contains one substrate phosphorylation step, yielding 1 ATP. It is assumed that in the conversion of isocitrate to keto glutarate, 1 NADPH is produced, 1 FADH$_2$ is generated by the conversion of succinate to fumarate, and 2 NADH$_2$ are produced during the two subsequent dehydrogenation steps. The subsequent oxidation of FADH$_2$ in the electron transfer chain involves one less proton translocation step than NADH$_2$. In the model, only NADH$_2$ is considered, therefore a correction must be made for FADH$_2$ by subtracting 1 ATP. Thus there is a net consumption of 2 ATP per dissimilation of one molecule of acetate [16], or 1 ATP per Cmol acetate, $\alpha_{21} = -1$.

Oxidative phosphorylation

*Thiomicrospira pantotropha* possesses both cytochrome aa$_3$ and cytochrome o as terminal oxidases [5]. The available literature [17] indicates that there are fewer proton
translocation steps involved in the reduction of oxygen via cytochrome o than when aa₃ is involved. However, the model cannot discriminate between the use of two terminal oxidases, thus the overall value for the ATP produced per two electrons for cytochromes aa₃ and o is given by δ. From experiments with other organisms, it was found that the ATP/2e will range from 1.5 to 3 [10, 17, 18].

\[ 0.5 \text{O}_2 + \text{NADH}_2 \xrightarrow{r_3} \text{H}_2\text{O} + \delta \text{ATP} \]  

(7.6)

Nitrification/denitrification

For a particular microorganism, specific biochemical reactions must be added to reactions \(r_1\) to \(r_3\). For \(T. \text{pantotropha}\) these are the nitrification and denitrification reactions. The basis of the modelling described in this article is the electron transfer chain of \(T. \text{pantotropha}\) [5]. Figure 7.1 shows a working model of the transport chain of \(T. \text{pantotropha}\) based on physiological measurements and preliminary cytochrome spectra of cells grown under various conditions [5] and on previous experiments with \(\text{Paracoccus denitrificans}\) [17]. There are some obvious differences from the cytochrome chain of \(P. \text{denitrificans}\) including the nitrite reductase of \(T. \text{pantotropha}\) being a copper enzyme, and that of \(P. \text{denitrificans}\) being a cytochrome cd. The branch points of the cytochrome chain of \(T. \text{pantotropha}\) have not yet been determined, and they have therefore been assumed to be similar to those of \(P. \text{denitrificans}\). Nitrification and aerobic denitrification by \(T. \text{pantotropha}\) may be explained by a bottleneck in the electron transfer somewhere between cytochrome c and oxygen via cytochrome aa₃, resulting in a greater degree of reduction in the electron transport chain, allowing electrons to flow to the reductases of the denitrification.

Nitrification of ammonia to nitrite

\[ \text{NH}_4 + 2 \text{O}_2 \xrightarrow{r_4} \text{NADH}_2 + \text{HNO}_2 + 2 \text{H}_2\text{O} + \alpha_{41} \text{ATP} \]  

(7.7)

The involvement of ATP in heterotrophic nitrification is not known. ATP is not necessary for the in vitro oxidation of ammonia or hydroxylamine by crude cell free extracts. However, NADPH₂ rather than NADH₂ is required by the ammonia monooxygenase of \(T. \text{pantotropha}\) [19]. In the TCA-cycle, 1 NADPH₂ can be produced per molecule of acetate, which can be used as a reductor in the synthesis of biomass. If this production route is not able to cope with the demand of the cell, NADPH₂ can also be produced from NADH₂ by a transhydrogenase which may require an amount of ATP ranging between 0 and 1 per NADPH₂ [16]. It may well be that due to nitrification, the cell is forced to use this energy expensive route. It is assumed that, as with the autotrophic ammonia oxidases, the two enzymes needed for heterotrophic nitrification, ammonia monooxygenase (AMO) and hydroxylamine
Figure 7.1 Schematic representation of the cytochrome chains devised to explain electron transport in *T. pantotropha*. Cyt. = cytochromes; (L) and (R) = arbitrary designations to distinguish 2 cyts. c552; cyt. c7 indicates one or more additional cytochrome c; UQ = ubiquinone; NaR = nitrate reductase; NiR = nitrite reductase; NoR = nitrous oxide reductase; AMO = ammonia monoxygenase; HoR = hydroxylamine oxidoreductase. The dashed line indicates uptake of NADH₂.
oxidoreductase (HoR) are located at the periplasmic side of the cell membrane [5], making active transport of ammonia and/or hydroxylamine unnecessary. The overall reaction including production of NADPH₂ is assumed to require a minimum of 1 ATP. Hence the value for $\alpha_{41}$ will be at least $-1$.

Nitrate reduction to nitrite

$$\text{HNO}_3 + \text{NADH}_2 \xrightarrow{r_5} \text{HNO}_2 + \text{H}_2\text{O} + \alpha_{61}\text{ATP}$$

(7.8)

Denitrification of nitrite

$$\text{HNO}_2 + 1.5 \text{NADH}_2 \xrightarrow{r_6} 0.5\text{N}_2 + 2\text{H}_2\text{O} + \alpha_{61}\text{ATP}$$

(7.9)

Stouthamer et al. [17] found that in *P. denitrificans* the reductions of nitrate to nitrite, nitrite to nitrous oxide and nitrous oxide to nitrogen all yield the same maximum ATP/2e, and that the value for each step is lower than that for oxygen. In analogy with these findings, the maximum theoretical values for $\alpha_{41}$ and $\alpha_{61}$ will be 1.67 and 2.51, respectively. However, *T. pantotropha* has a lower efficiency in proton translocation for oxygen and all the denitrifying steps (D. Castignetti, personal communication). On the basis of these results the value of $\alpha_{61}$ and $\alpha_{31}$ should be approximately half of these values. It may thus be concluded that $\alpha_{61} + \alpha_{61}$ will be between 1.0 and 1.5, and $\alpha_{41} + \alpha_{61}$ will be about $-0.2$ to $-1.2$.

7.4.2 Linear equation between observable conversion rates

The reactions $r_1$ to $r_8$ are termed ‘internal reactions’, and are based on biochemical knowledge and stoichiometry. These reactions occur within the cell and cannot be observed directly. However, the internal reaction rates can be related to the observable conversion rates outside of the cell. If, for *T. pantotropha*, the cell is represented as a box, the flows of substrate and product to and from the cell can be schematically given as shown by figure 7.2. Due to growth, nitrification and denitrification, ammonia, nitrite, nitrate, oxygen and acetate are consumed and biomass, nitrogen and carbon dioxide are produced. These conversion rates are related to the internal reaction rates. Knowledge of the internal reactions gives a direct relation between the observable conversion rates.

For steady state conditions in a fermentor, the conversion rate $r_i$ is given by the product of the dilution rate and the concentration difference between the incoming and outgoing streams to the fermentor:

$$r_i = D (C_{i,\text{out}} - C_{i,\text{in}})$$

(7.10)

$r_i$ is positive for production and negative for consumption of components. In the case of *T. pantotropha*, $r_5$, $r_{\text{NH}_3}$, $r_{\text{O}_2}$, and $r_{\text{HNO}_2}$ are negative, and $r_1$, $r_{\text{N}_2}$, and $r_{\text{HNO}_2}$ are positive. The conversion rates are related by a linear equation
Figure 7.2 Black box model of *Thiosphaera pantotropha* showing the relevant components entering and leaving the cell.

with coefficients composed of the parameters (stoichiometric coefficients) of the internal reactions. The observable conversion rates are determined by the internal reaction rates. If the conversion rates are expressed as a function of the internal reaction rates it follows that:

\[
\begin{align*}
    r_S &= -(1 + \alpha_{11}) r_1 - r_2 \\
    r_{\text{NH}_3} &= -0.25 r_1 - r_4 \\
    r_{\text{HNO}_2} &= r_4 + r_5 - r_6 \\
    r_{\text{N}_2} &= 0.5 r_6 \\
    r_X &= r_1 \\
    r_{\text{O}_2} &= -0.5 r_3 + r_2 \\
    r_{\text{CO}_2} &= \alpha_{11} r_1 + r_2 \\
    r_{\text{H}_2\text{O}} &= (0.5 - \alpha_{11}) r_1 - r_2 + r_3 + 2 r_4 + r_5 + 2 r_6 \\
    r_{\text{HNO}_3} &= -r_5
\end{align*}
\]  

(7.11)  
(7.12)  
(7.13)  
(7.14)  
(7.15)  
(7.16)  
(7.17)  
(7.18)  
(7.19)

As it is assumed that there is no net accumulation of NADH$_2$ and ATP, $r_{\text{NADH}_2} = 0$, $r_{\text{ATP}} = 0$:

\[
\begin{align*}
    r_{\text{NADH}_2} &= (2\alpha_{11} - 0.075) r_1 + 2 r_2 - r_3 - r_4 - \ r_5 - 1.5 r_6 = 0 \\
    r_{\text{ATP}} &= \left( \alpha_{12} + K + \frac{m_{\text{ATP}}}{\mu} \right) r_1 + \alpha_{21} r_2 + \delta r_3 + \alpha_{41} r_4 + \alpha_{51} r_5 + \alpha_{61} r_6 = 0
\end{align*}
\]  

(7.20)  
(7.21)
As there are fifteen reaction rates available (r₁ to r₆ and r₇ to r₉HNO₃) and eleven equations (r₇ to r₉ATP), the system can be described with four conversion rates. This results in a linear relation between the five conversion rates necessary to calculate the coefficients in the linear relation. The following conversion rates determined during the continuous culture experiments were selected: rₓ, rₛ, rNH₄, rHNO₂ and rHNO₃. If the internal reaction rates are expressed as functions of the conversion rates, it follows that:

\[
\begin{align*}
\text{r}_1 &= r_x \\
\text{r}_2 &= -(1 + \alpha_{11})r_x - r_s \\
\text{r}_3 &= -1.45r_x - 2r_s + 2.5r_{\text{NH}_4} + 1.5r_{\text{HNO}_2} + 2.5r_{\text{HNO}_3} \\
\text{r}_4 &= -0.25r_x - r_{\text{NH}_4} \\
\text{r}_5 &= -r_{\text{HNO}_2} \\
\text{r}_6 &= -0.25r_x - r_{\text{NH}_4} - r_{\text{HNO}_2} - r_{\text{HNO}_3}
\end{align*}
\]  

(7.22)

(7.23)

(7.24)

(7.25)

(7.26)

(7.27)

Substitution in the ATP balance and recalculation \( (r_x/C_x = \mu) \) finally gives:

\[
\frac{-r_s}{C_x} = \frac{-\left(\alpha_{12} + K\right) + \alpha_{21} + \alpha_{21}\alpha_{11} + 0.25\alpha_{41} + 0.25\alpha_{61} + 1.45\delta}{\alpha_{21} + 2\delta} \mu \\
+ \frac{2.5\delta - \alpha_{41} - \alpha_{61}}{\alpha_{21} + 2\delta} \left(\frac{-r_{\text{NH}_4}}{C_x}\right) + \frac{2.5\delta - \alpha_{41} - \alpha_{61}}{\alpha_{21} + 2\delta} \left(\frac{-r_{\text{HNO}_2}}{C_x}\right) \\
+ \frac{1.5\delta - \alpha_{61}}{\alpha_{21} + 2\delta} \left(\frac{-r_{\text{HNO}_3}}{C_x}\right) - \frac{m_{\text{ATP}}}{\alpha_{21} + 2\delta}
\]  

(7.28)

The specific acetate conversion rate \( (-r_s/C_x) \) is a linear function of the specific growth rate \( \mu \), the specific ammonia conversion rate \( (-r_{\text{NH}_4}/C_x) \), the specific nitrate conversion rate \( (-r_{\text{HNO}_3}/C_x) \) and the specific nitrite conversion rate \( (-r_{\text{HNO}_2}/C_x) \). We can write equation (7.28) as:

\[
y = a_1 x_1 + a_2 x_2 + a_3 x_3 + a_4 x_4 + b
\]  

(7.29)

The minus sign is incorporated in \( x_1, x_2, x_3 \) and \( x_4 \) because conversion rates for consumed components are negative. In this way \( y, x_1, x_2, x_3 \) and \( x_4 \) are positive numbers. The results of the experiments are also given as positive numbers. By using a linear regression procedure, it is possible to calculate the coefficients \( a_1, a_2, a_3 \) and \( a_4 \) which are composed of parameters involved in the ATP conversion of the internal reactions. In this case, Marquardt’s compromise method [20] was used. From these coefficients a number of stoichiometric coefficients \( (\alpha_{61}, (\alpha_{12} + K), etc.) \) can be determined. In this way, the ATP consumption or production during the specific reactions can be calculated and compared with biochemical data. It should be noted that the simple Pirt equation is no longer applicable.
Table 7.1 Analytical data for the aerobic, acetate-limited continuous cultures (data are from ref. [5, 9]). Present was Ac, NH₃ and O₂.

<table>
<thead>
<tr>
<th>D</th>
<th>ΔNH₃</th>
<th>ΔHNO₃</th>
<th>Cₓ</th>
<th>Cₛ,ₘₐ</th>
<th>(-r_{NH₃}/Cₓ)</th>
<th>(-r_{HNO₃}/Cₓ)</th>
<th>(-rₛ/Cₓ)</th>
</tr>
</thead>
</table>
| (1/ℎ) | (mol/m³) | (mol/m³) | (Cmol/m³) | (Cmol/m³) | (mol/Cmol h) | (mol/Cmol h) | (mol/Cmol h) |}
| .017 | 6.20 | -     | 7.55 | 50.6    | .014           | -               | .114      |
| .046 | 6.20 | -     | 6.42 | 37.2    | .044           | -               | .267      |
| .063 | 4.54 | -     | 9.29 | 40.0    | .031           | -               | .271      |
| .10  | 6.90 | -     | 7.13 | 40.0    | .097           | -               | .561      |
| .20  | 5.22 | -     | 11.6 | 40.0    | .090           | -               | .691      |
| .41  | 4.91 | -     | 11.7 | 40.0    | .173           | -               | 1.41      |

Table 7.2 Analytical data for the aerobic, acetate-limited continuous cultures (data are from ref. [5, 9]). Present was Ac, NH₃, HNO₃ and O₂.

<table>
<thead>
<tr>
<th>D</th>
<th>ΔNH₃</th>
<th>ΔHNO₃</th>
<th>Cₓ</th>
<th>Cₛ,ₘₐ</th>
<th>(-r_{NH₃}/Cₓ)</th>
<th>(-r_{HNO₃}/Cₓ)</th>
<th>(-rₛ/Cₓ)</th>
</tr>
</thead>
</table>
| (1/ℎ) | (mol/m³) | (mol/m³) | (Cmol/m³) | (Cmol/m³) | (mol/Cmol h) | (mol/Cmol h) | (mol/Cmol h) |}
| .017 | 2.25 | 8.60  | 6.54 | 40.0    | .006           | .922           | .104      |
| .044 | 1.67 | 13.4  | 8.11 | 40.0    | .009           | .073           | .217      |
| .086 | 1.25 | 15.7  | 8.19 | 40.0    | .013           | .165           | .420      |
| .14  | 0.95 | 16.6  | 8.11 | 40.0    | .016           | .286           | .691      |
| .17  | 0.95 | 17.3  | 8.27 | 40.0    | .020           | .356           | .822      |

7.4.3 Measurements aerobic experiments

The results of the aerobic chemostat experiments are given in tables 7.1 and 7.2. In all cases, the dissolved oxygen concentration was controlled at 80% air saturation. Gas analysis was not performed, so there was no check of mass balances. There was no accumulation of nitrite, so the linear relation between the conversion rates (7.29) reduces to

\[ y = a₁x₁ + a₂x₂ + a₃x₃ + b \]  

(7.30)

in which \( x₁ = \mu, x₂ = -r_{NH₃}/Cₓ \) and \( x₃ = -r_{HNO₃}/Cₓ \).

7.4.4 Results of fitting of the aerobic experiments

Fitting of the coefficients in the linear equation (7.30) gives \( a₁ = 1.83 \pm 0.09, \)
\( a₂ = 3.61 \pm 0.25, \) \( a₃ = 1.18 \pm 0.06 \) and \( b = 0.026 \pm 0.007 \), resulting in:
Table 7.3 Measured and calculated specific substrate utilization for the aerobic, acetate-limited continuous cultures. Present was Ac, NH₃ and O₂.

<table>
<thead>
<tr>
<th>D (h⁻¹)</th>
<th>(-\frac{r_s}{C_X}) (mol Cmol⁻¹ h)</th>
<th>(-\frac{r_s}{C_X}) (mol Cmol⁻¹ h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>measured</td>
<td>calculated</td>
</tr>
<tr>
<td>.017</td>
<td>.114</td>
<td>.108</td>
</tr>
<tr>
<td>.046</td>
<td>.267</td>
<td>.270</td>
</tr>
<tr>
<td>.063</td>
<td>.271</td>
<td>.252</td>
</tr>
<tr>
<td>.10</td>
<td>.561</td>
<td>.558</td>
</tr>
<tr>
<td>.20</td>
<td>.691</td>
<td>.717</td>
</tr>
<tr>
<td>.41</td>
<td>1.41</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Table 7.4 Measured and calculated specific substrate utilization for the aerobic, acetate-limited continuous cultures. Present was Ac, NH₃, HNO₃ and O₂.

<table>
<thead>
<tr>
<th>D (h⁻¹)</th>
<th>(-\frac{r_s}{C_X}) (mol Cmol⁻¹ h)</th>
<th>(-\frac{r_s}{C_X}) (mol Cmol⁻¹ h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>measured</td>
<td>calculated</td>
</tr>
<tr>
<td>.017</td>
<td>.104</td>
<td>.105</td>
</tr>
<tr>
<td>.044</td>
<td>.217</td>
<td>.225</td>
</tr>
<tr>
<td>.086</td>
<td>.420</td>
<td>.425</td>
</tr>
<tr>
<td>.14</td>
<td>.691</td>
<td>.679</td>
</tr>
<tr>
<td>.17</td>
<td>.822</td>
<td>.827</td>
</tr>
</tbody>
</table>

\[
\left(-\frac{r_s}{C_X}\right) = 1.83\mu + 3.61\left(-\frac{r_{NH_3}}{C_X}\right) + 1.18\left(-\frac{r_{HNO_3}}{C_X}\right) + 0.026 \quad (7.31)
\]

In tables 7.3 and 7.4, the measured specific conversion rates and the calculated specific acetate conversion rate are given. The dissolved oxygen concentration was controlled at 80% air saturation. It can be seen that the measured specific acetate conversion rate can be described with a linear relationship between the measured conversion rate. The coefficients in the linear equation (7.31) are composed of the parameters involved in the internal reaction rates as expressed by equation (7.28). From the results of the estimation and equation (7.28), the following relations can be calculated:
Using $a_1$:

\[(\alpha_{12} + K) = -0.83\alpha_{21} + \alpha_{21}\alpha_{11} + 0.25\alpha_{41} + 0.25\alpha_{61} - 2.21\delta \quad (7.32)\]

Using $a_2$:

\[\alpha_{41} + \alpha_{61} = -3.61\alpha_{21} - 4.72\delta \quad (7.33)\]

Using $a_3$:

\[\alpha_{51} + \alpha_{61} = -1.18\alpha_{12} + 0.14\delta \quad (7.34)\]

Using $b$:

\[m_{ATP} = -0.026(\alpha_{12} + 2\delta) \quad (7.35)\]

With equations (7.32)–(7.35), the aerobic experiments can be described. However, there is not enough information to calculate all of the stoichiometric coefficients. Besides $\alpha_{11}$, three stoichiometric coefficients, for example $\delta$, $\alpha_{41}$, and $\alpha_{51}$, still have to be chosen. With the chosen stoichiometric coefficients and equations (7.32)–(7.35), the other stoichiometric coefficients can be calculated. The results of the estimation will be evaluated after anaerobic denitrification has been dealt with.

### 7.5 Anaerobic denitrification of HNO₃

#### 7.5.1 The internal reaction rates

The same strategy as in the aerobic case can be applied to the anaerobic situation in which nitrate is the sole electron acceptor. In this case, nitrification ($r_4$) and aerobic oxidative phosphorylation ($r_3$) can be left out. The denitrification of nitrite and the nitrite formation from nitrate can be combined into one reaction rate, $r_7$, since nitrite formation from ammonia is unlikely to occur in the absence of oxygen. This results in the following balance equations:

**Biomass formation**

\[(1 + \alpha_{11})\text{CH}_2\text{O} + 0.25\text{NH}_3 \xrightarrow{r_1} (\alpha_{12} + K + \frac{m_{ATP}}{\mu}) \text{ATP} + \]

\[\text{CH}_{1.9}\text{O}_{0.8}\text{N}_{0.25} + \alpha_{11}\text{CO}_2 + (0.5 - \alpha_{11})\text{H}_2\text{O} + (2\alpha_{11} - 0.075)\text{NADH}_2 \quad (7.36)\]

**Substrate catabolism**

\[\text{CH}_2\text{O} + \text{H}_2\text{O} \xrightarrow{r_2} \text{CO}_2 + \alpha_{21}\text{ATP} + 2\text{NADH}_2 \quad (7.37)\]

**Denitrification**

\[\text{HNO}_3 + 2.5\text{NADH}_2 \xrightarrow{r_7} 0.5\text{N}_2 + 3\text{H}_2\text{O} + (\alpha_{51} + \alpha_{61})\text{ATP} \quad (7.38)\]
Linear equation between observable conversion rates

If the internal reaction rates are expressed as function of the observable conversion rates, it follows that:

\[
\begin{align*}
\tau_S &= -(1 + \alpha_{11}) r_1 - r_2 = 0 \\
\tau_{\text{NH}_3} &= -0.25 r_1 \\
\tau_{\text{HNO}_3} &= -r_7 \\
\tau_{\text{N}_2} &= 0.5 r_7 \\
\tau_X &= r_1 \\
\tau_{\text{CO}_2} &= \alpha_{11} r_1 + r_2 \\
\tau_{\text{H}_2\text{O}} &= (0.5 - \alpha_{11}) r_1 - r_2 + 3 r_7
\end{align*}
\]

No net accumulation of NADH$_2$ and ATP is assumed, \( r_{\text{NADH}_2} = 0 \) and \( r_{\text{ATP}} = 0 \):

\[
\begin{align*}
\tau_{\text{NADH}_2} &= (2\alpha_{11} - 0.075) r_1 + 2 r_2 - 2.5 r_7 = 0 \\
\tau_{\text{ATP}} &= (\alpha_{12} + K + \frac{m_{\text{ATP}}}{\mu}) r_1 + \alpha_{21} r_2 + \\
&\quad (\alpha_{51} + \alpha_{61}) r_7 = 0
\end{align*}
\]

There are 10 rates and 9 equations. The total system thus can be described with only one conversion rate. Two conversion rates are necessary to estimate the coefficients in the linear relationship between the conversion rates, here \( r_S \) and \( r_X \) are chosen.

If the internal reaction rates are expressed as function of the two measured conversion rates, \( r_S \) and \( r_X \), it follows that:

\[
\begin{align*}
\tau_1 &= r_X \\
\tau_2 &= -(1 + \alpha_{11}) r_X - r_S \\
\tau_7 &= -0.83 r_X - 0.8 r_S
\end{align*}
\]

Substitution in the ATP balance gives:

\[
\left( -\frac{m_{\text{ATP}}}{C_X} \right) = \left[ 0.8 (\frac{\alpha_{51} + \alpha_{61}}{\alpha_{21}}) \right] \mu - \\
\left[ (\frac{\alpha_{12} + K + \alpha_{21} \alpha_{11} + 0.83 (\alpha_{51} + \alpha_{61})}{0.8 (\alpha_{51} + \alpha_{61})}) \alpha_{21} \right]
\]

Equation (7.51) is a relation of the typical Pirt form

\[
y = a_1 x_1 + b
\]
Table 7.5 Analytical data for the aerobic, acetate-limited continuous cultures (data are from ref. [5, 9]). Present was Ac, HNO₃ and NH₃.

<table>
<thead>
<tr>
<th>D (h⁻¹)</th>
<th>ΔHNO₃ (mol m⁻³)</th>
<th>Cₓ (Cmol m⁻³)</th>
<th>Cₙ (Cmol m⁻³)</th>
<th>(-\frac{r_{\text{HNO₃}}}{Cₓ}) (mol Cmol⁻¹ h⁻¹)</th>
<th>(-\frac{r_{\text{NH₃}}}{Cₓ}) (mol Cmol⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>.040</td>
<td>20.3</td>
<td>3.61</td>
<td>40.0</td>
<td>.225</td>
<td>.443</td>
</tr>
<tr>
<td>.075</td>
<td>25.7</td>
<td>4.25</td>
<td>40.0</td>
<td>.454</td>
<td>.706</td>
</tr>
<tr>
<td>.090</td>
<td>25.9</td>
<td>3.87</td>
<td>40.0</td>
<td>.601</td>
<td>.930</td>
</tr>
<tr>
<td>.108</td>
<td>26.0</td>
<td>4.11</td>
<td>40.0</td>
<td>.683</td>
<td>1.05</td>
</tr>
<tr>
<td>.163</td>
<td>26.2</td>
<td>4.19</td>
<td>40.0</td>
<td>1.02</td>
<td>1.56</td>
</tr>
</tbody>
</table>

The coefficients $a₁$ and $b$ can be estimated from the measurements and a number of parameters ($\alpha_{12} + K$, $\alpha_{61} + \alpha_{61}$, etc.) can be determined. In this way, the ATP consumption or production in the specific reactions can be calculated and compared with biochemical knowledge.

7.5.3 Measurements anaerobic experiments

The NH₃ concentration was not measured during these experiments but it is assumed that all of the ammonia consumed was incorporated in biomass. Subsequent experiments have confirmed this [5]. With this assumption, the reduction degree balance [1] fits within 10%. The experimental data are given in table 7.5. There was no accumulation of nitrite.

7.5.4 Results of fitting of the anaerobic experiments

Fitting of only the coefficients in the linear equation (7.52) gives $a₁ = 9.15 \pm 0.28$ and $b = 0.07 \pm 0.03$, resulting in

$$\left( \frac{\dot{r}_{\text{NH₃}}}{Cₓ} \right) = 9.15 \mu + 0.07$$ (7.53)

In table 7.6, the measured specific reaction rates and the calculated specific acetate reaction rate are given. It can be seen from table 7.6 that the measured specific acetate reaction rate can be described with a linear relationship between measured conversion rates.

From the fitted coefficients and equation (7.51), the following can be calculated:

$$m_{\text{ATP}} = -0.056(\alpha_{51} + \alpha_{61}) - 0.07\alpha_{21}$$ (7.54)

$$-(\alpha_{12} + K) = -6.49(\alpha_{51} + \alpha_{61}) - 8.15\alpha_{21} + \alpha_{21}\alpha_{11}$$ (7.55)
Table 7.6 Measured and calculated specific substrate utilization for the anaerobic, acetate-limited continuous cultures. Present was Ac, HNO₃ and NH₃.

<table>
<thead>
<tr>
<th></th>
<th>( \frac{r_s}{C_X} ) measured</th>
<th>( \frac{r_s}{C_X} ) calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>.040</td>
<td>.443</td>
<td>.433</td>
</tr>
<tr>
<td>.075</td>
<td>.706</td>
<td>.753</td>
</tr>
<tr>
<td>.090</td>
<td>.930</td>
<td>.890</td>
</tr>
<tr>
<td>.108</td>
<td>1.05</td>
<td>1.05</td>
</tr>
<tr>
<td>.163</td>
<td>1.57</td>
<td>1.56</td>
</tr>
</tbody>
</table>

With equations (7.54) and (7.55), the anaerobic experiments can be described. Besides \( \alpha_{II} \), three other parameters, for example \( \alpha_{21}, \alpha_{51} \) and \( m_{ATP} \), still have to be estimated. The results of the estimation will be evaluated in the following section.

7.6 Evaluation of the results of the metabolic model in the aerobic and anaerobic continuous culture experiments

From the results of the anaerobic and aerobic experiments, the coefficients in the linear relationship between the flows could be estimated. The linear relations satisfactorily describe the measurements. The minimum number of conversion rates needed to describe the system is 1 for the anaerobic and 4 for the aerobic cases. The number of conversion rates needed to describe the system can also be calculated from the number of components involved in the reactions and the element balance. In this case, the cell is considered as a black box (see figure 7.2) and no information about the internal reaction rates is available (unstructured model). For the aerobic situation, 9 components are present and 4 elements, thus 5 conversion rates must be known. For the anaerobic case, 7 components are present and also 4 elements, so 3 conversion rates must be known. It can be seen that by the use of a structured model and definition of the internal reaction rates, the conversion rates necessary to describe the system are reduced by 1 for the aerobic and 2 for the anaerobic situations.

Another aspect of using structured rather than unstructured models is that it helps to attain insight into the metabolism of *T. pantotropha*. Furthermore, the results of the aerobic and anaerobic experiments can be combined by means of the stoichiometric coefficients involved in the ATP conversion.
From the values of the coefficients in the linear relations (7.31) and (7.53), the stoichiometric parameters involved in the internal reactions can be determined. However, the values for the different stoichiometric parameters cannot be calculated directly. Choices have to be made for a few parameters because the number of equations (7.32)–(7.35), (7.54) and (7.55), following from the values of the coefficients of the linear relation between the conversion rates, is less than the number of unknown parameters. For these choices, realistic values, as discussed in the introduction, based on published work can be chosen.

If the model describes aerobic nitrification/denitrification and anaerobic denitrification by *T. pantotropha* correctly, the stoichiometric parameters must be the same in both the aerobic and anaerobic situations. It must also be mentioned that the standard deviation in the calculated parameters can be high because of the propagation of error and the structure of the equations for the derivation of the parameters. This is especially true for the value of δ in equation (7.34) and for the value of \( \alpha_{51} + \alpha_{61} \), \( \alpha_{21} \) and consequently \( m_{\text{ATP}} \) in equation (7.54). These equations will not be used in the calculation of the stoichiometric coefficients. The relative errors of the coefficients of the other linear equations turned out to be relatively low, varying between 3 and 10 %, so the system can be described by the equations (7.32), (7.33), (7.35) and (7.55). When using \( \alpha_{11} = 0.3 \), see section 7.4.1, the model is consistent for the aerobic and the anaerobic cultures for \( \alpha_{51} + \alpha_{61} = 1.3 \), \( \alpha_{41} = -2 \), \( \delta = 0.9 \) and \( \alpha_{21} = -0.8 \), yielding:

\[
\begin{align*}
\alpha_{41} + \alpha_{61} & = -1.3 \pm 0.7 \\
 m_{\text{ATP}} & = -0.03 \pm 0.02 \\
 (\alpha_{12} + K) & = -1.9 \pm 0.4 \quad \text{(aerobic condition)} \\
 (\alpha_{12} + K) & = -2.2 \pm 0.4 \quad \text{(anaerobic condition)}
\end{align*}
\]

For \( \alpha_{41} = -2 \):

\[
\begin{align*}
\alpha_{61} & = 0.7 \pm 0.7 \\
\alpha_{51} & = 0.6 \pm 0.7
\end{align*}
\]

It can be concluded that the calculated stoichiometric coefficients have realistic values, but that the separate values of, for example, \( \alpha_{61} \) and \( \alpha_{61} \) cannot be determined accurately. Again, the model is consistent for the aerobic and anaerobic situations. Whether the theoretical values of the stoichiometric coefficients are proven to be correct by experimental verification cannot be concluded at this time. More accurate measurements and complete mass balances for the continuous cultures will be necessary.

An alternative approach which can be used to determine the stoichiometric coefficients involved in the ATP conversion, which has not been mentioned
before but was also considered, will be briefly reviewed here. Equations (7.28) and (7.51), the relationships between the observable conversion rates, can be rearranged. This result in relations in which the stoichiometric coefficients are separated from each other as much as possible, and the conversion rates combined, yielding the following equations:

For the aerobic case

\[
\begin{align*}
- \left( \alpha_{12} + K \right) \mu & + \alpha_{21} \left[ 1.3 \mu - \left( \frac{-r_x}{C_X} \right) \right] + \\
\delta \left[ 1.45 \mu - 2 \left( \frac{-r_x}{C_X} \right) + 2.5 \left( \frac{-r_{NH_4}}{C_X} \right) + 2.5 \left( \frac{-r_{NO_2}}{C_X} \right) + 1.5 \left( \frac{-r_{NO_3}}{C_X} \right) \right] + \\
\alpha_{41} \left[ 0.25 \mu - \left( \frac{-r_{NH_4}}{C_X} \right) \right] - \alpha_{51} \left( \frac{-r_{NO_2}}{C_X} \right) + \\
\alpha_{61} \left[ 0.25 \mu - \left( \frac{-r_{NH_4}}{C_X} \right) - \left( \frac{-r_{NO_2}}{C_X} \right) - \left( \frac{-r_{NO_3}}{C_X} \right) \right] - m_{ATP} & = 0
\end{align*}
\]  

(7.56)

For the anaerobic case

\[
\begin{align*}
- \left( \alpha_{12} + K \right) \mu & + \alpha_{21} \left[ 1.3 \mu - \left( \frac{-r_x}{C_X} \right) \right] + \alpha_{51} \left[ 0.83 \mu - 0.8 \left( \frac{-r_x}{C_X} \right) \right] + \\
\alpha_{61} \left[ 0.83 \mu - 0.8 \left( \frac{-r_x}{C_X} \right) \right] - m_{ATP} & = 0
\end{align*}
\]  

(7.57)

To obtain two linear equations in the form of

\[y = a_1 x_1 + a_2 x_2 + a_3 x_3 + a_4 x_4 + a_5 x_5 + b\]  

(7.58)

one of the (combinations of) conversion rates on the left hand side of equations (7.56) and (7.57) must be written as function of the other (combinations of) conversion rates. It can be seen that \( \left( \frac{-r_x}{C_X} \right) \) can not be written explicitly, as in the other approach, so \( \mu \) has been chosen to be written explicitly. The parameters \( x_1 \) to \( x_5 \) are combinations of measured conversion rates, being different for the aerobic and anaerobic cultures, and \( a_1 \) to \( a_5 \) and \( b \) are the coefficients involved in the ATP conversion, which are equal for the aerobic and anaerobic situation. \( \alpha_{11} \) was set to 0.3, \( a_1 = \frac{\alpha_{11}}{a_{12} + K} \), \( a_2 = \frac{\alpha_{11}}{a_{12} + K} \), \( a_3 = \frac{\alpha_{11}}{a_{12} + K} \), \( a_4 = \frac{\alpha_{61}}{a_{12} + K} \), \( a_5 = \frac{\delta}{a_{12} + K} \), \( b = m_{ATP} \).

In this way, the results of the aerobic and anaerobic experiments could be combined and fit together, directly resulting in the values for the stoichiometric coefficients. Moreover, for the anaerobic experiment the nitrate consumption data were also included. However, it appeared that the standard deviation in the resulting parameters was very high. This is due to the propagating error in the measured conversion rates, and also to the larger number of parameters to be fit (6 instead of 4). When only 3 stoichiometric
coefficients were fit ($\alpha_{51}$, $\alpha_{61}$ and $m_{\text{ATP}}$), and the others set to the values previously obtained by the other approach, the results were in agreement with those found by the other approach.

7.7 Conclusions

Aerobic nitrification/denitrification and anaerobic denitrification in an acetate limited continuous culture of *Thiosphaera pantotropha* can be described by linear equations between the various conversion rates. The stoichiometric coefficients concerned with ATP production and consumption can be calculated from the coefficients in the linear equations. For modelling of a steady state continuous culture, the use of a linear relation between the various conversion rates is sufficient. However, a biochemically structured model gives more insight into the physiology of the cell, and the results of the aerobic and anaerobic experiments can be combined. Choices for a few coefficients must be made because the number of unknown coefficients is higher than the number of equations derived from the coefficients. For these choices, realistic values obtained from the literature and from biochemical knowledge are used. The standard deviations in the calculated coefficients are high due to the deviation in the estimation of the coefficients in the linear equation, to the structure of the equations to derive the coefficients, and to the propagation of error in the calculations. For further and more accurate experimental validation complete mass balances, including gas analysis, must be made for continuous cultures.

The model is consistent for both the aerobic and the anaerobic conditions. The realistic choices: $\delta = 0.9$, $\alpha_{21} = -0.8$, $\alpha_{41} = -2$, $\alpha_{11} = 0.3$ and $\alpha_{51} + \alpha_{61} = 1.3$ give realistic results for the ATP requirement for biomass production, the ATP yield in the denitrification reactions and for the maintenance on ATP. These results are: $(\alpha_{12} + K) = -2.1 \pm 0.4$, $\alpha_{41} + \alpha_{61} = -1.3 \pm 0.7$, $\alpha_{61} = 0.7 \pm 0.7$, $\alpha_{51} = 0.6 \pm 0.7$ and $m_{\text{ATP}} = -0.03 \pm 0.02$.

It should be noted that experimental verification of biochemical stoichiometric coefficients has not yet been obtained using the presently available continuous culture data. However, by choosing realistic values for five stoichiometric coefficients acceptable (although inaccurate) values can be estimated for the remaining stoichiometric coefficients. The complete set of stoichiometric coefficients thus determined describes both aerobic as well as anaerobic performance.

7.8 Acknowledgements

The authors wish to thank Prof. D. Castignetti for providing us unpublished data of his work, and Dr. F.C. Boogerd for carefully reading this manuscript.
7.9 Nomenclature

\( a_1, a_2, b_1, b_2, c_1 \)  stoichiometric coefficients (mol Cmol\(^{-1}\))

\( a_1, \ldots, a_5 \)  coefficients in linear equation between conversion rates (mol Cmol\(^{-1}\))

\( b \)  intercept in linear equation between conversion rates (mol Cmol\(^{-1}\))

\( C_i \)  concentration of component \( i \) (mol m\(^{-3}\))

\( D \)  dilution rate (s\(^{-1}\))

\( K \)  ATP requirement for polymerization of precursors to biomass (mol Cmol\(^{-1}\))

\( m_i \)  maintenance coefficient on component \( i \) (mol Cmol\(^{-1}\) s\(^{-1}\))

\( r_i \)  conversion rate of component \( i \) or reaction rate number \( i \) (mol m\(^{-3}\) s\(^{-1}\))

\( t \)  time (s)

\( x_1, \ldots, x_5, y \)  conversion rates in linear equation between conversion rates (mol Cmol\(^{-1}\) s\(^{-1}\))

Greek symbols

\( \alpha_{11} \)  stoichiometric coefficient in biomass formation reaction (mol Cmol\(^{-1}\))

\( \alpha_{ij} \)  stoichiometric coefficient number \( j \) for ATP conversion in reaction number \( i \) (mol Cmol\(^{-1}\))

\( \delta \)  P/O ratio, ATP yield from oxydative phosphorylation (mol)

\( \mu \)  growth rate (s\(^{-1}\))

\( \Delta \)  difference sign for incoming and outgoing concentration

Subscripts

in  ingoing

out  outgoing

S  carbon substrate

X  biomass

7.10 Bibliography

Chapter 8

Determination of growth and coupled nitrification/denitrification by immobilized *Thiosphaera pantotropha* using measurement and modelling of oxygen profiles*

An oxygen microsensor in combination with mathematical modelling was used to determine the behaviour of immobilized *Thiosphaera pantotropha*. This organism can convert ammonia completely to nitrogen gas under aerobic conditions (coupled nitrification/denitrification) and denitrifies nitrate at highest rates under anaerobic conditions. Immobilization of *T. pantotropha* can result in aerobic and anaerobic zones inside the biocatalyst particle which will be advantageous for the conversion of ammonia and nitrate from waste water. However, information of the effects of immobilization on the physiology of *T. pantotropha* is necessary for the development of such a system. This chapter gives the extension of a model developed to describe the behaviour of chemostat cultures of *T. pantotropha* so that it can be used for immobilized cells. The original model was based on metabolic reaction equations, kinetic and diffusion equations have now been added. Experimental verification was carried out using a stirred tank reactor and a Kluyver flask. After immobilization in agarose, the cells were grown in the particles under continuous culture conditions for three days. After 24 hours the oxygen penetration depth showed a constant value of 100 μm, indicating that a steady state was reached. Light, electron and scanning electron micrographs showed that large colonies of cells were present in this 100 μm aerobic layer.

---

From the dynamics of the start-up phase, several parameters were determined from measurements of the oxygen concentration profiles made every few hours. The profiles simulated by the model were fitted to the measured data. The average value for the maximum specific growth rate was 0.52 h\(^{-1}\), and the maximum oxygen conversion rate was 1.0 mol Cmol\(^{-1}\) h\(^{-1}\). The maximum specific acetate uptake rate was 2.0 mol Cmol\(^{-1}\) h\(^{-1}\) and the Monod constant for acetate was 2.9 \(\times\) 10\(^{-2}\) mol m\(^{-3}\). The maximum specific nitrification rate was \(5.8 \times 10^{-1}\) mol Cmol\(^{-1}\) h\(^{-1}\), the amount of oxygen necessary for nitrification was 11\% of the total oxygen uptake rate.

Most of the kinetic parameters determined for the immobilized-cells were in good agreement with those for the suspended cells. Only the maximum specific growth rate was significantly higher, and the maximum specific nitrification rate was somewhat lower than for suspended cells.

The experimental results clearly show that an oxygen microsensor, in combination with mathematical modelling, can successfully be used to elucidate the kinetic behaviour of immobilized, oxygen consuming, cells.

8.1 Introduction

8.1.1 General introduction

*Thiosphaera pantotropha* was isolated from a denitrifying waste water treatment plant\([1]\). It is a facultative chemolithoautotroph, and nitrite and nitrate can both serve as electron acceptors aerobically as well as anaerobically\([2]\). Moreover, under aerobic conditions, and with an organic substrate provided, ammonia can be oxidized to nitrite, a phenomenon known as heterotrophic nitrification. However, because of simultaneous denitrification, nitrite generated by the nitrification does not accumulate in the cultures.

Because of this coupling of nitrification and denitrification, and thus the conversion of ammonia directly to nitrogen, *T. pantotropha* may be a suitable biocatalyst for the removal of ammonia from waste water. This process conventionally requires an (aerobic) autotrophic nitrification step followed by an (anaerobic) denitrification step. An organism which not only nitrifies and denitrifies, but which can also use the organic compounds normally present in the waste water (in contrast to autotrophic nitrifiers) has obvious attractions for effluent treatment. In such a process, immobilization can be advantageous. Immobilization in a gel results in gradients within the particle caused by the physiological activity of the cells and the diffusion limitations of the substrates. In the case of oxygen, this could result in aerobic and anaerobic zones inside the biocatalyst particle. Coupled nitrification/denitrification will
thus occur under aerobic conditions, while denitrification will occur at higher rates in the anaerobic region. That immobilization can be advantageous for the removal of ammonia from waste water has already been reported by other researchers[3]. However, in this case coimmobilized autotrophic nitrifiers and denitrifiers were used, for example Nitrosomonas europaea and Paracoccus denitrificans[4] in a polyelectrolyte complex. It was found that in an aerobically cultured system, nitrification and denitrification both occurred, with a higher maximum ammonia oxidation rate than had been measured for free cells.

This chapter shows the use of an oxygen microsensor, see [5], to measure the local oxygen concentration inside the biocatalyst particles containing immobilized, nitrifying/denitrifying T. pantotropha. A biochemically structured model based on intracellular reaction equations which had been developed for the description of the behaviour of suspended continuous cultures[6] was extended with kinetic and diffusion equations to describe the behaviour of the immobilized cells. By comparing the behaviour of the suspended and immobilized cells, the impact of immobilization on the physiology of the cells could be determined. This extended model will be presented in this chapter, together with examples of its use in predicting the oxygen consumption of the bacteria, and hence the oxygen profiles in the biocatalyst particles under various growth conditions. Fitting of the predicted profiles to the data from the measured profiles will allow the determination of the kinetic parameters of the immobilized T. pantotropha, and its comparison with the data of suspended cells. Moreover, the information obtained from the modelling and profile measurements can be used to determine the conditions most suitable for nitrogen removal of waste water by immobilized T. pantotropha.

8.1.2 Linear relation between net conversion rates

The model developed for the interpretation of data from acetate limited continuous cultures containing suspended cells, with oxygen and/or nitrate as electron acceptors and ammonia as the nitrogen source is described in ref. [6]. The modelling resulted in a relationship between several net conversion rates in which the consumption rate is considered to be negative and the production rate positive. For the acetate consumption rate, the relationship is as follows:

\[ r_a = -\frac{1}{Y_{ax}} r_X + \frac{1}{Y_{ax}} r_{NH_3} - m_a C_X \]  \hspace{1cm} (8.1)

For the oxygen consumption rate the relationship is:

\[ r_{o_2} = -\frac{1}{Y_{o_2}} r_X + \frac{1}{Y_{o_2}} r_{NH_3} - m_{o_2} C_X \]  \hspace{1cm} (8.2)
The yield coefficients in equations (8.1) and (8.1) are related to each other by stoichiometry:

\[
\frac{1}{Y_{ao}} = \frac{1}{Y_{ax}} - 1.225 \tag{8.3}
\]

\[
\frac{1}{Y_{ao}} = \frac{1}{Y_{an}} - 0.750 \tag{8.4}
\]

\[
m_{O_2} = m_a \tag{8.5}
\]

Fitting equation (8.1) to the measured data from acetate limited continuous cultures with suspended cells resulted in values for the yield and maintenance coefficients, leading to the following equation:

\[
r_a = -1.83r_X + 3.61r_{NH_3} - 0.026C_X \tag{8.6}
\]

Using equations (8.3)–(8.6) the following formula for the oxygen uptake can be calculated:

\[
r_{O_2} = -0.605r_X + 4.36r_{NH_3} - 0.026C_X \tag{8.7}
\]

The ammonia consumption rate, \(r_{NH_3}\), consists of the amount of ammonia used for nitrification to nitrite (which is directly converted to nitrogen (\(r_{N_2}\))) and the amount necessary for assimilation. The stoichiometric relationship is as follows:

\[
r_{NH_3} = -0.25r_X - r_{N_2} \tag{8.8}
\]

Substituting equation (8.8) in (8.6) gives:

\[
r_a = -2.73r_X - 3.61r_{N_2} - 0.026C_X \tag{8.9}
\]

Equation (8.9) can be rewritten to put \(r_X\) explicitly:

\[
r_X = -0.366r_a - 1.32r_{N_2} - 0.010C_X \tag{8.10}
\]

Substituting equation (8.8) in (8.7) gives:

\[
r_{O_2} = -1.70r_X - 4.36r_{N_2} - 0.026C_X \tag{8.11}
\]

### 8.1.3 Introduction of kinetic equations

During the experiments with immobilized cells, nitrate was not provided and oxygen served as electron acceptor (nitrite generated during nitrification was denitrified). The influence of varying substrate concentrations can be introduced by implementing kinetic equations in the linear relations between the net conversion rates. It is assumed that the rates of acetate and ammonia
uptake can be modelled with a Monod-type of relationship. Furthermore, it is assumed that oxygen-limited growth can be described by zero-order reactions because of the low value of the $K_{O_2}$ (1 to $2 \cdot 10^{-3}$ mol m$^{-3}$) [7]. This has also been found for other aerobic organisms [8]. These assumptions result in the following equations:

\[ \tau_X = \mu C_X \]  
\[ -r_a = q_{\text{max}}^a C_X \left( \frac{C_a}{C_a + K_a} \right) \left( \frac{C_{NH_3}}{C_{NH_3} + K_{a,NH_3}} \right) \]  

(8.12)  

(8.13)

Provided that acetate and ammonium are present in excess, the organism will convert acetate at its maximum uptake rate. For the nitrification rate, however, the situation is more complicated. There are indications that the nitrification rate is dependent on the oxygen concentration[2], with higher rates being reached at low, but not limiting, oxygen concentrations. As a first estimate, an inhibition equation which has been used to describe the substrate inhibition kinetics of several other microorganisms is introduced to express the effect of oxygen [9].

\[ r_{N_2} = q_{\text{max}}^{N_2} C_X \left( \frac{C_{O_2}}{(C_{O_2} + K_{O_2}) \left( 1 + \frac{C_{O_2}}{K_{m,O_2}} \right)} \right) \left( \frac{C_{NH_3}}{C_{NH_3} + K_{N_2,NH_3}} \right) \]  

(8.14)

It also appeared from the results of the continuous cultures with suspended cells that nitrification only accounts for 10% of the total oxygen consumption.

From equations (8.10) and (8.12)–(8.14) it follows that the maximum specific growth rate can be described as:

\[ \mu_{\text{max}} = 0.336 q_{\text{max}}^a - 1.32 q_{\text{max}}^{N_2} - 0.010 \]  

(8.15)

### 8.1.4 Oxygen profiles as a result of oxygen limitation

The oxygen profile in a biocatalyst particle is the result of both diffusion and consumption. For steady state conditions and zero-order reaction kinetics (ammonia and acetate in excess), neglecting the influence of oxygen on the nitrification rate as nitrification only accounts for 10% of the total oxygen consumption (see equations (8.11), (8.13) and (8.14)), the oxygen concentration as a function of the radius of the particle can be described by the following equation (see appendix A for greater detail):

\[ C_{O_2}(r) = \frac{1}{6} \frac{r^0_{O_2}}{D_e} \left( r^2 + \frac{2r^3}{r} - 3r^2_d \right) \]  

(8.16)
The assumption of a pseudo-steady state for the biomass concentration during a profile measurement seems justified when it is considered that a measurement takes about 8 minutes and, the minimum generation time of suspended *T. pantotropha* under similar conditions is about 1.5 h at 37°C[7]. A value for \( r_{O_2}^0 \), can be obtained as follows. The substrate concentration in the particle is calculated with a starting value for parameter \( r_{O_2}^0 \), and the measured value for the interface concentration \( C_I \). The calculated and measured profiles are compared, and the sum of squares of the differences between the calculated and measured substrate concentrations is minimized by adapting the value for \( r_{O_2}^0 \) using Marquardt’s algorithm[10].

If the internal oxygen profile in a biocatalyst particle with growing *T. pantotropha* is measured every few hours, the oxygen conversion rate can be determined for each time period. Using these conversion rates, the maximum specific growth rate for the immobilized cells can be calculated. For this, equations (8.11), (8.12) and (8.14) are used in combination with the integrated version of equation (8.12), the exponential growth equation:

\[
C_X(t) = C_X(0)e^{\mu_{max} t}
\]  

(8.17)

The result is relation (8.18):

\[
\ln \left( \frac{r_{O_2}^0(t)}{r_{O_2}^0(0)} \right) = \mu_{max} t
\]  

(8.18)

The maximum specific growth rate is independent of the value of the effective diffusion and yield coefficients. However, this procedure is only correct if these coefficients do not change as the internal biomass concentration increases. This can be checked by plotting the left hand term of equation (8.18) against the time when the results are obtained. The data points must lie on a straight line, with a slope representing the maximum specific growth rate. If it is not, the last data points on the slope must be omitted as the biomass concentration in the particles has then become too high to assume a constant effective diffusion coefficient.

### 8.1.5 Oxygen profiles as a result of acetate limitation

Under acetate limitation, the oxygen profile is dependent on the acetate concentration profile in the biocatalyst particle. Thus the shape of the oxygen curve under these conditions can provide information about the influence of substrates other than oxygen on the metabolism of immobilized *T. pantotropha*. Using a model equation describing the oxygen profile, information about the nitrification rate of the immobilized cells can also be obtained. This model is only valid if the cells are homogeneously distributed within the bead and ammonia is present in excess. Furthermore, it is assumed that
8.2 Materials and methods

8.2.1 Organism

Thiosphaera pantotropha LMD 82.5 was originally isolated from a denitrifying, sulphide-oxidizing waste water treatment system [2]. The bacterial strain used for the immobilized experiments was the same as that used for the continuous culture experiments [6].

8.2.2 Continuous cultures (suspended cells)

The chemostat was equipped with dissolved oxygen and pH control. The dissolved oxygen concentration was kept at 50% air saturation. The temperature was maintained at 37°C and the pH at 8.0. The medium supplied to the chemostat contained (in g l⁻¹): Na₂HPO₄, 0.4; KH₂PO₄, 0.15; NH₄Cl, 0.4; MgSO₄ · 7H₂O, 0.4, and 2 ml of a trace element solution [11]. As substrate, 20 mol m⁻³ acetate was supplied. The cells were harvested for immobilization and centrifuged at 6000 rpm for 20 minutes and were then ten times concentrated in a phosphate buffer (50 mol m⁻³, pH 8.0)

Oxygen is used during nitrification according to zero-order kinetics. The equation describing the oxygen concentration in the biocatalyst is as follows (see appendix B):

\[
C_{O_2}(r) = -0.620 \frac{D_{e,a}}{D_{e,O_2}} (C_{f,a} - C_a) - 0.620 \frac{k_{L,a}}{k_{L,O_2}} (C_{L,a} - C_{I,a}) - \frac{1}{6} \left( \frac{0.010C_X + 2.12r^0_{N_2}}{D_{e,O_2}} \right) (r_p^2 - r^2) - \left( \frac{0.010C_X + 2.12r^0_{N_2}}{k_{L,O_2}} \right) \frac{V_p}{A_p} \quad (8.19)
\]

Thus for the calculation of the internal oxygen profile, it is necessary to know the internal acetate profile. This acetate profile is calculated numerically using Monod kinetics and the diffusion/reaction equation (see equation A.1 appendix A). However, in contrast to oxygen, the surface acetate concentration cannot be measured, and it must thus be calculated using a value for the external mass transfer coefficient. From the acetate profile, the internal oxygen profile is calculated according to equation (8.19). This profile is compared with the measured profile and the difference between the calculated and measured oxygen concentrations is minimized by adapting the parameters using Marquardt's algorithm[10]. In this process the following parameters are estimated: the acetate uptake rate \(r^0_a\), the Monod constant for acetate \(K_a\) and \(0.010C_X + 2.12r^0_{N_2}\). Once the biomass concentration is known, the maximum specific acetate uptake rate \(q_{max}^a\) and the maximum specific nitrification rate \(q_{max}^{N_2}\) can be calculated.
8.2.3 Continuous culture reactors (immobilized cells)

A stirred tank reactor and a Kluyver flask [12] were used for the continuous culture experiments. The working volume was 50 ml. The dilution rate was 1.0 h\(^{-1}\); the temperature was maintained at 37°C and the dissolved oxygen concentration did not fall below 80% of air saturation. The medium supplied to the reactor had a pH of 8.0 and contained (in g l\(^{-1}\)): Na\(_2\)HPO\(_4\), 4.19; KH\(_2\)PO\(_4\), 1.5; NH\(_4\)Cl, 0.3; MgSO\(_4\) \cdot 7H\(_2\)O, 0.1, and 2 ml of a trace element solution [11]. The cultures were provided with 1 or 2 mol m\(^{-3}\) sodium acetate. The reactors contained 100 biocatalyst particles at the start of the experiment.

8.2.4 Immobilization procedure

For the immobilization, agarose was used (gel type 7, Sigma), because previous experiments had shown that agarose particles remained intact for the duration of an experiment, but could be easily penetrated by a microsensor [5]. 100 µl of a concentrated cell suspension was mixed on a whirl-mixer with 20 ml of a 5% (w/w) agarose suspension at 40°C. Thereafter, the agarose-cell mixture was injected into a perspex mould which contained 100 spherical holes with a diameter of 4.9 mm. After the injection, the mould was placed at 4°C for 30 minutes. This immobilization technique resulted in perfectly round spheres with uniform diameters and cell concentrations. All of the materials used for the immobilization procedure had either been sterilized, or disinfected where sterilization was not possible.

After the beads were removed from the mould, they were transferred to one of the continuous culture reactors. A single bead was used immediately for the first profile measurement. If the biocatalyst particles were not immediately used for a profile measurement, they were stored in buffer at 4°C.

8.2.5 Oxygen microsensor

The experiments were carried out with a Clark-type microsensor which was constructed according to the method described by Revsbech and Ward [13]. The diameter of the sensing tip of the sensor was about 15 µm. The sensor exhibited a standard current of 0.60 nA in air-saturated water at 37°C, and a linear response to varying oxygen concentrations. The zero oxygen reading was 2 \cdot 10^{-2} nA, independent of the environment or temperature. The sensor showed no measurable drift during the experiments after a stabilization time of one hour.

8.2.6 Microsensor measuring system

The measuring equipment was a modification of that described by Hooijmans et al. [5], a short summary is given here. The biocatalyst particle was placed in a small holder in a flow chamber maintained at 37°C. An air-saturated buffer
solution (oxygen concentration 0.21 mmol l⁻¹), with or without nutrients, was continuously circulated in the flow chamber at 3.5 ml s⁻¹ in order to provide a constant environment for the biocatalyst. Measurements were started after stabilization of the sample and positioning the microsensor 300 μm above the sample surface. The microsensor was mounted in a holder connected to a motor drive and micromanipulator in order to regulate the distance and speed of sensor movement. The speed of the sensor was 5 μm s⁻¹. The output signal from the sensor was amplified and recorded by a personal computer (IBM-PS/2). The data file created by the data acquisition program was transferred to a HP A900 computer which was used for calculations and data fitting. The data used were averages of 2 or 5 samples, taken at time intervals of 1 or 2 seconds, respectively, depending on the steepness of the measured gradients.

The acetate concentration in the medium was 1 mol m⁻³, the ammonia concentration was 5 mol m⁻³. By the use of a computer simulation, it was determined that these concentrations were high enough to ensure that only oxygen was rate limiting. For this computer simulation values for the Monod constant were used which were obtained for the free cells: \( K_{O_2} = 1 - 2 \cdot 10^{-3}\text{mol m}^{-3} \) [7], \( K_{a,\text{NH}_3} = 2 \cdot 10^{-4}\text{mol m}^{-3} \) and \( K_{N_a,\text{NH}_3} = 2 \cdot 10^{-2}\text{mol m}^{-3} \) (L.A. Robertson and J.G. Kuenen, unpublished data).

### 8.2.7 Analytical techniques

Acetate was determined with acetyl-coenzyme A synthetase using a test kit (Boehringer) or as total organic carbon with a TOCA master 915-B. Nitrite was determined using the Griess-Romijn reagent [14], ammonia was determined colorimetrically [15].

Protein was measured spectrophotometrically, by means of the microbiuret method [16]. The total organic carbon of washed cells was determined with a TOCA master 915-B. Carbon, hydrogen, oxygen and nitrogen were measured with a Perkin Elmer 240 C.

For (scanning) electron microscopy the cells in the biocatalyst were fixed using glutaraldehyde and stained with osmium tetroxide and ruthenium red.

### 8.2.8 Diffusion and mass transfer coefficients

In addition to the yield coefficients determined for the free cells, other coefficients were used in the calculations as follows. The value of the effective diffusion coefficient of oxygen in agarose (5% w/w) is, according to Hulst [17], 80% of its value in water (i.e. 2.3 \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1} at 37°C). The diffusion coefficient of acetate in water is 2.0 \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1} according to the Wilke-Chang relation [18]. As with oxygen, the effective diffusion coefficient of acetate in agarose was assumed to be 80% of its value in water, resulting in a value of 1.6 \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1}.

Previous measurements [19] showed that the thickness of the boundary layer for oxygen using a particle of 5 mm diameter and a liquid flow of
3.5 ml s\(^{-1}\) was 100 \(\mu\)m. This results in a external mass transfer coefficient for oxygen of \(2.9 \cdot 10^{-5}\) m s\(^{-1}\). If the same boundary layer thickness is assumed for acetate, the result is a mass transfer coefficient of \(2.0 \cdot 10^{-5}\) m s\(^{-1}\). For the situation in the reactor the mass transfer coefficient for oxygen was calculated using a literature correlation [20]. The value found was \(5 \cdot 10^{-5}\) m s\(^{-1}\). As can be seen this value is in good agreement with the value found for the flow chamber.

8.3 Results and discussion

8.3.1 Steady state situation

The experiment to determine quantitatively the behaviour of immobilized *Thiosphaera pantotropha* was performed twice. Two different reactors were used. For the first experiment a stirred tank reactor was used. Because of bad mixing and high sheer forces in this reactor a Kluvyer flask, which relies on air bubbles for mixing [12], was used for the second experiment. Both reactors were run for three days. After 24 hours the internal oxygen profiles did not change any more. Light, electron and scanning election micrographs showed that the distribution of cells had become heterogeneous. Due to large colonies of cells in the area towards the surface, the oxygen penetration depth was about 100 \(\mu\)m. Figure 8.1 shows an example of such a profile measurement. A

![Figure 8.1 Measured oxygen concentration profile (*) in an agarose bead at steady state, and the fitted curve. \(\Delta t = 24\) h, experiment 2.](image_url)
8.3 Results and discussion

Figure 8.2 Scanning electron micrographs from a section of a gel bead in steady state situation. $\Delta t = 24$ h, experiment 1. Bar represents 33 $\mu$m (left) and 1 $\mu$m (right).

Biocatalyst particle from the steady state was examined under the scanning electron microscope (figure 8.2). As can be seen, large colonies of cells developed near the surface of the particle, the cells being densely packed. The size of the colonies decreased towards the centre. As the area in the particle containing the large colonies was about 80 $\mu$m deep, this agreed very well with the oxygen penetration depth. Thus, the smaller colonies towards the centre were probably due to electron acceptor limitation. That the cells could not migrate through the agarose can be seen in figure 8.3. The cavities in the agarose gel were smaller than the diameter of the cells. No visible cell growth on the surface of the particle could be observed through a stereo-microscope, see figure 8.4.

It can be seen in figure 8.5 that some cells were forming the reserve material polyhydroxy-β-butyrate (PHB). This electron micrograph shows a section through a particle from a steady state, and reveals that mainly the cells at the outside of a colony near the surface of the bead contained PHB.

8.3.2 Specific growth rate of immobilized cells

For the determination of the specific growth rate of the immobilized cells, at approximately hourly intervals for a period of 6 or 7 hours a biocatalyst particle was removed from the reactor, and the oxygen concentration profile was measured in the flow chamber. The oxygen uptake rate ($r_{O_2}^0$) and the location where oxygen became depleted ($r_d$) were estimated by fitting of the measured oxygen concentration profile with the calculated profile (see equation (8.16)).
Figure 8.3 Scanning electron micrographs from a section of a gel bead in steady state situation. $\Delta t = 24$ h, experiment 1. Bar represents 0.33 $\mu$m. Agarose without cells (left) and with *T. pantotropha* (right).

Figure 8.4 Biocatalyst particle ($d_p = 4.9$ mm) in holder in flow chamber. Steady state situation ($\Delta t = 24$ h), experiment 1. Picture taken through the phototube of a stereomicroscope.
Figure 8.5 Electron micrograph from a section of a gel bead in steady state situation. $\Delta t = 24$ h, experiment 1. Bar represents about 1 $\mu$m. The white particles in the cells are PHB.

For experiment 1 duplicate measurements were performed, using two different beads. The variation in the oxygen uptake rate was maximal 10%. The measured and fitted oxygen concentration profiles for six consecutive times, the results of the Kluyver flask experiment, are shown in 8.6.

Using the oxygen uptake rates measured during the first few hours and equation (8.18), it was possible to calculate the maximum specific growth rate, $\mu_{\text{max}}$. For experiments 1 (stirred tank) and 2 (Kluyver flask), values of $0.45 \pm 0.05$ h$^{-1}$ and $0.58 \pm 0.02$ h$^{-1}$, respectively, were found. Figure 8.7 shows a plot of the results, the slope being $\mu_{\text{max}}$. As can be seen, the lines are straight until the last points selected, they do not curve away to the right. This means that, for the data points used, the yield and diffusion coefficients can be considered constant. Furthermore, it can be seen from the figure that the results of experiment 2 are described better by a linear relation than those of experiment 1. This is probably because of better mixing in the Kluyver flask than in the stirred tank reactor. For the suspended cells, the value of the specific growth rate determined under controlled conditions (80% air saturation, pH 8.0) with acetate and ammonia was $0.42$ h$^{-1}$ [Robertson and Kuenen, in preparation].

8.3.3 Biomass concentration in the biocatalyst particle

For each time interval, the biomass concentration was calculated using the determined oxygen conversion rate, the maximum specific growth rate, maintenance requirement (as derived from chemostat data) and the amount of
Figure 8.6 Measured oxygen concentration profiles in agarose beads containing immobilized *T. pantotropha* (experiment 2), and the fitted curves. (*0 h; (o) 1 h; (+) 2 h; (#) 3 h; (⊕) 4.5 h; (●) 6.8 h.

Figure 8.7 Logarithmic value of normalized oxygen conversion rate as function of time. The lines are calculated using linear regression. The slopes represent the maximum growth rate. (Δ, ----) experiment 1; (+, ------) experiment 2.
Table 8.1 Calculated biomass concentration ($C_X$) and oxygen penetration depth ($r_p - r_d$) as a function of measuring time $t$.

<table>
<thead>
<tr>
<th>$t$ (h)</th>
<th>$C_X$ (Cmol m$^{-3}$)</th>
<th>$r_p - r_d$ (mm)</th>
<th>$C_X$ (Cmol m$^{-3}$)</th>
<th>$r_p - r_d$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>experiment 1</td>
<td>experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.27</td>
<td>2.5</td>
<td>0.32</td>
<td>2.5</td>
</tr>
<tr>
<td>1</td>
<td>0.52</td>
<td>2.5</td>
<td>0.47</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>0.90</td>
<td>2.5</td>
<td>0.89</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>2.5</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>3.5</td>
<td>2.3</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.5</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>5.25</td>
<td>2.5</td>
<td>1.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.75</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>0.41</td>
</tr>
<tr>
<td>7.5</td>
<td>11</td>
<td>0.63</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PHB formation has not been included.

Oxygen necessary for nitrification (see equations (8.11) and (8.12)). As will be described later, the amount of oxygen used for nitrification was determined from the oxygen profiles obtained under acetate limitation, and appeared to be about 10% of the total oxygen consumption. The possibility of PHB occurring in the cells has not been included in the calculated biomass concentration. The result can be seen in table 8.1. Because agarose cannot be dissolved without heating, it was not possible to compare the calculated biomass concentration with measurements. Thus only the initial biomass concentration, which was determined from the cell suspension used for immobilization, was estimated. For experiment 1, this initial concentration was 0.56 Cmol m$^{-3}$, and for experiment 2 it was 0.59 Cmol m$^{-3}$. By comparing these values with the concentrations determined from the profile measurements (table 8.1, experiments 1 and 2) it can be seen that the latter are somewhat lower, possibly due to the death of some of the inoculum during immobilization.

Table 8.1 also includes the oxygen penetration depth for each time period. Once the penetration depth became smaller than 2.45 mm (after approximately 3.5 hours), the cells near the centre of the particle became oxygen depleted, and therefore ceased to grow. At this point the distribution of the cells in the bead started to become heterogeneous.

Using the calculated biomass concentration, the maximum specific oxygen uptake rate could be calculated from the oxygen profiles found in beads that were oxygen limited. These values were 0.90 mol Cmol$^{-1}$ h$^{-1}$ (experiment 1), and 1.1 mol Cmol$^{-1}$ h$^{-1}$ (experiment 2), respectively. These numbers are in agreement with the number found for the continuous culture experiments.
run at $D = 0.41 \text{ h}^{-1}$. For the suspended cells the maximal oxygen uptake rate was $1.0 \text{ mol Cmol}^{-1} \text{ h}^{-1}$ [6].

### 8.3.4 Influence of acetate limitation on oxygen profiles

The influence of acetate limitation on the oxygen profiles was determined using low ($0.1 \text{ mol m}^{-3}$, $0.2 \text{ mol m}^{-3}$ and $0.3 \text{ mol m}^{-3}$) acetate concentrations in the medium in the flow chamber. The biocatalysts used were taken out of the fermentor at 3.5 h and 3.3 h for experiments 1 and 2, respectively, to guarantee a homogeneous distribution of the cells in the particle. For experiment 1, duplicate measurements were performed, using 2 particles for each acetate concentration. For experiment 2, duplicates were not made, but each measurement was done with a new particle and fresh medium in order to prevent suspended growth in the medium of the flow chamber. The measured oxygen concentration profiles were fitted to the calculated ones using the model described in appendix B. The parameters that were fitted were $r_0$, $K_a$ and $(0.010 C_X + 2.12 r_N^0)$.

Figure 8.8a and 8.8b gives an example of the results of a fit for an acetate concentration of $0.1 \text{ mol m}^{-3}$ and $0.3 \text{ mol m}^{-3}$, including the calculated internal acetate and oxygen concentration profiles and measured oxygen concentration profile. As can be seen the fit is very accurate.

Table 8.2 gives the parameters resulting from the fit procedure. For both experiments, the biomass concentration used to express the values per Cmol was calculated by interpolation using the data shown in figure 8.7. The biomass concentrations at the time of sampling were $1.7 \text{ Cmol m}^{-3}$ for experiment 1, and $1.8 \text{ Cmol m}^{-3}$ for experiment 2. The maximum specific acetate uptake rate ($q_{\text{max}}^a$) was $2.1 \pm 1.0 \text{ mol Cmol}^{-1} \text{ h}^{-1}$ (experiment 1) and $1.8 \pm 0.9 \text{ mol Cmol}^{-1} \text{ h}^{-1}$ (experiment 2), respectively. For the chemostat culture (80% air saturation, $D = 0.41 \text{ h}^{-1}$) a value of $1.4 \text{ mol Cmol}^{-1} \text{ h}^{-1}$ was

<table>
<thead>
<tr>
<th>Acetate (mol m$^{-3}$)</th>
<th>$q_{\text{max}}^a$ (mol Cmol$^{-1}$ h$^{-1}$)</th>
<th>$K_a \cdot 10^{-2}$ (mol m$^{-3}$)</th>
<th>$0.010 + 2.12 r_N^0$ (mol Cmol$^{-1}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>exp. 1</td>
<td>exp. 2</td>
<td>exp. 1</td>
<td>exp. 2</td>
</tr>
<tr>
<td>0.1</td>
<td>2.5</td>
<td>1.4</td>
<td>4.1</td>
</tr>
<tr>
<td>0.2</td>
<td>1.8</td>
<td>2.9</td>
<td>3.5</td>
</tr>
<tr>
<td>0.3</td>
<td>1.6</td>
<td>1.1</td>
<td>–</td>
</tr>
</tbody>
</table>

Different acetate concentrations were used. The parameter values for experiment 1 are averages over two profiles per acetate concentration.
Figure 8.8a Measured oxygen concentration profile (+) in an agarose bead as a result of the calculated acetate concentration profile (experiment 2), and the fitted curve. 0.1 mol m\(^{-3}\) acetate, the dashed line represents the corresponding acetate profile.

Figure 8.8b Measured oxygen concentration profile (+) in an agarose bead as a result of the calculated acetate concentration profile (experiment 2), and the fitted curve. 0.3 mol m\(^{-3}\) acetate, the dashed line represents the corresponding acetate profile.
measured.

The maximum specific nitrification rate \((q_{\text{max}}^{N})\), calculated from \((0.010 \ C_X + 2.12 \ r_{N_2}^0)\), was \((0.53 \pm 0.15) \cdot 10^{-1}\) mol Cmol\(^{-1}\) h\(^{-1}\) (experiment 1) and \((0.62 \pm 0.08) \cdot 10^{-1}\) mol Cmol\(^{-1}\) h\(^{-1}\) (experiment 2), respectively. Compared with the value for the suspended cells, \(0.71 \cdot 10^{-1}\) mol Cmol\(^{-1}\) h\(^{-1}\) (continuous culture, \(D = 0.41\) h\(^{-1}\) [6]), the nitrification rate is the same. Furthermore, the amount of oxygen necessary for nitrification can be compared with the total oxygen demand of the immobilized cells. For this, the nitrification rate was expressed as mol O\(_2\) : Cmol\(^{-1}\) h\(^{-1}\) \((= 2.12 r_{N_2}^0)\) rather than mol NH\(_3\) : Cmol\(^{-1}\) h\(^{-1}\). For experiment 1 the amount of oxygen necessary for nitrification was 0.18 mol O\(_2\) m\(^{-3}\) h\(^{-1}\), the total oxygen demand was 1.5 mol O\(_2\) m\(^{-3}\) h\(^{-1}\), (situation in which oxygen is limiting). Thus 12% of the oxygen taken up by the cells was used for nitrification. For experiment 2, the same calculation was performed and resulted in a value of 11% of the total oxygen uptake being used for nitrification. This is approximately the same as the value obtained for suspended cells (10%) [6].

The Monod constant for acetate was \((3.8 \pm 2.4) \cdot 10^{-2}\) mol m\(^{-3}\) for experiment 1, and \((2.0 \pm 1.3) \cdot 10^{-2}\) mol m\(^{-3}\) for experiment 2. For the suspended cells in batch culture a value of 2.0 \cdot 10^{-2}\) mol m\(^{-3}\) was measured [L.A. Robertson and J.G. Kuenen, unpublished data].

As an indication for the accuracy of the results, it is possible to calculate the maximum specific acetate uptake rate from the maximum specific oxygen uptake rate, resulting from the profiles in which oxygen is limiting, by combining equations (8.6) and (8.7). The results are 1.5 mol Cmol\(^{-1}\) h\(^{-1}\) (experiment 1) and 1.8 mol Cmol\(^{-1}\) h\(^{-1}\) (experiment 2). For experiment 2 this is in agreement with the q resulting from the fit of the oxygen profiles limited by acetate, for experiment 1 the value is about 30% lower.

It is also possible to calculate the maximum specific growth rate using the parameters resulting from the fit of the acetate limited profiles, and equation (8.15). The result is a value of \(\mu_{\text{max}}\) of 0.59 h\(^{-1}\) (experiment 1) and 0.51 h\(^{-1}\) (experiment 2). For experiment 2, the result is somewhat lower than the \(\mu_{\text{max}}\) determined from the oxygen profiles as function of time, for experiment 1 the value is high. However, these calculated values are inaccurate considering the high standard deviation for \(q_{\text{max}}^{N}\).

The results are summarized in table 8.3 which shows all of the kinetic parameters determined for the immobilized as well as the suspended cells.

### 8.4 Conclusion

The combination of mathematical modelling and measurements with an oxygen microsensor is an accurate approach to elucidate the behaviour of immobilized, growing, oxygen-consuming cells. By measuring oxygen concentration...
Table 8.3 Kinetic parameters determined for suspended cells and immobilized cells.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>( \mu_{\text{max}} ) (h(^{-1}))</th>
<th>( q_{\text{O}_2}^{\text{max}} ) (mol (Cmol h(^{-1})))</th>
<th>( q_{\text{ac}}^{\text{max}} ) (mol (Cmol h(^{-1})))</th>
<th>( q_{\text{N}_2}^{\text{max}} ) (mol (Cmol h(^{-1})))</th>
<th>( K_a ) (mol m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspended cells batch</td>
<td>0.42</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2 \cdot 10^{-2}</td>
</tr>
<tr>
<td>Suspended cells continuous culture</td>
<td>--</td>
<td>1.0</td>
<td>1.4</td>
<td>0.71 \cdot 10^{-1}</td>
<td>--</td>
</tr>
<tr>
<td>( D = 0.42 ) h(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immobilized cells experiment 1</td>
<td>0.45 (0.59(^1))</td>
<td>0.90</td>
<td>2.1 (1.5(^2))</td>
<td>0.53 \cdot 10^{-1}</td>
<td>3.8 \cdot 10^{-2}</td>
</tr>
<tr>
<td>Immobilized cells experiment 2</td>
<td>0.58 (0.51(^2))</td>
<td>1.1</td>
<td>1.8 (1.8(^2))</td>
<td>0.62 \cdot 10^{-1}</td>
<td>2.0 \cdot 10^{-2}</td>
</tr>
</tbody>
</table>

\( \mu_{\text{max}} \) and \( q_{\text{O}_2}^{\text{max}} \) resulted from fitting the oxygen profiles in which oxygen was limiting; \( q_{\text{ac}}^{\text{max}} \), \( q_{\text{N}_2}^{\text{max}} \) and \( K_a \) resulted from fitting oxygen profiles in which acetate was limiting. \(^1\) = calculated using results oxygen profiles limited by acetate, \(^2\) = calculated using oxygen profiles limited by oxygen.

Profiles during several time intervals in agarose beads containing growing *T. pantotropha* and using different substrate concentrations, much information about the kinetics and growth behaviour of the immobilized cells could be determined. This information included the maximum specific oxygen uptake rate (1.0 mol Cmol\(^{-1}\) h\(^{-1}\)), the maximum specific acetate uptake rate (2.0 mol Cmol\(^{-1}\) h\(^{-1}\)), the Monod constant for acetate (2.9 \cdot 10^{-2} mol m\(^{-3}\)), the maximum specific nitrification rate (0.58 \cdot 10^{-1} mol Cmol\(^{-1}\) h\(^{-1}\)) and the maximum specific growth rate (0.52 h\(^{-1}\)). Light, electron and scanning electron micrographs were taken for a qualitative impression of the steady state situation, and were in agreement with the results of the oxygen profile measurements. By comparison of the behaviour of the suspended and immobilized cells it appeared that there was no significant difference between them. However, this does not mean that the same result will be found when other organisms or other carrier material is used.

8.5 Acknowledgements

The authors wish to thank Prof. J.G. Kuenen for his participation in the discussions.
8.6 Nomenclature

- \( A_p \) surface particle (m^2)
- \( C_i \) concentration of component i (mol m^{-3})
- \( D \) dilution rate (s^{-1})
- \( D_{e,i} \) effective diffusion coefficient for component i (m^2 s^{-1})
- \( d_p \) diameter of the gel bead (m)
- \( k_{L,i} \) external mass transfer coefficient for component i (m s^{-1})
- \( K_i \) Monod constant for component i (mol m^{-3})
- \( K_{in,i} \) inhibition constant for component i (mol m^{-3})
- \( m_i \) maintenance coefficient for component i (mol C mol^{-1} s^{-1})
- \( q_{\text{max}}^i \) maximum specific rate of component i (s^{-1})
- \( r \) radial distance (m)
- \( r_p \) radius particle (m)
- \( r_d \) location where substrate concentration becomes zero (m)
- \( r_i \) net conversion rate of component i in a reactor (mol m^{-3} s^{-1})
- \( r_i^0 \) net conversion rate of component i in a gel bead (mol m^{-3} s^{-1})
- \( t \) time (s)
- \( V_p \) volume particle (m^3)
- \( Y_{i,j} \) yield factor for component j on component i (-)

**Greek symbols**

- \( \mu \) specific growth rate of the biomass (s^{-1})
- \( \mu_{\text{max}} \) maximum specific growth rate of the biomass (s^{-1})

**Subscripts**

- \( a \) acetate
- \( I \) at the interface
- \( L \) in the liquid
- \( o \) oxygen
- \( x \) biomass
- \( n \) ammonia

8.7 Bibliography


Appendix A

Calculation of the oxygen consumption rate from an internal oxygen profile

The internal substrate concentration profile for zero-order reaction kinetics is described by the following steady state differential equation:

$$\frac{d}{dr} \left( r^2 \frac{dC}{dr} \right) = -\frac{r^0}{D_e} r^2$$  \hspace{1cm} (A.1)

with its boundary conditions:

$$r = r_p, \quad \frac{dC}{dr} = \frac{k_L}{D_e} (C_L - C_I)$$  \hspace{1cm} (A.2a)

$$r = r_d, \quad C = 0$$  \hspace{1cm} (A.2b)

The differential equation can be solved analytically resulting in the following relationship:

$$C(r) = \frac{r^0}{6D_e} \left( r^2 + \frac{2r^3_d}{r} - 3r^3_d \right)$$  \hspace{1cm} (A.3)

The location where the substrate concentration becomes zero, $r_d$, can be calculated by substituting the surface concentration $C_I$, which can be measured with the microsensor, in equation (A.3):

$$\frac{2}{3} r^3_d - \frac{2r^3}{3} r_p + \frac{1}{3} r^3_p - \frac{2C_I D_e r_p}{r^0} = 0$$  \hspace{1cm} (A.4)
Appendix  B

Calculation of the influence of acetate on the metabolism of *T. pantotropha*

The relationship between the oxygen consumption rate and the acetate consumption rate is given by the following equation:

\[
r_{O_2}^0 = \frac{1}{Y_{oa}} r_a^0 - \frac{1}{Y_{oN_2}} r_{N_2}^0 - m_a \left( 1 - \frac{1}{Y_{ox}} + \frac{1}{Y_{an}} \right) C_X \tag{B.1}
\]

The relation between these yield coefficients and the coefficients used in the other conversion rates mentioned in the chapter are as follows:

\[
\frac{1}{Y_{oa}} = \left( \frac{1}{Y_{ox}} + \frac{1}{Y_{an}} \right) \tag{B.2}
\]

\[
\frac{1}{Y_{oN_2}} = \frac{1}{Y_{an}} \left( \frac{1}{Y_{ox}} + \frac{1}{Y_{an}} - 1 \right) \tag{B.3}
\]

Using the coefficients that were determined for the free cells lead to:

\[
r_{O_2}^0 = 0.620 r_a^0 - 2.12 r_{N_2}^0 - 0.010 C_X \tag{B.4}
\]

The amount of substrate converted is transported from the medium, so the oxygen and acetate consumption rate are given by:

\[
r_{O_2}^0 = k_{L,o_2} \frac{A_p}{V_p} (C_{L,o_2} - C_{I,o_2}) \tag{B.5}
\]

\[
r_a^0 = k_{L,a} \frac{A_p}{V_p} (C_{L,a} - C_{I,a}) \tag{B.6}
\]
When substituting equations (B.5) and (B.6) in equation (B.4) it follows that:

\[ k_{L,\text{O}_2} \left( C_{L,\text{O}_2} - C_{I,\text{O}_2} \right) = 0.620 k_{L,a} \left( C_{L,a} - C_{I,a} \right) - \left( 0.010 C_X + 2.12 r_{N_2}^0 \right) \frac{V_p}{A_p} \]  

(B.7)

The internal concentration profile is given by the following equation with boundary conditions:

\[ \frac{d}{dr} \left( r^2 \frac{dC_a}{dr} \right) = -\frac{r^0}{D_e} r^2 \]  

(B.8)

with boundary conditions:

\[ r = r_p, \quad C_{O_2} = C_{I,\text{O}_2} \]  

(B.9a)

\[ r = 0, \quad \frac{dC_{O_2}}{dr} = \frac{dC_a}{dr} = 0 \]  

(B.9b)

When substituting equation (B.8) in equation (B.4) it follows that:

\[ \frac{D_{e,\text{O}_2}}{r^2} \frac{d}{dr} \left( r^2 \frac{dC_{O_2}}{dr} \right) - 0.620 \left( \frac{D_{e,a}}{r^2} \frac{d}{dr} \left( r^2 \frac{dC_a}{dr} \right) \right) - \left( 0.010 C_X + 2.12 r_{N_2}^0 \right) = 0 \]  

(B.10)

The differential equation can be solved analytically, resulting in relationship (B.11). Hereby it is assumed that oxygen is used for nitrification according to zero-order kinetics, and ammonia is present in excess.

\[ 0.620 D_{e,a} \left( C_{I,a} - C_a \right) + D_{e,\text{O}_2} \left( C_{O_2} - C_{I,\text{O}_2} \right) + \frac{1}{6} \left( 0.010 C_X + 2.12 r_{N_2}^0 \right) \left( r_p^2 - r^2 \right) = 0 \]  

(B.11)

Substituting equation (B.7) in (B.11):

\[ C_{O_2}(r) = -0.620 \frac{D_{e,a}}{D_{e,\text{O}_2}} \left( C_{I,a} - C_a \right) - 0.620 \frac{k_{L,a}}{k_{L,\text{O}_2}} \left( C_{L,a} - C_{I,a} \right) - \frac{1}{6} \left( 0.010 C_X + 2.12 r_{N_2}^0 \right) \left( r_p^2 - r^2 \right) - \left( 0.010 C_X + 2.12 r_{N_2}^0 \right) \frac{V_p}{A_p} \]  

(B.12)
Summary

When an enzyme or cell, a so-called biocatalyst, is immobilized in a carrier, the overall behaviour can be affected. This effect is due to diffusion limitation of substrate or product which takes place in and around the carrier, and to possible changes of the enzyme or cell itself. For the enzymes these changes can be caused by conformational alteration of the enzyme or sterical hindrance of the substrate, for cells chemical or physical stress can play a role. When the influence of diffusion on the conversion rate is known and corrected for, the effect of immobilization on the enzyme or cell itself can be quantified. This information is indispensable for the development of a process in which immobilized biocatalysts are used.

For the work presented in this thesis a self-made oxygen microsensor was used. With an oxygen microsensor, which has a tip diameter of about 5 to 10 μm, local oxygen concentrations inside a carrier containing a biocatalyst can be measured, giving information about the behaviour of the immobilized biocatalyst. By comparison with the behaviour of the biocatalyst in suspension the influence of immobilization can be determined.

The application of an oxygen microsensor for measuring oxygen profiles in a gel bead containing an oxygen consuming enzyme is presented. The results obtained with the microsensor were accurate and reproducible. Hereafter it was possible to use the oxygen microsensor for the determination of the intrinsic kinetic parameters of an immobilized, oxygen reducing enzyme. The model enzyme used was L-lactate 2-monoxygenase, immobilized in agarose-gel beads. The reaction kinetics could be well described by the Michaelis-Menten equation. Different particle diameters and enzyme concentrations were used. From the internal oxygen concentration profiles the intrinsic kinetic parameters were determined by fitting simulated profiles to the measurements. The resulting parameters appeared to be independent of particle radius or enzyme loading used, proving the method to be reliable. The intrinsic kinetic parameters were also compared with the parameters of the suspended enzyme. It appeared that due to immobilization the maximum conversion rate of the enzyme, as well as the Michaelis-Menten constant, decreased.

In the following study the same model enzyme and immobilization material was used. Two different experimental methods of determining the in-
trinsic kinetic parameters were used. The first one, based on a steady state,
consisted of oxygen profile measurements in the biocatalyst particles using
an oxygen microsensor. The second one consisted of batch conversion exper-
iments using a biological oxygen monitor to measure the change of oxygen
concentration in the liquid phase caused by the immobilized biocatalysts. For
the dynamic method it was assumed that the concentration profile in the
bead was in pseudo-steady state with respect to the liquid oxygen concen-
tration. The data resulting from the dynamic method were processed using two
different mathematical methods. There was no significant difference in the
intrinsic kinetic parameters obtained from the two experimental methods and
two different mathematical methods. The main difference in the application
of the mathematical methods was the computer calculation time needed.

One of the parameters that influence the concentration profile inside a
biocatalyst bead is the external mass transfer resistance. Boundary layer
measurements with an oxygen microsensor are described outside a spherical
biocatalyst particle in a liquid flow. Different fluid velocities and particle
diameters were used. The measured thickness of the boundary layer appeared
to be smaller than the thickness calculated using literature correlations. A
possible explanation is that the local liquid velocity existing in the measuring
equipment was higher than the superficial velocity used in the calculations.
No experimental distinction could be made between the results obtained with
different particle diameters. The reason being the fact that the influence of
the diameter is too small at high Reynolds numbers, while at low Reynolds
numbers the measuring error was too large due to vortices. However, one can
conclude that care must be taken when using correlations from the literature
to predict external mass transfer resistance.

Beside the influence of immobilization on the behaviour of an enzyme
the influence on metabolically active, growing cells was also studied. Oxygen
concentration profiles in carrageenan-gel beads and slabs containing growing,
immobilized *Escherichia coli* B (pTG201) were measured with an oxygen
microsensor. The oxygen penetration depth in the gel decreased with time,
eventually reaching a steady state value of approximately 100 μmeter for
both gel beads and slabs. A reaction-diffusion model employing zero-order
cell growth kinetics was found to provide an excellent fit to the experimental
concentration data, resulting in a value for the specific growth rate of the
immobilized cells. This growth rate was close to the growth rate measured
for free-cell suspensions.

After having studied the effect of immobilization on *E. coli*, an organ-
ism with a more complex metabolism, namely *Thiosphaera pantotropha*, was
selected. *T. pantotropha*, isolated from a waste water treatment plant, is capa-
bile of aerobic heterotrophic nitrification and both aerobic and anaerobic den-
itrification. These phenomena have been studied in acetate-limited aerobic
and anaerobic continuous cultures supplied with ammonia and nitrate. Be-
cause *T. pantotropha* is able to convert ammonia completely to nitrogen gas under aerobic conditions, and can also use nitrate as electron acceptor under anaerobic conditions, immobilization can be advantageous when the organism is used for waste water treatment. Immobilization will result in aerobic and anaerobic zones in the carrier. However, the effect of immobilization on the physiology of the cells must be known before such a system can be designed for use in waste water treatment. Due to the complexity of the metabolism of the organism it was necessary to develop a model describing the relationship between the several conversion rates for the cells in suspension. The model was build on intra-cellular reaction equations, based on knowledge of the biochemical pathways. The measurable conversion rates are related through a linear equation on the basis of the specified intra-cellular reaction rates. The coefficients in the linear equations are composed of parameters in the ATP production and consumption by the microorganism, and were estimated from the continuous culture measurements. It was shown that with realistic values for these parameters, the metabolically structured model describes the aerobic as well as the anaerobic experiments. This model was then extended with kinetic and diffusion equations to describe the behaviour of immobilized *T. pantotropha*. For the experiments the cells were immobilized in agarose-gel beads. The cells were grown in the beads under continuous culture conditions. After 24 hours the oxygen penetration depth showed a constant value of about 100 μmeter. From the dynamics of the start-up phase, several parameters were determined from measurements of oxygen concentration profiles made every few hours, by fitting the model equations to the measurements. The model gave a very accurate fit of the measurements. The parameters included the maximum specific growth rate, the maximum specific oxygen conversion rate, the maximum specific acetate conversion rate, the Monod constant for acetate and the maximum specific nitrification rate. Most of them were in good agreement with those determined for the suspended cells, only the maximum specific growth rate was significantly higher, and the maximum specific nitrification rate was somewhat lower than those for the suspended cells.

It can be concluded that the use of an oxygen microsensor in combination with mathematical modelling is suitable to obtain quantitative information about the behaviour of immobilized enzymes and/or cells. This is, of course, only true under the condition that oxygen plays a significant role in the systems used. This is the situation for many biological processes, but not for all of them. The research for the development of different types of microsensors is on its way, leading to a broader range of applications in the future. An important condition for the use of microsensors to measure concentration profiles is that the carrier is suitable for penetration with a microsensor. There were virtually no practical problems with agarose, carrageenan however was more difficult to penetrate and in the case of alginate the material deformed too much to be used for accurate measurements.
Samenvatting

Wanneer een enzym of cel, ook wel biokatalysator genoemd, in een drager wordt geïmmobiliseerd kan hierdoor de katalyserende werking beïnvloed worden. Dit verschijnsel wordt veroorzaakt door diffusielimiteer van substraat of produkt in en om de drager, of door verandering van de kinetiek van de door het enzym of de cel gekatalyseerde reaktie. Wat enzymen betreft kan dit het gevolg zijn van sterische hindering van het substraat of verandering van de conformatie van het enzym, wat de cellen betreft kan chemische of fysische stress een rol spelen. Wanneer de invloed van diffusie op de conversiesnelheid bekend is, is het mogelijk hiervoor te corrigeren en de invloed van immobiliseren op het enzym of de cel zelf te bepalen. Deze kennis is nodig voor het ontwikkelen van processen die gebruik maken van geïmmobiliseerde biokatalysatoren.

Voor het werk dat in dit proefschrift wordt gepresenteerd werd gebruik gemaakt van een zelfgemaakte zuurstof-microsensor. Met een zuurstof-microsensor, waarvan de punt een diameter heeft van 5 tot 10 μmeter, is het mogelijk om in een geïmmobiliseerd systeem plaatselijk de zuurstof concentratie te meten. Dit levert informatie op over het gedrag van de geïmmobiliseerde biokatalysator. Bij vergelijking met het gedrag van de biokatalysator in oplossing kan de invloed van immobilisatie bepaald worden.

De toepasbaarheid van een microsensor om zuurstof profielen te meten in gel-bolletjes die een zuurstof consumerend enzym bevatten wordt gepresenteerd. De resultaten die verkregen werden bleken nauwkeurig en reproduceerbaar te zijn. Vervolgens werd een zuurstof-microsensor gebruikt om de intrinsieke kinetische parameters van een geïmmobiliseerd enzym te bepalen. Het model-enzym dat hiervoor werd gebruikt was L-lactaat 2-monoxygenase, geïmmobiliseerd in gel-bolletjes van agarose. De reactiekinetiek van het enzym kon met de Michaelis-Menten snelheidsvergelijking beschreven worden. Verschillende deeltjesdiameters en enzymconcentraties werden gebruikt, wat verschillende concentratie gradienten in de drager opleverde. De intrinsieke kinetiekparameters werden bepaald door gecommueerde concentratieprofielen te fitten aan de gemeten profielen. Het bleek dat de parameters die hieruit resulteerden onafhankelijk waren van de straal van de deeltjes en gebruikte enzymconcentratie. Dit vormde een bewijs voor de betrouwbaarheid van de methode. De intrinsieke kinetiekparameters werden
vervolgens vergeleken met de parameters van het enzym in oplossing. Ten gevolge van het immobiliseren nam de maximale omzettingssnelheid en de Michaelis-Menten constante voor zuurstof van het enzym af.

Hierna werd hetzelfde immobilisatiemateriaal en modelenzym toegepast om de intrinsieke kinetiekparameters te bepalen door gebruik te maken van twee verschillende experimentele methoden. De eerste methode was gebaseerd op een stationaire toestand en bestond uit het meten van zuurstofgradienten in de biokatalysator deeltjes met een zuurstof-microsensor. De tweede methode bestond uit het meten met een biologische zuurstof monitor van de verandering van de zuurstofconcentratie in de vloeistoffase die veroorzaakt wordt door de geïmmobiliseerde biokatalysatoren. Om uit deze dynamische metingen de intrinsieke parameters te berekenen werd verondersteld dat het concentratieprofiel in de bol in pseudo-steady state was met betrekking tot de zuurstofconcentratie in de vloeistof. Voor de berekening werden twee verschillende mathematische methoden gebruikt. Zowel de twee verschillende experimentele methoden als de verschillende rekenmethoden leverde geen significant verschil op voor de intrinsieke kinetiekparameters. Het verschil in de toepassing van de twee rekenmethoden was de rekentijd die de computer nodig had.

Een van de parameters die de concentratiegradient in een biokatalysator-deeltje beïnvloedt is de externe stoftransportlimiet. De grensnaag om een biokatalysatorbolletje dat zich in een vloeistofstroom bevindt, kan gemeten worden met een zuurstof-microsensor. Verschillende vloeistofsnellen en deeltjesdiameters werden gebruikt. Het bleek dat de dikte van de grensnaag gemeten met behulp van de microsensor kleiner was dan wanneer deze berekend werd met behulp van literatuurcorrelaties. Een mogelijke verklaring hiervoor kan zijn dat de vloeistofsnellheid in de meetopstelling plaatselijk hoger was dan de superficiële snelheid die in de correlaties gebruikt werd. Voor verschillende deeltjesdiameters werd geen verschil in grensnaagdikte geconstateerd. Theoretisch beschouwd is de afhankelijkheid van de diameter bij een hoog Reynoldsgetal klein, bij een laag Reynolds getal bleek de meetfout te groot te zijn ten gevolge van het ontstaan van vortices. Het is echter duidelijk dat men voorzichtig moet zijn bij het toepassen van literatuurcorrelaties om externe stoftransportlimiet te voorspellen.

Afgezien van de invloed van immobilisatie op het gedrag van een enzym is ook de invloed op metabolisch actieve, groeiende cellen bestudeerd. Zuurstof concentratiegradienten, gemeten in bollen en platen van carrageenamet geïmmobiliseerde, groeiende, *Escherichia coli B* (pTG201), worden beschreven. De bollen en de platen werden onder continuicultuur omstandigheden gebracht. De indringdiepte van zuurstof in de gel nam in de loop van de tijd af tot een stabiele waarde van 100 μmeter werd bereikt, voor zowel de bollen als de platen. Een gekoppeld reactie-diffusie model, waarin de kinetiek met een nulde-orde vergelijking beschreven werd, leverde een uitstekende fit op van de
gemeten concentratiegradienten. Hieruit werd de specifieke groeisnelheid van de geïmmobiliseerde cellen bepaald. De waarde van de groeisnelheid van de geïmmobiliseerde cellen lag dicht in de buurt van die van de gesuspendeerde cellen.

Na het effect van immobilisatie op *E. coli* bestudeerd te hebben, werd een organism met een complexer metabolisme, namelijk *Thiosphaera pantotropha*, geselecteerd. *T. pantotropha*, geïsoleerd uit een afvalwaterzuiveringsinstallatie, is in staat tot aerobe heterotrope nitrificatie en tot zowel aerobe als anaerobe denitrificatie. Deze processen zijn in acetaat-gelimiteerde aerobe en anaerobe continu culturen bestudeerd, waarbij ammoniak en/of nitraat werd toegediend. Omdat *T. pantotropha* in staat is ammoniak in stikstofgas om te zetten onder aerobe condities, en onder anaerobe condities nitraat als electronenaacceptor te gebruiken, kan immobiliseren gunstig zijn wanneer het organism gebruik wordt voor de verwijdering van ammonium en nitraat uit afvalwater. Immobilisatie zal resulteren in het ontstaan van een aerobe en een anaerobe zone in het dragermateriaal. Het is echter nodig om te weten wat het effect is op de fysiologie van het organism voordat een dergelijk systeem toegepast kan worden. Ten gevolge van de complexiteit van het metabolisme van het organism was het nodig om een model te ontwikkelen dat de relatie tussen de verschillende omzettingssnelheden voor de cellen in oplossing beschrijft. Het model is gebaseerd op reactievergelijkingen die op intra-cellulair niveau plaatsvinden. De omzettingssnelheden die buiten de cel gemeten kunnen worden zijn aan elkaar gerelateerd op basis van de intra-cellulaire reactiesnelheden. De aldus ontstane lineaire vergelijkingen bevatten coëfficiënten die opgebouwd zijn uit parameters die een rol spelen in de ATP produktie en consumptie van het organism. De coëfficiënten werden bepaald uit de continuicultuur metingen. Het bleek dat met realistische waarden voor de parameters het metabolisch gestрукureerde model zowel aerobe als de anaerobe experimenten goed beschreef. Vervolgens werd het model uitgebreid met diffusie- en reactievergelijkingen om het gedrag van de geïmmobiliseerde *T. pantotropha* te kunnen beschrijven. Een geconcentreerde celsuspensie werd geïmmobiliseerd in bollen van agarose-gel, en onder continuicultuur omstandigheden gebracht. Na 24 uur was de indringdiepte voor zuurstof, die ongeveer 100 μmeter bedroeg, constant. Diverse kinetiekparameters konden uit de gemeten zuurstofprofielen bepaald worden door de profielen te fitten met modelvergelijkingen, die de profielen goed beschreven. De resulterende parameters omvatten de maximale specifieke groeisnelheid, de maximale specifieke zuurstof conversiesnelheid, de maximale specifiek acetaat conversiesnelheid, de Monod-constante voor acetaat en de maximale specifieke nitrificatiesnelheid. De meeste parameters waren in overeenstemming met de parameters bepaald voor de gesuspendeerde cellen. De maximale specifieke groeisnelheid was echter significant hoger, en de maximale specifieke nitrificatiesnelheid was iets lager dan voor de gesuspendeerde cellen.
Geconcludeerd kan worden dat het gebruik van een zuurstof-microsensor in combinatie met modelvergelijkingen geschikt is om kwantitatieve informatie te verkrijgen over het gedrag van geimmobiliseerde enzymen en cellen. Een voorwaarde is dat zuurstof bij deze processen een rol van betekenis speelt. Dit is in veel biologische systemen het geval, hoewel niet in alle. Het onderzoek met betrekking tot de ontwikkeling van microsensoren staat echter niet stil en zal in de toekomst leiden tot een breder toepassingsgebied. Een belangrijke voorwaarde voor het gebruik van microsensoren om concentratieprofielen te meten, is dat met de microsensor in het dragemateriaal geprikt kan worden. Voor agarose leverde dit geen problemen op, voor carrageenan echter was dit praktisch al moeilijker te realiseren en voor alginaat was de vervorming van het materiaal te groot om nauwkeurig te kunnen meten.
Dankwoord

Een promotieonderzoek is nooit het werk van één persoon. Daarom wil ik hier de gelegenheid benutten om diegenen te bedanken, die aan dit promotiewerk een bijzondere bijdrage hebben geleverd. Als eerste wil ik mijn promotor Karel Luyben bedanken. Karel, ik ben je zeer erkentelijk dat je me de mogelijkheid hebt geboden om zelf richting aan het onderzoek te geven.

Bert Geraats heeft binnen dit project een bijzondere plaats ingenomen. Na zijn stageperiode van drie maanden is hij bij de vakgroep gebleven om zijn vervangende dienstplicht te vervullen. Bert, ik zal mij onze samenwerking als enorm stimulerend en constructief blijven herinneren.

Cor Ras bedankt, je was als maker van de zuurstof-microsensoren onmisbaar voor het onderzoek.

Ik wil Ed van Niel, Lesley Robertson en Gijs Kuenen bedanken voor de, hoewel in het begin moeilijke, uiteindelijk toch succesvolle samenwerking op het ‘Thiosphaera’-gebied. De interesse en begeleiding van Sef Heijnen speelde hierbij een belangrijke rol. Lesley, ook bedankt voor de taalkundige correctie van mijn engelse teksten.

De twee afstudeerders Marc Stoop en Mieke Boon en de wetenschappelijk medewerker Jacques Potters wil ik bedanken voor hun bijdrage in de opstartfase van het onderzoek.

Aan de samenwerking met de amerikaanse post-doc. Cathy Briasco en Ph.D. student Huang Jun van de Universiteit van Compiègne denk ik met veel plezier terug. Cathy, I am very glad to have met you, and that we had the opportunity of working together.

Curriculum vitae


Sinds 1 februari 1990 is de auteur werkzaam bij het Institute for Hydraulic and Environmental Engineering te Delft.

---
