Doctoral Thesis

Growth characteristics of Acinetobacter johnsonii 210A under single and dual nutrient limitation with special reference to carbon, nitrogen and phosphorus

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GROWTH CHARACTERISTICS OF
ACINETOBACTER JOHNSONII 210A UNDER
SINGLE AND DUAL NUTRIENT LIMITATION
WITH SPECIAL REFERENCE TO CARBON, NITROGEN AND PHOSPHORUS

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH
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presented by
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Zürich, 1999
Thank you’s

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all the sports people with whom I shared many sweaty and painful lunch breaks as well as sunny hours by the pool,

and everybody else who has given me nice and not so nice times at EAWAG

******** you know who you are ********

Finally, I am thankful to the Swiss National Science Foundation and EAWAG for financing my research.
“Now is always the right time
With something positive on your mind
Whenever something pulls you down
Just get back and hold your ground”

Curtis Mayfield, 1996
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CDW</td>
<td>cell dry weight (biomass)</td>
</tr>
<tr>
<td>COD</td>
<td>chemical oxygen demand</td>
</tr>
<tr>
<td>C$_O$/N$_O$ ratio</td>
<td>carbon to nitrogen ratio in medium feed</td>
</tr>
<tr>
<td>C$_O$/P$_O$ ratio</td>
<td>carbon to phosphorus ratio in medium feed</td>
</tr>
<tr>
<td>C$_O$/O$_2$ ratio</td>
<td>carbon to oxygen ratio in medium feed</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>double-distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic-acid</td>
</tr>
<tr>
<td>DOC</td>
<td>dissolved organic carbon</td>
</tr>
<tr>
<td>DOT</td>
<td>dissolved oxygen tension</td>
</tr>
<tr>
<td>EBPR</td>
<td>enhanced biological phosphorus removal</td>
</tr>
<tr>
<td>kHz</td>
<td>kilo hertz</td>
</tr>
<tr>
<td>n.d.</td>
<td>not detected</td>
</tr>
<tr>
<td>orthoP-P</td>
<td>phosphorus contained in orthophosphate</td>
</tr>
<tr>
<td>PHB</td>
<td>poly-(R)-3-hydroxybutyrate</td>
</tr>
<tr>
<td>PHA</td>
<td>poly-(R)-3-hydroxyalkanoate</td>
</tr>
<tr>
<td>$^{31}$P-NMR</td>
<td>$^{31}$P nuclear magnetic resonance</td>
</tr>
<tr>
<td>PolyP</td>
<td>polyphosphate</td>
</tr>
<tr>
<td>PQQ</td>
<td>pyrrolo-quinoline-quinone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic-acid</td>
</tr>
<tr>
<td>rev min$^{-1}$</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>$S_0$</td>
<td>substrate concentration in medium feed</td>
</tr>
<tr>
<td>vvm</td>
<td>volume of air per volume of culture liquid and minute</td>
</tr>
<tr>
<td>wt%</td>
<td>weight %: grams per 100 grams</td>
</tr>
</tbody>
</table>
Summary

In many natural as well as engineered ecosystems microbial biomass production is limited by the availability of the macro-nutrients carbon, nitrogen or phosphorus. Overloading ecosystems with these nutrients results in increased biomass production and its subsequent microbial degradation leads to anaerobic conditions and with this to the death of higher organisms. Therefore, these nutrients must be eliminated from the sewage during wastewater treatment. Today, mainly biological processes are used to achieve this goal. Phosphorus is removed by a relatively new process named “enhanced biological phosphorus removal” (EBPR) which is based on alternating aerobic and anaerobic growth conditions of the sludge which lead to a cyclic accumulation and reutilisation of the bacterial reserve polymers PHA (poly-(R)-3-hydroxyalkanoate), glycogen and polyphosphate. These storage polymers are usually accumulated when an imbalanced nutrient supply limits microbial division. However, nutrient imbalance and polymer formation have not been linked in EBPR.

For a long time the assumption that only one nutrient at a time may limit microbial biomass production has prevailed (based on von Liebig’s “law of the minimum”). However, in the last ten to fifteen years experimental evidence has been presented that microbial biomass production may be limited by more than one nutrient at a time. This evidence stems mainly from continuous culture experiments with defined mineral growth media of different carbon to nitrogen ratios. When the $C_0/N_0$ ratio in the medium feed was varied three different growth regimes were observed: distinct limitations by either carbon or nitrogen alone and, in-between, a transition zone of varying width where both, carbon and nitrogen were limiting growth at the same time. The extension of this transition zone was dependent on ability of the cells to adapt their composition to either carbon or nitrogen limitation and thereby changing their composition. However, data on multiple nutrient limitation with nutrients other than carbon
and nitrogen are still scarce. Further experimental data are needed to verify the general significance of this phenomenon.

In this work, *Acinetobacter johnsonii* 210A was chosen as a model organism to study multiple-nutrient-limited growth in continuous culture during steady-state as well as transient growth. The organism was originally isolated from an EBPR performing wastewater plant and is known to show distinct changes of cellular composition when grown under phosphorus and carbon limitation, respectively.

In a first step the mineral medium used for cultivation of *Acinetobacter johnsonii* 210A was optimised and the growth behaviour on different carbon substrates which are known to be present in EBPR was characterised in batch culture (acetate, (iso)butyrate, ethanol, (iso)butanol, and mixtures thereof). Growth on (iso)butanol was characterised by the excretion of large amounts of the oxidation product, butyrate. No acetate was excreted when the cells grew on ethanol. When mixtures of acetate with butyrate, ethanol or butanol, were supplied, acetate was always used preferentially. Also in these mixtures butanol was partly excreted as butyrate. Glucose was no substrate for *Acinetobacter johnsonii* 210A, but when supplied with PQQ (pyrrolo-quinoline-quinone) the cells were able to oxidise glucose to gluconate. However, they were not able to use the derived electrons for the production of additional biomass.

When *Acinetobacter johnsonii* 210A was grown in continuous culture with different ratios of carbon and nitrogen, carbon and phosphorus, and carbon and oxygen, respectively, dual-nutrient-limited growth was observed. The extension of the dual-nutrient-limited growth regime was significant only for carbon and phosphorus since the organism was capable to change its phosphorus content by a factor of 3. PHB (poly-(R)-3-hydroxybutyrate) was only accumulated under single-phosphorus-limited growth conditions. When changing the \( C_0/O_2 \) or the \( C_0/N_0 \) ratio in the medium feed the extension of the growth regime remained small due to the fact that the cellular growth yields were similar under carbon, nitrogen, and oxygen limitation.
The response of *Acinetobacter johnsonii* 210A to rapidly changing nutrient availability was investigated in transient experiments in continuous culture. During transients from carbon to oxygen limitation the cells showed no sign of PHB accumulation as is known from EBPR. When a culture was shifted from carbon limitation to phosphorus limitation a lower specific growth rate and a reduced capacity to reduce INT (iodonitrotiazolium-chloride) were observed alongside with the accumulation of PHB indicating reduced cell fitness when phosphorus was limiting growth.

In different experiments of the present work *Acinetobacter johnsonii* 210A lowered its cellular phosphorus content drastically under phosphorus limitation. The cellular RNA content dropped to less than 2% of the cellular dry weight. Moreover, the capacity to oxidise substrate as well as to resume growth in nutrient rich medium was clearly reduced compared to carbon-limited cells.

Although *Acinetobacter johnsonii* 210A did not respond to oxygen or nitrogen limitation it was capable of quickly adapting to phosphorus limitation and can therefore be considered a suitable model organism to study further aspects of phosphorus limitation in Gram-negative bacteria.
Zusammenfassung


Ausmass dieser Doppellimitation hing prinzipiell davon ab, wie ausgeprägt die Änderung der Zellzusammensetzung als Folge von Kohlenstoff- oder Stickstofflimitation war. Daten zu anderen Nährstoffen fehlen heute noch weitgehend. Es ist deshalb notwendig, die experimentelle Datenbasis zu vergrössern, um eine Aussage über die generelle Bedeutung des Phänomens Doppellimitation machen zu können.

In der vorliegenden Arbeit diente *Acinetobacter johnsonii* 210A als Modellorganismus, um die Doppellimitation in kontinuierlicher Kultur sowohl unter Gleichgewichtsbedingungen als auch unter transienten Wachstumsbedingungen zu untersuchen. Das Bakterium stammt aus Klärschlamm einer Anlage mit Erhöhter Biologischer Phosphorelimination. Frühere Untersuchungen haben zudem gezeigt, dass der Organismus die Zellzusammensetzung an Kohlenstoff- und Phosphorlimitation anzupassen vermag.


Wurden *Acinetobacter johnsonii* 210A im Nährmedium verschiedene Verhältnisse von Kohlenstoff und Stickstoff, Kohlenstoff und Phosphor und
Kohlenstoff und Sauerstoff angeboten, konnte tatsächlich Doppellimitation beobachtet werden. Im Falle der Kombination Kohlenstoff und Phosphor war diese Wachstumszone sehr ausgeprägt, da das Bakterium in der Lage war, seinen Phosphorgehalt um einen Faktor drei zu variieren. PHB (Polyhydroxybutyрат) wurde nur unter Phosphorlimitation eingelagert. Bei der Kombination C₀/O₂ und C₀/N₀, waren die Doppellimitationszonen schmal, da die Biomasseausbeuten zwischen Kohlenstoff-, Stickstoff- und Sauerstofflimitation sehr ähnlich waren.


In verschiedenen Experimenten führte Phosphorlimitation zu einer drastischen Reduktion des zellulären Phosphor-Gehalts in *Acinetobacter johnsonii* 210A. Der zelluläre RNA-Gehalt sank unter 2% des totalen Zelltrockengewichts. Mehr noch, die Kapazität, Substrat zu oxidieren sowie die Fähigkeit in nährstoffreichem Medium zu wachsen, waren bei phosphorlimitierten Zellen klar beeinträchtigt.

SECTION 1

GENERAL INTRODUCTION
1.1 Nutrient limitation: a common feature in the microbial world

Physico-chemical parameters such as nutrient availability determine life in natural as well as in engineered ecosystems. The diversity of micro-organisms with respect to their nutritional abilities is so great that they are found in every known ecosystem from the very nutrient rich human intestines to the very nutrient poor surface of snow or the deep subsurface environments. Furthermore, micro-organisms show a high degree of flexibility when growing in environments with fluctuating nutrient concentrations. It is therefore not surprising that micro-organisms play an important role in the cycling of the key nutrients carbon, nitrogen and phosphorous in terrestrial as well as aquatic ecosystems (Azam et al., 1983).

Due to both microbial and chemical processes virtually all natural ecosystems are most of the time depleted of one or more of the key nutrients. As a consequence microbial growth in these ecosystems is nearly always nutrient-limited. This does not necessarily mean that such ecosystems are oligotrophic. The term “nutrient-limited” simply refers to an imbalanced nutrient availability.

In engineered systems nutrient limitations are imposed onto micro-organisms as part of the means to optimise a desired process. The production of secondary metabolites such as antibiotics is an important biotechnological process. Often, nitrogen and/or phosphorus deficient growth media are used to

---

1 The term “nutrient-limited growth” is generally used to describe two different aspects of microbial growth. On one hand the availability of a nutrient controls the growth rate. This kinetic control is usually described by an extended Monod-equation (Monod, 1942; Baltzis & Fredrickson, 1988; Chesbro et al., 1990; Bae & Rittmann, 1996; Kovarova-Kovar & Egli, 1998). On the other hand the term "nutrient-limited growth" is used to describe the stoichiometric aspect of growth namely the amount of biomass that can be produced by a microorganism from a given amount of a nutrient (von Liebig, 1840). In the following work attention is solely given to this stoichiometric aspect.
grow the organisms and to stimulate metabolite formation (Calam, 1986). In order to obtain maximum biosynthesis rates for specific metabolites fermentation processes have to be steered along an optimum path which also includes limitation by selected nutrients. During wastewater treatment the availability of nutrients also plays a key role since the composition of the inflowing wastewater with respect to the relative amounts of the different nutrients determines the effectiveness of the carbon, nitrogen or phosphorus elimination processes (Egli & Zehnder, 1994). It may even be necessary to modify the composition in order to optimise the elimination process. An example for such a procedure is the addition of carbon substrate to pre-treated wastewater in order to ensure the complete elimination of nitrogen (Egli & Zehnder, 1994; Purtschert, 1997).

Consequently, if one wants to understand the behaviour of microbial populations in natural or in man-made environments it is necessary to study the way in which micro-organisms accommodate to nutrient-limited conditions.

1.1.1 General microbial responses to nutrient limitation

The ability of micro-organisms to grow in environments short of one or more essential nutrients is a result of their potential to change their composition. On a short time-scale these changes mainly occur at the phenotype level (reviewed in Harder & Dijkhuizen, 1983). Generally, in nutrient-limited environments, micro-organisms must optimise their uptake and conversion efficiency of those nutrients which are limiting growth. The uptake can be improved by either producing more of the uptake enzymes or the synthesis of special high affinity uptake systems. For nutrients which diffuse through the cell membrane the regulation of intracellular enzymes plays a key role. The enzymes involved in the early steps of the metabolism must be efficient in removing the compounds, thus ensuring a gradient between intracellular and extracellular concentrations. Concomitantly with these responses the excretion of side products and the accumulation of storage polymers are often observed when micro-organisms are
growing nutrient-limited. Particularly aspects of reserve material accumulation will be of interest in this thesis (Tab. 1.1).

<table>
<thead>
<tr>
<th>Growth limiting nutrient</th>
<th>Reserve polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>polyP</td>
</tr>
<tr>
<td>N</td>
<td>lipids (incl. PHA, PHB), glycogen, polyP, carbohydrates</td>
</tr>
<tr>
<td>P</td>
<td>lipids (incl. PHA, PHB), glycogen</td>
</tr>
<tr>
<td>O2</td>
<td>PHA, PHB</td>
</tr>
</tbody>
</table>

Table 1.1: Reserve polymers accumulated by micro-organisms during growth under various nutrient limitations; adapted from Schlegel, 1985.

The list in Table 1.1 is by no means meant to be exhaustive but rather to give an overview about the ecologically most significant reserve materials that are accumulated by micro-organisms. Microbial storage polymers that are accumulated as a response to a nutrient imbalance have two main functions for the cell: they serve as intracellular sources of certain elements, as energy storage materials, or both (for an overview see Dawes & Senior, 1973; Schlegel, 1985).

1.1.2 Growth limitation by major nutrients carbon, nitrogen or phosphorus

Often, growth is limited by one of the major nutrients carbon, nitrogen or phosphorus. In order to understand the special cellular responses to limitations by one of these nutrients it is necessary to know to which cellular components they are allocated and what is the function of these components in a cell. Together, these elements make up over 50% of bacterial dry weight. The carbon content of microbial biomass is between 40wt% and 50wt%, around 10wt% is nitrogen and roughly between 1.5wt% and 6wt% is phosphorus (Pirt, 1975;
Herbert, 1976; Schlegel, 1985; Gottschalk, 1988). Carbon is found in all cell components, while nitrogen and phosphorus can clearly be attributed to certain cell constituents as it is shown in Figure 1.1. Nitrogen is assimilated to be incorporated mostly into protein, nucleic acids, and cell wall polymers. Phosphorus is mainly used for the synthesis of RNA and DNA.

![Figure 1.1](image)

**Figure 1.1:**
Composition of dry weight biomass of Gram-negative bacteria; data for the overall composition of major cell components was taken from Schlegel (Schlegel, 1985); the composition of individual macromolecules was taken from different sources (Herbert, 1976; Gottschalk, 1988).

Carbon limitation

Complex, overall changes in metabolism and major changes in cellular composition are often observed when cells are growing substrate-limited. Examples for all types of responses described in the previous section are found in the literature (Harder & Dijkhuizen, 1983). Generally, when the availability of the carbon substrate is limiting growth, a high carbon conversion efficiency can be observed and the diversion of substrate into extracellular products is minimised (Harder & Dijkhuizen, 1983). Interestingly, there is also evidence for
an increased or even altered catabolic versatility during growth under substrate-limited conditions (reviewed in Egli, 1995). In numerous studies it has been shown that the regulation of substrate uptake systems is tightly coupled to the extracellular concentration of substrates (Tempest, 1970; Harder & Dijkhuizen, 1983). An example for this is the uptake of glucose in *Pseudomonas aeruginosa*: during substrate-limited growth a high affinity system is active which allows the organism to take up glucose from the medium down to micromolar concentrations. When glucose is available in excess, it is oxidised to gluconate and 2-keto-gluconate in the periplasmatic space and these two compounds are then taken up separately. The affinity for glucose with this system is much lower, only about 1 mM (Harder & Dijkhuizen, 1983). Substrate limitation also affects the cellular composition of many micro-organisms. Very often an increased phosphorus content can be observed. The accumulation of large amounts of phosphorus is known from almost all living cells (Dawes & Senior, 1973; Kulaev, 1979; Kulaev & Vagabov, 1983). Usually, phosphorus is stored in cells as linear chains of condensed orthophosphate residues stabilised by Mg$^{2+}$, Ca$^{2+}$ and K$^+$ (Kulaev, 1979; Kulaev & Vagabov, 1983; Bonting, 1993). The polyphosphate chains reach lengths of several thousands of residues (Kulaev, 1979).

**Nitrogen limitation**

When micro-organisms are growing under nitrogen limitation the flux of the non-limiting carbon substrate is not tightly balanced and this may lead to the excretion of low molecular weight metabolites, extracellular polymers or the accumulation of intracellular reserve materials (Dawes & Senior, 1973; Tempest & Neijssel, 1978 Harder & Dijkhuizen, 1983; Schlegel, 1985). Due to the high content of reserve materials apparently lower cellular protein contents are often observed. Changes in external concentrations of nitrogen are known to have an effect on the cytoplasmatic ammonia-assimilating enzymes in micro-organisms (Harder & Dijkhuizen, 1983). Both, an enhanced synthesis of the ammonia-capturing enzymes as well as synthesis of a different pathway with a higher
affinity for ammonia assimilation have been observed in micro-organisms (Harder & Dijkhuizen, 1983; Gräzer-Lampart et al., 1986).

Phosphorus limitation

Phosphorus limitation affects cellular reproduction since it restricts nucleic acid synthesis (Alton & Koch, 1974; Cooney et al., 1976; Cooney & Wang, 1976). Additionally, the composition of the cell wall in Gram-positive bacteria and the cell membrane in Gram-negative bacteria are affected in such a way that phosphorus containing components are replaced by other, non-phosphorus containing ones that are able to fulfil the same function (Tempest, 1969; Harder & Dijkhuizen, 1983). In Bacillus subtilis teichonic acids which are contained in the cell wall were found to be replaced by the phosphorus-free teichuronic acids when the organism was deprived of phosphorus. Similarly, in Pseudomonas fluorescens, a Gram-negative marine organism, the phospholipids which normally are part of the cytoplasmic membrane were absent when the cells were grown under phosphate limitation (for an overview see Harder & Dijkhuizen, 1983). Phosphorus-limited growth may lead to metabolite excretion or accumulation of storage materials such as PHB (Cooney et al., 1976a; Ackermann et al., 1995). An increased production of the exoenzymes extracellular ribonuclease and alkaline phosphatase has been observed in Bacillus licheniformis (Harder & Dijkhuizen, 1983). Major phosphorus transport systems with low and high affinity for phosphate are expressed in response to the extracellular phosphate concentration. Such uptake systems are known from a number of bacteria including Acinetobacter johnsonii 210A (Rosenberg, 1987; van Veen et al., 1993a).

1.2 Multiple nutrient limitation

In 1840 Justus von Liebig formulated the basis for the understanding of biological nutrition with the "law of the minimum" which was based on observations from plant growth (von Liebig, 1840). It states that only one nutrient limits the maximum quantity of biomass that can be produced in a
biological system with a certain amount of nutrients. This concept was also taken on by microbiologists and found its way into the design of growth media. Still today, most growth media for batch and continuous cultivation in the laboratory are designed such that one nutrient terminates growth and ultimately determines the maximum amount of biomass that can be built (batch culture) or that the continuous addition of a nutrient controls the specific rate of growth and the steady-state concentration of biomass (continuous culture) (Monod, 1942; Monod, 1950; Tempest, 1970; Mason & Egli, 1993). Recently it has been shown that Liebig's concept must be extended because studies with pure cultures in the laboratory as well as studies of natural systems, in particular of aquatic ecosystems, have clearly shown that bacterial growth may well be limited by more than one nutrient at a time (Paerl, 1982; Egli, 1995). Clear experimental proof on the basis of culture parameters, cell composition and physiology has been presented for combinations of carbon and nitrogen, carbon and phosphorus as well as nitrogen and phosphorus (Panikov, 1979; Egli & Quayle, 1984; Egli & Quayle, 1986; Gräzer-Lampart et al., 1986; Pengerud et al., 1987; Minkevich et al., 1988; Duchars & Attwood, 1989; Rutgers et al., 1990; Egli, 1991; Lucca et al., 1991; Durner, 1998; Zinn, 1998). Indications for dual-nutrient-limited growth with combinations of C/Zn, C/O₂, C/K and C/vitamins have also been reported (Harrison, 1972; Lawford et al., 1980; Egli, 1982). In addition, the phenomenon was treated on a purely theoretical basis in a number of studies (for an overview see Egli, 1995). Today, even data on triple-nutrient-limited growth (C/N/P) are available for Pseudomonas oleovorans in continuous culture (Zinn, 1998).

1.2.1 Dual-nutrient-limited growth with the key nutrients carbon, nitrogen and phosphorus

Carbon, nitrogen, and phosphorus are governing microbial growth in many ecosystems (Paerl, 1982; Egli, 1995). Consequently, limitations by these nutrients have been the focus of many investigations. Most data on dual-nutrient-limited growth were collected for the nutrients carbon and nitrogen.
Experimentally, a continuous culture was supplied with a mineral medium in which the amount of the carbon substrate ($C_0$) was increased stepwise while all other nutrients, including nitrogen ($N_0$) remained constant. After each increase a steady-state was awaited and the biomass as well as the residual nutrients were measured. In Figure 1.2 the results of a chemostat experiment to assess dual-($C/N$)-limited growth in *Hyphomicrobium ZV620* are shown. Clearly, three distinct growth regimes were found: carbon-limited growth at low carbon to nitrogen ratios in the medium feed, an intermediate growth regime where both, nitrogen and carbon were used up completely, and nitrogen-limited growth at high $C_0/N_0$ ratios. Both, in bacteria and yeast, it was observed that the physiology of cells grown under dual-nutrient-limitation differed significantly from single-nutrient-limited grown cells (Egli, 1982; Gräzer-Lampart *et al.*, 1986). During dual-nutrient-limited growth the cells were characterised by a variable cell composition with respect to the enzyme activities as well as structural elements such as storage polymers (Egli, 1991).

**Figure 1.2:**
Growth of *Hyphomicrobium ZV620* with methanol and $\text{NH}_4^+$ ($S_0=20 \text{ mg L}^{-1} \text{N}$) in the chemostat at a constant dilution rate ($D=0.054 \text{ h}^{-1}$), as a function of the of the carbon to nitrogen ratio in the medium feed ($C_0/N_0$ ratio) (adapted from Gräzer-Lampart *et al.*, 1986).
Experimental data obtained with *Klebsiella pneumoniae* in chemostat culture under C and N limitation at different growth rates was re-examined and theoretically analysed (Egli & Schmidt, 1988). This analysis revealed that dual-nutrient-limited growth was far more pronounced at low growth rates (Fig. 1.3).

Furthermore, dual-(C/N)-limited growth was not confined to steady-state cultures but also found under transient growth conditions (Cooney & Wang, 1976b; Egli, 1982; Zinn, 1998). This finding is especially interesting since many natural ecosystems are characterised by fluctuations in nutrient availability.

![Figure 1.3](image)

**Figure 1.3:**
Extension of double-nutrient-limited growth regimen (grey area), as a function of the $C_0/N_0$ ratio in the medium feed and the growth rate, for a continuous culture of *Klebsiella pneumoniae*. Glycerol was used as carbon and energy source and $\text{NH}_4^+$ as nitrogen source (adapted from Egli, 1991).

Dual nutrient limitation with phosphorus as one nutrient has not been studied so well although phosphorus is known to be central for microbial productivity in many aquatic ecosystems and is, therefore, a key player in a complex and important food chain (Paerl, 1982; Vadstein & Olsen, 1989). It is not surprising that the early studies were conducted with aquatic organisms (Panikov, 1979; Pengerud et al., 1987). Similar to the results obtained with carbon and nitrogen, dual-(C/P)-limited growth was observed at a wide range of $C_0/P_0$
ratios. Also during dual-(C/P)-limited growth many micro-organisms accumulated storage polymers (Tab. 1.2).

All these results show that micro-organisms are very versatile in adapting their phenotype to nutrient-limited environmental conditions. Moreover, the relatively new yet basic findings of dual-nutrient-limited growth make it obvious that there is still need for research of microbial physiology in order to reach a better understanding of the microbes in engineered as well as natural environments.

<table>
<thead>
<tr>
<th>organism</th>
<th>dilution rate [h⁻¹]</th>
<th>dual (C/P) limitation [C₀/P₀ g g⁻¹]</th>
<th>reserve polymer</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida utilis</td>
<td>0.3</td>
<td>35.1-142.9</td>
<td>glycogen, fat</td>
<td>Lucca et al., 1991</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>0.2</td>
<td>35.1-142.9</td>
<td>glycogen, fat</td>
<td>Lucca et al., 1991</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>0.1</td>
<td>70.2-142.9</td>
<td>glycogen, fat</td>
<td>Lucca et al., 1991</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>0.17</td>
<td>at 76</td>
<td>polyP</td>
<td>Hao &amp; Chang, 1987</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>0.08</td>
<td>at 152</td>
<td>polyP</td>
<td>Hao &amp; Chang, 1987</td>
</tr>
<tr>
<td>Acinetobacter johnsonii 210A</td>
<td>0.1</td>
<td>12.9-64.5</td>
<td>polyP, PHB</td>
<td>Bonting et al., 1992a</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>0.03</td>
<td>140-300</td>
<td>PHA</td>
<td>Pengerud et al., 1987</td>
</tr>
<tr>
<td>Pseudomonas oleovorans</td>
<td>0.2</td>
<td>34-88.1</td>
<td></td>
<td>Zinn, 1998</td>
</tr>
</tbody>
</table>

Table 1.2:
Literature data for dual-(C/P)-limited growth of different micro-organisms in continuous culture. Borders between carbon-limited, dual-(C/P)-limited and phosphorus-limited growth are expressed in C₀/P₀ ratios of the medium feed; ¹no data on polyP available; ²no information given.
1.3 Polymer accumulation during Enhanced Biological Phosphorus Removal (EBPR)

Storage polymers are important features in many wastewater treatment systems (van Loosdrecht et al., 1997a). Yet, their importance has only been accepted in the EBPR process which has been a focus for microbiologists and wastewater engineers for more than 20 years (Jenkins & Tandoi, 1991; Randall, 1992; van Loosdrecht et al., 1997b). Excessive uptake of phosphate from aerated sludge was first reported from India in the late 1950's (Srinath et al., 1959). It was soon verified that this phosphate uptake was a biological process (Levin & Shapiro, 1965). While initial research mainly focused on the phosphate uptake during the aerobic phase, the significance of the anaerobic phase and the phosphate release were only recognised several years later (Randall et al., 1970). In the mid 1970's the research on the microbiology of this process was intensified because the responsible organisms for it were still largely unknown. In 1975 Fuhs and Chen described an isolation procedure for bacteria on which most subsequent work relied (Fuhs & Chen, 1975). Since then, several different models have been presented to describe the biochemistry of biological phosphorus removal. They were mostly based on results from sludge directly or on data obtained with bacteria of the obligat aerobic genus Acinetobacter which are frequently found in EBPR plants. The scheme depicted in Figure 1.4 represents the model which is generally accepted at the time of writing (Mino et al., 1987; Wentzel et al., 1991; Smolders et al., 1995).

In the whole process the storage polymers PHA and polyP as well as glycogen play important roles. EPBR plants show characteristic patterns of polymer accumulation and degradation during an operational cycle (Fig. 1.4). Such a cycle starts with an anaerobic phase in which the bacteria take up acetate and release orthophosphate into the bulk water. Intracellularly, PHB is built up at the expense of polyP and glycogen. During this phase, no growth occurs. Then an aerobic phase follows which is characterised by an uptake of
phosphorus and growth of the bacterial biomass. Intracellularly the glycogen pool is restored and polyP is accumulated.

**Figure 1.4:**
Scheme of an operation cycle in the EBPR process. Depicted is a bacterium during the anaerobic and the subsequent aerobic phase. PolyP: polyphosphate, PHB: poly-β-hydroxybutyrate, Glyc: glycogen (simplified from Smolders et al., 1995)

Although models for EBPR are largely based on *Acinetobacter* there is growing evidence today that also other bacteria are involved in the process. On one hand this evidence is based on *in situ* analyses of the microbial communities of EBPR plants which revealed only small numbers of *Acinetobacter* (Manz et al., 1994; Wagner et al., 1994; Amann et al., 1998). On the other hand physiological studies with pure cultures of *Acinetobacter* have
failed to show glycogen accumulation during aerobic growth or high rates of phosphate release under anaerobic growth conditions, both of which are important processes in EBPR plants (Deinema et al., 1985; Mino et al., 1987; Kortstee et al., 1994; van Loosdrecht et al., 1997c). However, attempts to isolate a strain which shows all characteristics from the EBPR process have failed so far. Therefore, \textit{Acinetobacter} still remains the best suitable organism to study selected microbiological aspects of EBPR in pure culture.

1.3.1 Model organism \textit{Acinetobacter johnsonii} 210A

Among the different \textit{Acinetobacter} species that have been isolated from EBPR plants \textit{Acinetobacter johnsonii} 210A is the one which has been studied most extensively. Its phosphorus metabolism has been studied in detail (van Groenestijn, 1988; Bonting, 1993; van Veen et al., 1993a). The two polymers polyphosphate and PhIB have also been investigated in batch and continuous culture (van Groenestijn et al., 1989; Bonting et al., 1992a). Accumulation of polyP occurs under nitrogen, sulphur, and carbon limitation (van Groenestijn et al., 1989; Bonting et al., 1992a). When polyP is accumulated the cellular P content can be as high as 12% of the cell dry weight (measured at 15°C) (Deinema et al., 1985). PolyP occurs in one or two large or a number of small granules in the cells. The average chain length as determined by gel electrophoresis was found to be ca. 700 residues (Bonting, 1993). Mg$^{2+}$ and Ca$^{2+}$ which are to some extent exchangeable, serve mostly as counterions (Bonting, 1993). Several enzymes of the polyP-metabolism in \textit{Acinetobacter johnsonii} 210A have been identified and characterised. The enzyme responsible for the formation of polyP, however, has not yet been identified. No polyphosphate-kinase activity was found \textit{in vitro} (van Groenestijn et al., 1988; van Niel et al., 1999). PolyP is degraded via a polyP-AMP phosphotransferase, which has been purified (Bonting et al., 1992b). As to the function of polyP in the cells it has been shown that its hydrolysis helps keeping the ATP-level high during anaerobic conditions thus allowing the cells to survive periods of anaerobiosis (van Groenestijn et al., 1987). Therefore, at least in this case, it serves as an
energy reserve. When grown under phosphorus limitation *Acinetobacter johnsonii* 210A is known to accumulate PHB up to 25% of its cell dry weight (Bonting *et al.*, 1992a). This finding is consistent with reports on other *Acinetobacter* strains which also accumulate PHB (Vierkant *et al.*, 1990; Weltin, 1996). Not much is known about the environmental conditions under which the organism accumulates PHB. So far only phosphorus limitation has been identified as a trigger for PHB accumulation (Bonting *et al.*, 1992a). There are indications that PHB is formed neither under nitrogen nor under oxygen limitation (Deinema *et al.*, 1985; van Groenestijn *et al.*, 1989). Furthermore, it is not known if PHB can serve as an endogenous carbon source when cells are carbon starved or as a sink for reduction equivalents as this has been proposed for *Azotobacter beijerinckii* (Senior & Dawes, 1971).

### 1.4 Scope of thesis

As outlined in the previous section the influence of nutrient availability on the accumulation and degradation of bacterial reserve materials has been studied for many years. Today, it is generally accepted that nutrient imbalances play a key role in the metabolism of the most important bacterial reserve polymers PHA, polyP, glycogen and carbohydrates. Yet, this knowledge has only very recently made its way into the examination of biotechnological processes such as EBPR, in which polyP, PHA and glycogen play a key role. Up to now, EBPR research has focused mainly on two aspects: microbiologists have tried to isolate the organisms responsible for the process since the very beginning, while for wastewater engineers the more practical aspects, namely plant design, optimal running conditions etc., have been of prime interest. The current models of EBPR have been developed mainly by engineers and the influence of nutrient availability on the observed dynamics of the storage polymers has never been investigated. The same holds true for research carried out with pure cultures of *Acinetobacter johnsonii* 210A.
For a long time, research on nutrient imbalances or in fact nutrient limitation was carried out assuming the existence of only one single growth-limiting nutrient at a time. Only recently has the new concept of dual-nutrient-limited growth been established. Experimental data especially regarding the significance of dual nutrient limitation for biotechnological processes are still scarce.

Therefore, the influence of single and dual nutrient limitation on the physiology of *Acinetobacter johnsonii* 210A was studied with respect to limitation by the macro-nutrients carbon, nitrogen and phosphorus. These nutrients are the major components of bacterial biomass. Two of them, carbon and phosphorus are also directly involved in the process of enhanced biological phosphorus removal. Additionally, the physiology under energy- and oxygen limited growth conditions was of interest because of the occurrence of these growth regimes in the EBPR process.

The questions tackled in this thesis were as follows:

• Can *Acinetobacter johnsonii* 210A grow on different carbon substrates simultaneously? How does the addition of an external energy source influence the growth behaviour on an energy-limited substrate?

• How does *Acinetobacter johnsonii* 210A behave under energy- or oxygen-limited growth conditions? Can the accumulation of PHB be observed?

• Does *Acinetobacter johnsonii* 210A exhibit dual-nutrient-limited growth with respect to the elements nitrogen, phosphorus and carbon?

• Is there a difference between steady-state conditions and transient growth as it is likely to occur during wastewater treatment?

The materials and methods used in this thesis are described in detail in the next section. The thesis consists of three result sections each tackling one or more of the questions listed above.
In section 3 the growth behaviour of *Acinetobacter johnsonii* 210A on different substrates as well as substrate mixtures is presented. Acetate-limited growth in continuous culture was examined with or without the addition of glucose and PQQ as external energy source.

In section 4 the results from shift experiments in continuous culture are presented. The shifts were performed from carbon to oxygen and from carbon to phosphorus limitation, respectively. In these experiments the ability of *Acinetobacter johnsonii* 210A to respond to changing nutrient availability was examined with special attention given to the accumulation and degradation of the reserve polymers PHB and polyP.

Section 5 describes the physiology of *Acinetobacter johnsonii* 210A growing in continuous culture with media of different $C_0/N_0$, $C_0/P_0$, and $C_0/O_2$ ratios, respectively. Dual-nutrient-limited growth was assessed at different growth rates and the results discussed within the concept of Egli (Egli, 1991).

General conclusions which can be drawn from the results of the experiments are presented at the end of the thesis and in an outlook suggestions for further experiments are made.
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Section 2

Materials & Methods
2.1 Organism, media, and cultivation conditions

2.1.1 Organism

*Acinetobacter johnsonii 210A* was a gift from Dr. G. Kortstee, Agricultural University of Wageningen, the Netherlands. The organism was spread out on complex medium agar plates prepared as described in section 2.1.3 and grown at 30°C until single colonies were easily visible (approximately after 48h). A single colony was picked and stock cultures were immediately prepared as described below.

2.1.2 Stock culture preparation

For the preparation of stock cultures *Acinetobacter johnsonii 210A* was grown in batch culture with complex medium prepared as described in section 2.1.3 (50mL of culture medium in 500mL Erlenmeyer flasks) on a rotary shaker at 30°C. When the exponential growth phase was reached aliquots of 1mL were transferred into sterile cryotubes containing 1mL of glycerol. The cells were mixed with the glycerol and immediately frozen at -80°C.

2.1.3 Media

All chemicals for media preparation were of analytical grade and purchased from Fluka (Buchs, Switzerland) unless otherwise stated.

Complex media

For growth of stock cultures and inocula complex medium pH 7.0 was used which consisted of 10g bactotryptone, 5g yeast extract (both from Biolife, Milano, Italy), and 5g NaCl per L of nanopure water prepared from deionised water using a Seralpur Pro90 device (Allhüser, Ransbach-Baumbach, Germany).

Purity of the chemostat culture was checked on complex medium agar plates which were prepared as follows: 0.5g yeast extract, 1.0g bactotryptone (both from Biolife, Milano, Italy), and 0.2g glucose were dissolved in 1L of nanopure
water. The pH was adjusted to 7.0 with 10N NaOH. Then 15g L\(^{-1}\) of agar (Merck, Darmstadt, Germany) was added and the mixture autoclaved.

**Minimal media**

Three different minimal media were used to cultivate *Acinetobacter johnsonii* 210A. The exact compositions are given in Table 2.1 and 2.2. The procedure for preparation of all three media was the same: first the basic salts medium was mixed, acidified to pH 3.5 with concentrated HCl and heat sterilised. After cooling down, 400μL L\(^{-1}\) of Fe(II)EDTA solution and 200μL L\(^{-1}\) trace element solution were added through a sterile nylon-filter of 0.2μm (Schleicher&Schuell, Dassel, Germany). The batch and fed-batch culture media were amended with 10% [v/v] of concentrated phosphate-buffer pH 7.0 and well mixed. Finally, the pH of the complete medium was adjusted to 7.0 with 10M NaOH.
<table>
<thead>
<tr>
<th>Element</th>
<th>Theoretical yield coefficient&lt;sup&gt;1&lt;/sup&gt; (Y&lt;sub&gt;x&lt;/sub&gt;)</th>
<th>medium A</th>
<th>medium B</th>
<th>medium C</th>
<th>medium D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. in medium [g L&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>Excess factor F&lt;sub&gt;e&lt;/sub&gt;</td>
<td>Conc. in medium [g L&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>Excess factor F&lt;sub&gt;e&lt;/sub&gt;</td>
<td>Conc. in medium [g L&lt;sup&gt;-1&lt;/sup&gt;]</td>
</tr>
<tr>
<td>B</td>
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<td>2.58 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<tr>
<td>N</td>
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<td>2.1</td>
<td>2.6 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
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<tr>
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</table>

Table 3.1:
Analysis and comparison of mineral media used to cultivate *Acinetobacter johnsonii* 210A in batch and continuous culture. Theoretical excess factors for elements are listed with respect to carbon (F<sub>e</sub>) for carbon-limited medium. <sup>1</sup> data from Pirt, 1975; <sup>2</sup> up to 1.5 g L<sup>-1</sup> C; medium A: van Groenstijn *et al.*, 1987; medium B: van Groenstijn *et al.*, 1989; medium C: Bonting, 1993; medium D: this study; n.a.: not added.
Table 2.2:
Composition of concentrated stock solutions used to prepare the minimal media.

For minimal medium agar plates 1.5% [w/v] of agar (Merck, Darmstadt, Germany) was added to batch type minimal medium.
2.1.4 Inocula

Inocula for chemostat experiments were prepared in the following way: 100mL of complex medium in a 300mL Erlenmeyer flask was inoculated with 2mL of stock culture. The culture was incubated on a rotary shaker at 30°C overnight. 60mL of the resulting cell suspension were used to inoculate the bioreactor.

Inocula for batch experiments were always pregrown in Erlenmeyer flasks at 30°C on a rotary shaker under the same conditions as used later in the experiment. The inoculated volume for batch cultures varied between 1 and 5% [v/v].

2.1.5 Continuous culture

In carbon/nitrogen and carbon/oxygen limitation experiments as well as the carbon/phosphorus transient experiments the cells were grown in a 1.5L bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 1L. The temperature was set to 30°C and the pH held constant at pH 7 ± 0.1 with 1M NaOH. The dissolved oxygen tension (DOT) was monitored continuously by a DOT-electrode (Mettler Toledo, Urdorf, Switzerland). DOT was never below 30% of air saturation: this was ensured by aeration (pressurised air at 0.9vvm, (volume of air per volume of culture liquid and minute)) and stirring at 700 - 800rev min⁻¹.

In phosphorus-limited cultures a fast corrosion of the steel heating device (reaching directly into the culture) was observed. This is the reason why in carbon/phosphorus limitation experiments the cells were grown in a 1L double-walled glass jar bioreactor with a working volume of 0.9L (Schmizo AG, Zofingen, Switzerland). The temperature was maintained at 30°C by circulating water and the pH held constant at pH 7±0.1 with 1M NaOH. The air was supplied aseptically through a 0.2μm PTFE-filter (Gelman Sciences, Ann Arbor, Michigan, USA). The culture was stirred with a Teflon-coated bar on a magnetic stirrer.
For the carbon/nitrogen limitation experiments the medium was prepared as described in section 2.1.3. The C\textsubscript{0}/N\textsubscript{0} ratio of the medium feed was increased during the experiment by adding different volumes of a concentrated Na-acetate solution (200g L\textsuperscript{-1}) to the medium reservoir through a sterile 0.2\textmu m nylon-filter (Schleicher&Schuell, Dassel, Germany). When media of low phosphorus content were used, KH\textsubscript{2}PO\textsubscript{4} was first dissolved in a small volume of ddH\textsubscript{2}O and then added to the medium reservoir through a sterile 0.2\textmu m nylon-filter after autoclaving (Schleicher&Schuell, Dassel, Germany). Two media with different concentrations of phosphorus were used in the transient experiments. The high phosphate medium contained 100mg L\textsuperscript{-1} phosphorus while to the low phosphate medium no phosphate was added. The P-concentration in the latter was always below 0.25mg P L\textsuperscript{-1}.

Steady-state samples were taken after a minimum of 5 volume changes when the biomass and residual nutrient concentrations remained constant. For checking culture purity samples were drawn from the chemostat aseptically every second day and diluted in sterile 10mM MgCl\textsubscript{2}. The uniformity of the cells was checked under the light microscope. Additionally, an aliquot of the diluted sample was plated out on complex medium agar and incubated for 2 days at 30°C or until colonies were visible. The agar plates were then checked for colony uniformity.

2.1.6 Batch and fed-batch cultures

Batch and fed-batch cultures were grown either in 500mL Erlenmeyer flasks containing 100mL of growth medium on a rotary shaker at 30°C or in a 1.5L bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 1L (details as described above). The medium was prepared as described in Table 2.1 and 2.2.

Carbon-fed-batch experiments were started with varying concentrations of PO\textsubscript{4}\textsuperscript{3-} in the medium (see Tab. 2.1). The Erlenmeyer flasks were inoculated and additional carbon was fed to the bacterial culture by adding a concentrated Na-
acetate solution (200g L⁻¹, pH 7) at a concentration of 1g acetate-C L⁻¹. After selected time intervals samples were aseptically drawn from the culture with a syringe. The pH was checked regularly and if necessary readjusted to 7.0 with concentrated HCl.

2.2 Analysis of biomass, cellular components and culture parameters

2.2.1 Sample treatment

Samples for routine analysis of biomass, cellular components and nutrients were withdrawn aseptically from the culture (batch or continuous) and immediately put on ice. 100mL were required for a complete analysis of the culture, including bacterial dry weight.

2.2.2 Biomass

For routine biomass determination a defined volume of the culture was centrifuged at 4°C (12 min, 14'000xg). The resulting pellet was washed once with Tris-HCl 50mM pH 7.6, transferred into tarred glass tubes and dried at 105°C to constant weight. The weight of the glass tubes was determined after they had cooled down to room temperature in an exsiccator. All measurements were done in triplicate, the standard deviation was below 5%.

As a fast method to estimate the biomass concentration of a culture the optical density was measured at 546nm (OD₅₄₆nm) using an Uvikon 860 spectrophotometer (Kontron Instruments, Basel, Switzerland). When necessary samples were diluted with 10mM MgCl₂ down to an OD₅₄₆nm of less than 0.5 units.

When the sample volume in an experiment was limited a rough estimation of biomass was possible by converting the optical density to dry weight according to the equation depicted below (Fig. 2.1).
Figure 2.1:
The relationship between optical density at 546nm and cell dry weight (CDW). The data points were measured in chemostat experiments at growth rates of 0.07h⁻¹, 0.3h⁻¹, and 0.35h⁻¹, respectively.

2.2.3 Cell composition

The carbon and nitrogen content of freeze-dried biomass was measured in a Carlo Erba EA1108 CHNS-(O) elemental analyzer (Carlo Erba, Milan, Italy). For analysis a known amount of cells were transferred into a tin capsule and burned at 1000°C, the produced gases were first reduced and then completely oxidised to CO₂, SO₂, H₂O, and NO₂. These products were separated over a chromatography column and quantified with a thermal conductivity detector. Sulphanilamide was used as a standard.

2.2.4 Poly-(R)-3-hydroxybutyrate (PHB)

The PHB content of the cells was determined by acidic hydrolysis and subsequent measurement of 3-hydroxybutyric acid by ion chromatography (for a reference see Hesselmann et al., 1999). 5mL of bacterial culture were centrifuged (4°C, 15min, 12'000xg), washed once with Tris-HCl 50mM pH 7.6, resuspended in 1mL of distilled water, transferred into 10mL-pyrex-vials and
stored at -20°C until further analysis. The collected samples were thawed and 1mL of concentrated H$_2$SO$_4$ was added to each tube under continuous stirring. The cells were then hydrolysed in a water bath at 90°C for 90min. After hydrolysis the samples were transferred to volumetric flasks and diluted with nanopure water to concentrations between 2 and 20mg L$^{-1}$ of 3-hydroxybutyric acid (for the description of the analysis of 3-hydroxybutyric acid see section 2.3.1). To determine the recovery rate of this PHB extraction method activated sludge was spiked with commercially available PHB (Fluka, Buchs, Switzerland). Extraction and analysis of 3-hydroxybutyric acid resulted in the recovery of 88% of the added PHB (Hesselmann et al., 1999).

2.2.5 Polyphosphate (polyP)

Polyphosphate was measured in freeze-dried biomass by solid-state $^{31}$P-NMR. 10 - 50mg of freeze dried cells were homogenised and directly used for analysis. Single-pulse $^{31}$P-NMR spectra with proton decoupling were measured on a Bruker ASX400 NMR spectrometer at ambient temperature using a 4-mm magic angle spinning (MAS) probe head. Typical conditions for recording spectra were as follows: MAS rate 8 kHz, recycle delay 5s; spectral width 98kHz; pulse length 3μsec (45 degree).

With this method the percentage of cellular P present as polyP can be determined. In order to get an absolute value it was necessary to determine the total P-content of the cells by the chemical method described in section 2.3.4. Having this value at hand the polyP content can easily be calculated.

2.2.6 RNA

The total RNA content of the biomass was measured by the orcinol reaction method. In this method the purine-bound ribose moieties of RNA react with acidified orcinol to form a green chromogen. Extraction and subsequent analysis of RNA was performed according to Methods for General and Molecular Bacteriology (1994). In contrast to the original protocol the heating
time was set to 20 min instead of 30 min. This change was introduced since no further color development occurred after 20 min of incubation, and, according to Herbert and coworkers (1971), the sensitivity for ribose is greatest after a heating time of 20 minutes. As a standard AMP was tested in concentrations between 5 and 100 µg AMP mL⁻¹ (Fig. 2.2).

![graph](image)

**Figure 2.2:**
Calibration curve for AMP in concentrations between 5 and 100 µg mL⁻¹ AMP.

The amount of RNA was calculated from the results using reference data from the literature (Herbert et al., 1971; Nultsch, 1986):

\[
\text{RNA [µg mL}^{-1}]\text{]} = \frac{[\text{AMP}] \times 0.331}{0.256}
\]

[AMP]: concentration of AMP in µg mL⁻¹ (see Fig. 2.2)
0.331: ribose-part of AMP [w/w]
0.256: purine-bound ribose part [w/w] of *E. coli* RNA, containing 81% rRNA and 19% soluble RNA (Neidhardt et al., 1990)

As a comparison, commercially available yeast RNA (purchased from ICN Pharmaceuticals Inc., Costa Mesa, Ca, USA) was also tested between 20 and 200 µg mL⁻¹ NaRNA. Interestingly, the calibration with AMP using the above
conversion factors, and direct calibration with RNA resulted in two significantly different calibration curves (Fig. 2.3). Consequently, in all measurements, RNA was used as a standard.

![Figure 2.3: Calibration curves for RNA measurements. The calibration with AMP results in amounts which are a factor 4.45 lower than those obtained with yeast RNA.](image)

Hexose is known to react also with the orcinol reagent (Herbert et al., 1971). Since the extraction procedure for RNA also extracts all DNA from the cells interference by deoxyribose must be considered. To quantify a possible interference commercially available *E. coli* DNA (purchased from ICN Pharmaceuticals Inc., Costa Mesa, Ca, USA) was also measured. Deoxyribose gave 36% of the color of ribose. This was more than stated in the description of the method in "Methods for General and Molecular Bacteriology" (1994). All the RNA measurements were therefore corrected assuming a DNA content of 5wt%.
In order to check the accuracy and especially the extraction efficiency of the method employed, *E. coli* ML30 was grown in batch culture on complex and minimal medium, harvested in the exponential growth phase, and analyzed for RNA. The RNA content of *E. coli* ML30 was 25.4 wt% for $\mu=1.0h^{-1}$, 19.5 wt% for $\mu=1.38h^{-1}$, and 20.8 wt% for $\mu=1.45h^{-1}$, respectively. From the literature the RNA content of *E. coli* at a growth rate of $1.0h^{-1}$ is reported to be 20 wt% and 24 wt% at $\mu=1.45h^{-1}$ (Bremer & Dennis, 1987). Hence, the numbers obtained in this work are within the same range as those reported in the literature.

### 2.2.7 INT activity measurements

The activity of the cells’ respiratory chain can be measured by adding INT (iodonitrotrazolium-chloride) as an artificial electron acceptor. Oxidised INT penetrates the cell wall and accepts electrons from the respiratory chain. Its reduced form precipitates as an INT-formazan crystal which can be extracted from the cells with a solvent. The absorption of the reduced INT can be measured at 460 nm. In this report a modified method originally proposed by Al-Awadhi and coworkers for *Bacillus* sp. NCIB 12522 was used (Al-Awadhi et al., 1988).

The reaction mixture was prepared in 2.2 mL Eppendorf tubes. Each tube contained 0.2 mL Tris-HCl buffer (0.5M, pH 7.2), 0.01 mL Na-acetate (0.25 g L$^{-1}$, pH 7.0), 0.2 mL INT-solution (0.2%). To start the reaction 1.0 mL of bacterial culture ($OD_{566}=0.75-1.6$) was added, the tube shortly vortexed to ensure mixing and incubated at 30°C. After 60 min the reaction was stopped with 0.2 mL of 37% formaldehyde. The Eppendorf tubes were centrifuged at 12'000 xg for 5 min and the supernatant removed. To the pellet 2 mL of dimethylsulfoxide and 10 μL of concentrated HCl were added. The mixture was sonified for 3 min followed by the extraction of the reduced INT-salt for 20 min at room temperature in the dark. The suspension was then centrifuged at 12’000 xg for 5 min and the extracted INT-formazan complex was measured at 460 nm. As a reference the same assay
was performed with cells which had been boiled for 10min and, therefore, should have had no activity.

### 2.2.8 Maximum oxygen uptake measurements

Oxygen uptake experiments were carried out in a Clark-type oxygen probe at 30°C (Rank Brothers, Cambridge, United Kingdom) in a reaction chamber of 2mL at 30°C. A sample of culture liquid was withdrawn from the chemostat, centrifuged for 5min at 12’000xg and the pellet was washed once with 50mM Tris-HCl, pH 7.6. Then the pellet was resuspended in mineral medium (pH 7.0, without carbon source) to an OD_{546nm} between 0.15 and 0.25. The OD value was measured and later used for calculating the specific oxygen uptake per unit of biomass. In this suspension the endogenous oxygen uptake was measured. Maximum oxygen uptake of the cells was determined upon the addition of 10µL of a concentrated Na-acetate solution (200g L^{-1}) to give a final concentration of 1g L^{-1} Na-acetate in the reaction chamber.

### 2.3 Chemical analysis of nutrients and substrates

#### 2.3.1 Acetate, butyrate, and 3-hydroxybutyrate

The concentration of the three organic acids was measured by ion chromatography using a Dionex DX 500 chromatographic system (Dionex, Olten, Switzerland). The column was a Dionex Ionpac AS10, 0-4mm, together with a guard column Ionpac AG 10, 0-4mm. Diluted NaOH served as eluent and was delivered to the system at a flow rate of 1mL min^{-1}. The elution program was a linear gradient of NaOH from 7.6 to 9.5mM in 10.5min. During this period the weakly absorbing organic ions were eluted, including various cell metabolites at small concentrations. After this gradient the column was flushed with 150mM NaOH for 10min to remove the strong adsorbing ions such as SO_{4}^{2-}. Finally, an equilibration at the initial concentration (7.6mM) for 10min was performed before the next run was started. A typical chromatogram is shown in Figure 2.4. The initial peak at 4.9min marks the arrival of the sample. The
flushing of the column with 10mM NaOH appears in the chromatogram as a broad peak between 15min and 25min.

Detection limits for acetate, butyrate, and 3-hydroxybutyrate were 0.4mg L\(^{-1}\) with an injection volume of 20\(\mu\)L.

The identity of the main metabolite from batch cultures growing with butanol was verified with mass spectrometry. The samples were prepared for mass spectrometry as follows: Culture supernatant was loaded on the ion chromatograph. The unknown component appeared as a single peak and the fraction containing this peak was collected. An identical fraction without the aforementioned peak was used as a reference. For mass spectroscopic analysis, direct infusion experiments with the samples collected from the ion chromatograph were performed using a Harvard Syringe Pump model “22” (Harvard Instruments, Gams, Switzerland) at a flow rate of 20\(\mu\)L \(\text{min}^{-1}\). A Platform LC model (Micromass, Manchester, United Kingdom) was used with electrospray interface operated in negative ion mode. The needle voltage was set to 2.3kV and the cone voltage was either 16 or 100 V. A range between 30
and 300 Da was scanned with a speed of 1s per scan. Nitrogen gas was used as nebuliser and drying gas at a flow rate of 500L h⁻¹ and a temperature of 150°C.

2.3.2 Ethanol and butanol

Both alcohols were measured on a gas chromatograph type Carlo Erba 4200 (Milano, Italy) equipped with an FID detector. For separation a 2m glass column packed with Tenax TA, 80/100 with a 2mm inside diameter (Machery & Nagel, Düren, Germany) was used. The analysis was performed at 145°C, the injector temperature being 250°C and a FID temperature of 255°C. As a carrier gas helium was used at a flow rate of 13mL min⁻¹. The injection volume was 0.7μL and concentrations could be quantified down to 3mg L⁻¹ ethanol or butanol.

2.3.3 Ammonium

Ammonium in the culture supernatant was measured by the indophenol method described by (Scheiner, 1976). The linear range of the assay was between 0.2 and 2mg L⁻¹ N and the detection limit 0.15mg L⁻¹ N. Immediately before analysis samples were diluted with nanopure water to concentrations below 2mg L⁻¹ N.

2.3.4 Orthophosphate (orthoP) and total phosphate (TotalP)

Orthophosphate was measured by a modified ascorbic acid method (APHA, 1976). In this method orthophosphate, ammoniummolybdate and potassium antimon oxtartrate react under acidic conditions to form a complex. This complex is reduced by ascorbic acid and the product can be analysed spectrophotometrically at 880nm. The assay was linear between 0.07 and 0.7mg L⁻¹ P.

For TotalP analysis 1mL of chemostat culture was mixed with 9mL of a 5% potassium peroxodisulfate solution and autoclaved at 121°C for 2h. Then the orthoP content of the hydrolysate was determined as described above. When low phosphorus containing cultures were analyzed up to 5mL of culture
were mixed with potassium peroxodisulfate to a final volume of 10mL. TotalP measurements were done in triplicate.

2.3.5 DOC

Dissolved organic carbon was measured using a Tocor 2 carbon analyser (Maihak AG, Hamburg, Germany). The concentration range for analysis was between 0 and 100mg L\(^{-1}\) C and samples were diluted with nanopure water when necessary. Prior to analysis the samples were acidified to pH 2 with concentrated HCl and dissolved CO\(_2\) was removed by purging with N\(_2\) for 10min.

2.3.6 Glucose and gluconate

Gluconate and glucose were both measured enzymatically with commercially available enzyme tests (Boehringer Mannheim, Germany). The detection limit for gluconate was 0.5mg L\(^{-1}\) using a sample volume of 2mL. Glucose was measured using the GOD-Period\(^\text {TM}\) method (Boehringer Mannheim, Germany). The samples were analysed at 420nm (instead of 560nm as recommended by the manufacturer). This modification resulted in a higher sensitivity for glucose (detection limit 1-2 mg L\(^{-1}\) glucose).

2.3.7 Analysis of CO\(_2\) and O\(_2\)

CO\(_2\) and O\(_2\) in the effluent gas stream from the bioreactors were measured online using a gas chromatograph (type GC-8A, Shimadzu & Co, Tokyo, Japan). The effluent gas line was connected directly to the GC (automated sampling), which was equipped with two parallel packed columns and a thermal conductivity detector. The columns were packed as follows: one with a molecular sieve of 5A 80/100, and the other with Porapak Q 80/100 (both from Brechbühler AG, Schlieren, Switzerland). Helium served as carrier gas. The injector and detector temperatures were set to 150°C and the oven temperature to 80°C.
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SECTION 3

GROWTH OF

ACINETOBACTER JOHNSONII 210A IN BATCH
AND CONTINUOUS CULTURE:
USE OF SINGLE AND MIXED SUBSTRATES
Abstract

The growth characteristics of Acinetobacter johnsonii 210A were investigated in the presence of single and mixed carbon substrates, both, in batch and chemostat culture. Initially, the mineral medium for growth of Acinetobacter johnsonii 210A was optimised and the maximum specific growth rate, as a function of the pH, was determined in batch culture at 30°C with acetate as sole source of carbon and energy. Acinetobacter johnsonii 210A grew faster at pH 7.0 where it reached a maximum specific growth rate of 0.81h⁻¹. The maximum specific growth rates on other substrates were lower: 0.51h⁻¹ on ethanol, 0.38h⁻¹ on isobutanol, 0.36h⁻¹ on butanol, 0.3h⁻¹ on malate, and 0.25h⁻¹ on butyrate. Growth on isobutanol and butanol was characterised by the excretion of metabolites. In the case of isobutanol and butanol the main metabolites were the corresponding carboxylic acids. Acinetobacter johnsonii 210A was also grown in batch culture with mixed substrates. These mixtures were combinations of acetate together with a second substrate, namely butyrate, butanol and ethanol, respectively, initially present at equal amounts (circa 250mg L⁻¹ carbon). During cultivation with mixtures acetate was always used preferentially but the maximum specific growth rate (0.81h⁻¹) was not reached. It was also tested whether supplying Acinetobacter johnsonii 210A with a substrate that can be oxidised but not assimilated might enhance the growth yield on acetate. Therefore, Acinetobacter johnsonii 210A was grown continuously on acetate with the addition of glucose and PQQ at a molar ratio of 0.93 (Mol acetate : Mol glucose). Although the cells oxidised more than half of the glucose to yield gluconate no increase in biomass yield was observed.

Key words: Acinetobacter, mixed substrate utilisation, batch culture, growth yield, PQQ
3.1 Introduction

The design of growth media is a powerful tool to control and manipulate bacterial growth. Be it in biotechnological processes or in basic research, knowledge about the influence of the composition of the culture medium used for cultivation on the growth behaviour of micro-organisms is essential. In the laboratory well defined growth media are usually used to study microbial physiology. The design of such growth media is based on information about the elemental growth yields \( (Y_{XE}) \) which describe how much biomass can be produced from a certain element supplied to the micro-organism. Growth yields vary of course with growth conditions such as specific growth rate, type of growth-limitation etc. Nevertheless, under similar conditions they arc fairly constant among many micro-organisms (Pirt, 1975; Egli & Fiechter, 1981). When no or little metabolites are excreted these yields are usually a reflection of the elemental composition of bacteria. For heterotrophic microbes the only exep tion is carbon for which the growth yield depends also on the energy content (degree of reduction), because it serves also as an energy source and is always partly respired to CO\(_2\).

The carbon to energy ratio of a carbon substrate reflects its energy content. Usually, the substrates are divided into energy deficient and energy surplus substrates depending mostly on the substrate's degree of reduction (Linton & Stephenson, 1978). Thus, growth under substrate limitation may be carbon- or energy-limited, depending on the substrate. Energy-limited compounds are highly oxidised (for example short chain organic acids such as oxalate) and maximum biomass yields for such carbon sources are in the range of 0.1 to 0.4g CDW per g\(^{-1}\) substrate-C. In contrast, with more reduced substrates such as ethanol or butanol constant carbon growth yields of 1.4g CDW g\(^{-1}\) substrate-C have been reported (Linton & Stephenson, 1978).

In wastewater treatment plants microorganisms are growing with a wide range of energetically different substrates. *Acinetobacter johnsonii* 210A, which is often considered to be a model organism for EBPR, is capable to use
several short chain organic acids as growth substrates. However, nothing is known about the influence of mixtures of substrates on the growth behaviour of *Acinetobacter johnsonii* 210A.

In continuous culture, growth yield and growth rate can be improved by mixing substrates with different carbon:energy ratios if the compounds are used simultaneously (van Verseveld *et al.*, 1979; Müller *et al.*, 1983). Moreover, it is even possible to separate biomass production and energy generation completely by adding an external energy source which cannot be assimilated, e.g. as reported for *Pseudomonas oxalaticus* OX1 growing with acetate and formate (Dijkhuizen & Harder, 1979) or for *Acinetobacter* species growing with acetate and glucose (Müller & Babel, 1986; van Schie *et al.*, 1987).

With respect to the utilisation of additional energy it was found that *Acinetobacter* species are unable to use glucose or gluconate as a carbon source although a membrane-bound glucose dehydrogenase is synthesised constitutively. This glucose dehydrogenase often lacks the cofactor PQQ (Bouvet & Bouvet, 1989; Duine, 1991). When some *Acinetobacter* strains grow in the presence of glucose and PQQ they are able to gain reducing equivalents from oxidising the sugar to the corresponding acid but are not able to assimilate it (Müller & Babel, 1986; van Schie *et al.*, 1987).

In this work the media previously used to cultivate *Acinetobacter johnsonii* 210A in carbon-limited continuous culture (van Groenestijn *et al.*, 1987; van Groenestijn *et al.*, 1989; Bonting, 1993) were analysed with respect to the theoretical excess of each element over carbon. Analysis was done with the help of elemental growth yields which were taken from Pirt (Pirt, 1975). Based on these calculations a new medium was constructed and tested. The maximum specific growth rate dependent on the pH was determined with acetate as a substrate. To obtain information on the growth behavior of *Acinetobacter johnsonii* 210A with mixed substrates as they occur in wastewater treatment plants, batch experiments with mixtures of acetate and butyrate, acetate and ethanol, and acetate and butanol were performed. Finally, it was tested or not
whether in our strain of *Acinetobacter* the growth yield with acetate as the primary, energy-limited substrate, may be improved by the addition of an external energy source in the form of glucose and PQQ.

### 3.2 Results

#### 3.2.1 Medium analysis

The carbon-limited mineral media which had been used to cultivate *Acinetobacter johnsonii* 210A were analysed for their contents of the different elements (van Groenestijn *et al.*, 1987; van Groenestijn *et al.*, 1989; Bonting *et al.*, 1992a). Medium analysis was performed as described by Egli and Fiechter (Egli & Fiechter, 1981). Theoretical excess factors (F) for each element with respect to carbon were calculated from the medium composition according to the formula $F_C = \frac{Y_X}{Y_{XC}} \frac{C_X}{C_C}$. where $Y_X$ is the growth yield for the element of interest, $C_X$ its concentration in the growth medium, $Y_{XC}$ the growth yield for carbon and $C_C$ the carbon concentration in the growth medium. For the whole analysis, $Y_{XC}$ was set at 1.1g CDW g\(^{-1}\) substrate-C and $C_C$=1.0g L\(^{-1}\). Resulting values of $F_C$ less than 1 indicate a theoretical limitation, whereas factors exceeding 1 mark a theoretical excess of an element over carbon. In Table 3.1 the excess factors $F_C$ for the different medium components are listed. Column A to C show data from the previously used media. In medium A and B especially the trace elements copper, zinc, and manganese have to be considered critical with respect to an undesired limitation. In medium C all excess factors are above 1 but nevertheless cobalt might be critical especially when taking into consideration that it forms hardly soluble salts with many anions.

As a consequence of this analysis carbon-limited mineral medium D was designed (Tab. 3.1). The concentrations of the elements were adjusted such that the theoretical excess factors over carbon were roughly between 5 for bor and 25 for trace elements such as copper, cobalt or zinc. In order to minimise the danger of running into an unexpected limitation even at higher carbon concentrations also $\text{Na}_2\text{EDTA}$ was added to the growth medium at a
concentration of 0.02mM (equimolar to the trace elements). The maximum specific growth rate on the new mineral medium supplemented with acetate was found to be $0.81\text{h}^{-1}$ in batch culture at 30°C. The biomass yield for carbon ($Y_{xc}$ in gCDW/gC) was 1.02 at $\mu=0.375\text{h}^{-1}$ and 1.05 at $\mu=0.585\text{h}^{-1}$ (see also section 5).

In an earlier study the temperature optimum for batch growth of Acinetobacter johnsonii 210A was determined and it was found to be between 25°C and 30°C (van Groenestijn et al., 1989). From the same study a biomass yield $Y_{xc}$ of 1.0g g$^{-1}$ (at $\mu=0.55\text{h}^{-1}$) and a maximum specific growth rate of only $0.69\text{h}^{-1}$ at pH 7.0 and 25°C were reported. Therefore, the newly composed medium was given preference in all experiments since it led to a better performance of the organism in batch culture and high growth yields in continuous culture.
<table>
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<td>2.58 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>8.6</td>
<td>2.50 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>8.3</td>
</tr>
<tr>
<td>N</td>
<td>8.75</td>
<td>2.62 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.1</td>
<td>2.6 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.3</td>
<td>2.6 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.3</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>K</td>
<td>59.5</td>
<td>3.8 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>20.6</td>
<td>1.15 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>6.2</td>
<td>3.88 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
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<td>3.88 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
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<td>1.0</td>
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<td>1.0</td>
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</tr>
</tbody>
</table>

Table 3.1:
Analysis and comparison of mineral media used to cultivate *Acinetobacter johnsonii* 210A in batch and continuous culture. Theoretical excess factors for elements are listed with respect to carbon (F<sub>e</sub>) for carbon-limited medium. 1) data from Pirt, 1975; 2) up to 1.5g L<sup>-1</sup> C; **medium A**: van Groenstijn *et al.*, 1987; **medium B**: van Groenstijn *et al.*, 1989; **medium C**: Bonting, 1993; **medium D**: this study; n.a.: not added.
3.2.2 Growth with single carbon substrates in batch culture

Growth with acetate

With this new medium the maximum specific growth rate of *Acinetobacter johnsonii* 210A was determined with acetate as sole source of carbon and energy at pH values between 6 and 9 (Fig. 3.1).

![Graph showing the effect of pH on maximum specific growth rate](image)

**Figure 3.1:**
Effect of pH on the maximum specific growth rate of *Acinetobacter johnsonii* 210A in batch culture at 30°C. In all experiments mineral medium with acetate as sole carbon and energy source was used. The standard deviation in 3 independent experiments performed at pH 7 was 4.2%.

Between pH 6 and pH 7 the maximum specific growth rate increased rapidly. The optimum pH for growth of *Acinetobacter johnsonii* 210A was pH 7.0 at which the maximum specific growth rate was 0.81 h⁻¹. At pH values above 7.5 precipitations were visible in the culture medium. Most likely the slow decrease of the maximum specific growth rate towards higher pH values was due to trace element limitation caused by the formation of insoluble salts.
Growth with other C-sources

The maximum specific growth rate at pH 7.0 was also determined for butyrate, DL-malate, isobutanol, butanol, and ethanol respectively (Tab. 3.2). Batch cultures supplied with butanol, isobutanol, and malate often showed a similar growth pattern: After inoculation cells grew initially to an optical density of approx. 0.3 and then stopped. After a short lag phase, growth continued at a slower rate which is listed in Table 3.2 as \( \mu_{\text{max}} \). This suggested a transient accumulation of metabolites formed from the primary carbon source which might be used as growth substrates subsequently. The transient accumulation and utilisation of these metabolites are presented in more detail in Figures 3.3 to 3.5. It became obvious that batch cultures with the afore mentioned carbon sources were themselves “multi-substrate-systems” and single-substrate-growth was only the case when ethanol was used. In batch cultures growing with butanol the major excreted metabolite was identified with mass spectrometry and it was shown to be the corresponding acid, butyrate. The metabolite from the batch on isobutanol coeluted with isobutyrate on the ion chromatograph which suggests that also in this case the first step in isobutanol metabolism was the oxidation to the corresponding acid. In the batch culture supplied with malate a similar growth behaviour was observed, suggesting also the transient accumulation of a product, but no attempt was made to identify the excretion product (Fig. 3.2).
Figure 3.2:
Batch growth of *Acinetobacter johnsonii* 210A on DL-malate. The initial growth rate was 0.3h⁻¹. In a second growth phase the cells continued to grow at 0.05h⁻¹.

<table>
<thead>
<tr>
<th>substrate</th>
<th>( \mu_{\text{max1}} ) [h⁻¹]</th>
<th>( \mu_{\text{max2}} ) [h⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>butyrate</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>DL-malate</td>
<td>0.30</td>
<td>0.05</td>
</tr>
<tr>
<td>isobutanol</td>
<td>0.26</td>
<td>0.26 (isobutyrate)</td>
</tr>
<tr>
<td>isobutanol</td>
<td>0.38</td>
<td>0.21 (isobutyrate)</td>
</tr>
<tr>
<td>butanol</td>
<td>0.18</td>
<td>0.36 (butyrate)</td>
</tr>
<tr>
<td>ethanol</td>
<td>0.51</td>
<td>-</td>
</tr>
<tr>
<td>isobutyrate</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2:
Maximum specific growth rate of *Acinetobacter johnsonii* 210A on different substrates at pH 7.0 and 30°C. Column A gives the initial maximum specific growth rate found on the original substrate while in column 2 the maximum specific growth rates of the second exponential growth phase are listed, when known, the excreted metabolite serving as a main growth substrate is given in brackets. ¹) preculture grown in batch on acetate; ²) preculture grown continuously on acetate at \( \mu=0.3h^{-1} \); ³) preculture grown continuously on isobutanol at \( \mu=0.1h^{-1} \); ⁴) preculture grown in batch on ethanol.
When *Acinetobacter johnsonii* 210A grew on butanol large quantities of butyrate were excreted into the medium (Fig. 3.3). After 7.1h butanol had disappeared from the medium and the butyrate concentration reached its peak (Fig. 3.3a). At this point 54% of the utilised butanol had accumulated as butyrate in the culture. The butyrate concentration showed a plateau for approx. 0.5h and then decreased to undetectable quantities. Acetate was also excreted by the cells but its concentration remained negligible (maximum conc. 16mg L\(^{-1}\) C). The specific growth rate during the first growth phase when butanol was oxidised was 0.18h\(^{-1}\). Growth clearly accelerated during the second growth phase on butyrate where a specific growth rate of 0.36h\(^{-1}\) was observed (Fig. 3.3b). In order to check for other metabolites the DOC concentration was analysed and compared to the total carbon from individually measured compounds, i.e. butanol, butyrate and acetate (Fig. 3.3c). The three compounds analysed always made up at least 90% of the total carbon present in the culture medium. Therefore, growth on a further unidentified metabolite is not likely.

The biomass was determined gravimetrically at the end of the batch only (528mg CDW L\(^{-1}\)) due to the limited total sample volume available (estimates made with the help of the optical density in conjunction with the calibration curve in the materials and methods section (which was based on data from continuous culture) led to an overestimation of the biomass concentration. Therefore, the biomass produced was estimated with the help of the final OD vs. biomass ratio, assuming a linear behaviour between biomass and OD over the whole concentration range). During the first 6.1h 431mg L\(^{-1}\) butanol-C were utilised, 41% of which (193mg L\(^{-1}\) C) led to the formation of 216mg L\(^{-1}\) CDW, resulting in an overall growth yield of 1.12g CDW per gram of butanol-C.
Figure 3.3: Biomass formation and excretion of butyrate during batch growth of *Acinetobacter johnsonii* 210A on butanol. The substrate was supplied at an initial concentration of 470 mg L\(^{-1}\) C. Cells for inoculation were pregrown in continuous culture on acetate at D=0.3 h\(^{-1}\).
The influence of the inoculum on batch growth of *Acinetobacter johnsonii* 210A on isobutanol was examined (Fig. 3.4). In Figure 3.4a the results obtained from a batch culture in which the cells had been pregrown on isobutanol are given whereas in Figure 3.4b a batch culture inoculated with cells pregrown on acetate is shown. The influence of growth conditions used for cultivation of the inoculum is obvious. In the first batch (Fig. 3.4a) the cells had been accustomed to growth on isobutanol and isobutyrate was already present in the inoculum (not shown). In this case no lag phase was observed before the cells started to grow. When cells were pregrown on acetate (Fig. 3.4b) - a lag phase of almost 4h was observed before growth started. The different precultivation not only had an effect on the lag phase but also on the exponential growth phase and on the maximum specific growth rate. In Figure 3.4b growth clearly stopped at an OD$_{546\text{nm}}$ of 0.3 and was resumed only after 1h at a much lower growth rate. In contrast to this, with cells adapted to isobutanol the maximum specific growth rate was constant during the whole exponential growth phase (Fig. 3.4a). In both batch cultures isobutyrate appeared in the medium (Fig. 3.4a, b). In the batch inoculated with iso-butanol grown cells the highest concentration of acid was found after 3.25h (38mg L$^{-1}$ isobutyrate-C). However, by that time only about 25% of the end-biomass had been built. Thus, growth during the remaining time of the batch was not with isobutyrate only but the cells either grew with isobutanol and isobutyrate simultaneously or even with further excreted metabolites which were not detected in this experiment.
Figure 3.4:
Batch growth of *Acinetobacter johnsonii* 210A on isobutanol. The initial substrate concentration was 500mg L\(^{-1}\) isobutanol-C; a) cells pregrown with isobutanol in continuous culture at \(\mu=0.1\) h\(^{-1}\); b) cells pregrown in batch culture with acetate; c) calculation of maximum specific growth rates \(\mu_{\text{max}}\).
3.2.3 Growth with mixtures of substrates

*Acinetobacter johnsonii* 210A was grown in batch culture with different mixtures of compounds, supplied as carbon and energy substrates. As the primary substrate acetate was used to which ethanol, butyrate, or butanol were added, respectively. In all experiments the cells used for inoculation had been grown continuously on acetate at a specific growth rate of $\mu = 0.3 \, \text{h}^{-1}$. Biomass estimates were made with the measurement from the batch culture growing with butanol as described in the last section. Biomass yields are given in grams of dry biomass per gram of substrate-carbon.

**Acetate/Butanol**

Growth of *Acinetobacter johnsonii* 210A in batch culture supplied with a mixture of acetate (270mg L$^{-1}$ C) and butanol (200mg L$^{-1}$ C) resulted in sequential utilisation of the two carbon sources (Fig. 3.5).

![Figure 3.5:](Image)

**Figure 3.5:**
Batch growth of *Acinetobacter johnsonii* 210A with a mixture of acetate and butanol in mineral medium at 30°C. The initial concentrations of acetate and butanol were 270mg L$^{-1}$ C and 200mg L$^{-1}$ C, respectively. The inoculum was pregrown in continuous culture on acetate at $D = 0.3 \, \text{h}^{-1}$. 
Acetate was consumed without lag phase and it was used to completion within 4.75h. During this time the concentration of butanol stayed constant. Without apparent lag phase the cells were able to switch from growth with acetate to growth with butanol. When growing with butanol they excreted a part of the butanol as butyrate. 6 hours after inoculation most of the butanol was used up and the concentration of butyrate in the culture medium reached its peak. On acetate the cells grew at a rate of 0.53h$^{-1}$; growth on butanol/butyrate, which occurred at least partly simultaneously, was significantly slower with a $\mu$ of 0.14h$^{-1}$. The biomass yield on acetate was 1.36g g$^{-1}$, the yield on butanol was 0.85g g$^{-1}$. The overall yield of the experiment was 1.1 g g$^{-1}$.

**Acetate/Ethanol**

Batch growth of *Acinetobacter johnsonii* 210A on a mixture of acetate (260mg L$^{-1}$ C) and ethanol (350mg L$^{-1}$ C) showed that the cells used these substrates sequentially (Fig. 3.6).

On acetate a maximum specific growth rate of 0.64h$^{-1}$ was observed. The elemental growth yield for carbon during growth on acetate was 0.89g g$^{-1}$. After a short lag phase the cells started to grow on ethanol on which they reached a maximum specific growth rate of 0.43h$^{-1}$.
Figure 3.6:
Batch growth of *Acinetobacter johnsonii* 210A with a mixture of acetate (260mg L⁻¹ C) and ethanol (350mg L⁻¹ C) in mineral medium at 30°C. The cells were pregrown in continuous culture with acetate at D=0.3h⁻¹.

The biomass yield on ethanol was 1.05g g⁻¹. From the ethanol concentration profile during the initial growth phase with acetate, it was not clear whether the cells already started to utilise ethanol while growing on acetate. In the late exponential phase a little bit of acetate was excreted into the growth medium.

**Acetate/Butyrate**

When *Acinetobacter johnsonii* 210A was grown with a mixture of acetate (240mg L⁻¹ C) and butyrate (215mg L⁻¹ C) the cells used the two substrates sequentially (Fig. 3.7). Within 3.7h 90% of the acetate was utilised and the culture reached an OD₅₄₆ nm of 1.6. The maximum specific growth rate during growth on acetate was 0.57h⁻¹. After depletion of acetate from the medium the cells started to utilise butyrate without apparent lag. The biomass showed a short lag of 0.5h after which exponential growth continued at a lower growth rate (0.16h⁻¹).
Figure 3.7:
Batch growth of *Acinetobacter johnsonii* 210A with a mixture of acetate (240 mg L\(^{-1}\) C) and butyrate (215 mg L\(^{-1}\) C) in mineral medium. The inoculum was derived from a continuous culture growing on acetate at \(\mu = 0.1\) h\(^{-1}\).

Also in this batch, most biomass was produced from acetate while the consumption of butyrate only resulted in a very low increase in biomass concentration. The growth yield on acetate was 1.2 g g\(^{-1}\), the yield on butyrate 0.57 g g\(^{-1}\).

### 3.2.4 Glucose as an additional energy source for *Acinetobacter johnsonii* 210A

To test whether or not *Acinetobacter johnsonii* 210A was able to utilise additional energy for biomass synthesis, the organism was grown with a mixture of acetate and glucose/PQQ. The experiment started off from an acetate-limited continuous culture (1.5 g L\(^{-1}\) sodium acetate) run at D=0.242 h\(^{-1}\) or D=0.375 h\(^{-1}\). Then the medium was supplemented with 3.6 g L\(^{-1}\) or 2.2 g L\(^{-1}\) of D-glucose and the culture was run until another steady-state was reached. After reaching steady-state, 0.6 mg L\(^{-1}\) of PQQ was added to the growth medium. The oxidation of glucose was followed by measuring its disappearance and the production of gluconate as is shown in Figure 3.8 for D=0.375 h\(^{-1}\). Acetate was measured both
at the start and the end of the experiment to verify carbon-limited growth. Biomass formation was followed by measuring the optical density at 546nm. Similar data were obtained at $D=0.242 \text{h}^{-1}$ (not shown).

**Figure 3.8:**
Oxidation of glucose by an acetate-limited continuous culture of *Acinetobacter johnsonii* 210A growing at $\mu=0.375 \text{h}^{-1}$. After the start-up of the experiment, 2 steady-states were measured, the first one with acetate as only carbon source ①, in order to ensure that the culture was growing carbon-limited. The second one with acetate and glucose to verify that no growth occurred on glucose ②. At $t=0\text{h}$ the experiment was started by adding PQQ in excess.
The addition of glucose to an acetate-limited continuous culture of *Acinetobacter johnsonii* 210A had no significant effect on the concentration of biomass in the reactor (Fig. 3.8). As expected, *Acinetobacter johnsonii* 210A did not grow on glucose. Immediately upon addition of PQQ the cells started to oxidise glucose and gluconate appeared in the fermenter (Fig. 3.8). 1.4g L\(^{-1}\) glucose was oxidised to gluconate and appeared as gluconate, no other products were excreted by the cells. This was verified by measuring the dissolved organic carbon concentration which added up perfectly to that of glucose and gluconate.

The elemental growth yield for carbon was identical at 1.02g g\(^{-1}\) in the medium with acetate only and that supplemented with glucose. At D=0.242h\(^{-1}\) an increase of biomass concentration was observed, however this increase was within the range of analytical inaccuracies of the dry weight determination (Tab. 3.3). The most important parameters of the experiments at D=0.242h\(^{-1}\) and D=0.375h\(^{-1}\) are summarised in Table 3.3.

<table>
<thead>
<tr>
<th>glucose [mM]</th>
<th>glucose oxidised [mM]</th>
<th>acetate:glucose ratio(^1) [Mol/Mol]</th>
<th>D [h(^{-1})]</th>
<th>(Y_{X/Ac})(^2)</th>
<th>(Y_{X/AcGlu})(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.2</td>
<td>6.6</td>
<td>2.8</td>
<td>0.375</td>
<td>1.02</td>
<td>1.02</td>
</tr>
<tr>
<td>20</td>
<td>14.4</td>
<td>1.3</td>
<td>0.242</td>
<td>0.88</td>
<td>1.02</td>
</tr>
</tbody>
</table>

**Table 3.3:**
Growth of *Acinetobacter johnsonii* 210A with a mixture of acetate and glucose in continuous culture at 30\(^{\circ}\)C. In both experiments acetate was supplied at a concentration of 18.5mM. Glucose was never oxidised completely but a residual concentration of 1g L\(^{-1}\) glucose remained in the culture. All measurements were taken during steady-state. \(^1\)the effective ratio used by the bacteria; \(^2\)yields are given in g cell dry weight per gram of acetate-C; \(Y_{X/Ac}\): biomass yield on acetate; \(Y_{X/AcGlu}\): biomass yield on the mixture of acetate and glucose.
3.3 Discussion

3.3.1 Medium analysis

In this work growth of *Acinetobacter johnsonii* 210A with single and mixed substrates was examined in batch and continuous culture. Since it is known that the composition of the medium can affect both the specific rate of growth as well as the growth yield with respect to the limiting substrate the mineral medium used by van Groenestijn and co-workers to cultivate *Acinetobacter johnsonii* 210A was analysed (Pirt, 1975; van Groenestijn *et al.*, 1987; van Groenestijn *et al.*, 1989; Bonting *et al.*, 1992a). Analysis of the media with help of excess factors revealed shortages of the trace elements copper, zinc, and manganese which could possibly lead to undesired limitations. Therefore, a new mineral medium was constructed and used in batch and continuous cultivation. Compared to former results, this mineral medium supported a significantly higher maximum specific growth rate on acetate (0.81 h⁻¹, compared to 0.69 h⁻¹) as well as an equally high biomass yield (1.05 g g⁻¹). It is unlikely that the temperature difference between the two experiments was the reason for the different maximum growth rates because van Groenestijn and co-workers showed that the maximum specific growth rate was nearly identical at 25°C and 30°C (van Groenestijn *et al.*, 1989). Growth yields obtained in the new medium were in the same range as those formerly reported although trace element limitation is known to lead to diminished growth yields at high dilution rates. It must, therefore, be concluded that trace element limitation was unlikely to have occurred in the range of growth rates tested by van Groenestijn and co-workers.

3.3.2 Growth on single substrates

The maximum growth rate was determined with different substrates. Clearly, acetate was the carbon source which led to the highest specific growth rate at pH 7.0 (0.81 h⁻¹). Other substrates showed maximum specific growth rates between 0.25 h⁻¹ and 0.51 h⁻¹. Growth on butanol and isobutanol was characterised by the excretion of metabolites and a sequential growth pattern.
In the case of butanol part of the primary substrate was excreted as butyrate during a first growth phase. The extensive formation of products from short chain alcohols is a well known phenomenon and has been reported from different types of bacteria: acetic acid bacteria, Gram-positive bacilli, as well as mixed bacterial cultures (Al-Awadhi et al., 1988; Gottschalk, 1988). In our experiments *Acinetobacter johnsonii* 210A never excreted large amounts of acetic acid. This is consistent with earlier findings where the excretion of C₄-carboxylic acids was observed: during growth on malate *Acinetobacter calcoaceticus* excretes oxalacetate which is also metabolised to yield biomass (Dolin & Juni, 1978). Batch growth with DL-malate also led to two growth phases in *Acinetobacter johnsonii* 210A. However, in our case the growth rate was much lower in the second growth phase which is in contrast to the results by Dolin & Juni who observed about the same growth rates on malate and on oxalacetate. It is also known from an earlier study by Bonting that *Acinetobacter johnsonii* 210A grows on D-malate (Bonting, 1993), in the presented work a racemic mixture was used. Maybe some of the L-malate supported growth after exhaustion of D-malate at a much lower growth rate.

### 3.3.3 Growth on mixed substrates

In wastewater treatment systems a whole range of organic compounds is present which can be used as growth substrates by bacteria. In EBPR plants mainly short chain organic acids are produced in the first (anaerobic) phase and serve as substrates for the organisms present. Although *Acinetobacter johnsonii* 210A is often considered to be a model organism for part of EBPR it is not known to date how the organism copes with mixtures of substrates. Batch cultures of *Acinetobacter johnsonii* 210A with mixtures of acetate and butyrate, acetate and ethanol, and acetate and butanol, respectively, always showed a sequential utilisation pattern with acetate being used preferentially. This is in accordance with the literature where it is stated that diauxic growth is a common phenomenon for mixed carbon substrate utilisation in batch culture (at high concentrations). An indication for the preferential utilisation of acetate was also
observed by van Groenestijn & Deinema (van Groenestijn & Deinema, 1985). When *Acinetobacter johnsonii* 210A grew on valerate, propionate was excreted pointing to a preferential consumption of acetate generated through β-oxidation of valerate (van Groenestijn & Deinema, 1985).

### 3.3.4 Influence of preculture

Growth behaviour of *Acinetobacter johnsonii* 210A (i.e. initial growth rate and lag-phase) in batch culture with isobutanol varied depending on the cultivation condition of the inoculum. The influence of the preculture on the initial growth rate was surprising: a higher rate was observed in the batch culture with the acetate grown inoculum. A lag-phase was observed in batch cultures with isobutanol when the inoculum had grown on acetate while cells pregrown on isobutanol started growing without delay. In both batch cultures isobutyrate appeared in the culture medium, although the concentration was higher in the batch inoculated with cells grown on acetate (pregrown in batch). This indicates an imbalance between the initial alcohol dehydrogenase and the subsequent catabolic enzymes in acetate grown cells (also observed in batch cultures with mixed substrates). As expected, in cells pregrown on isobutanol, the excreted amount of isobutyrate was smaller.

In the batch cultures with mixed substrates only acetate pregrown inocula were used. Acetate was always utilised without lag. This is in accordance with observations from batch cultures of *Methyllobacterium* strain DM4 growing with mixtures of acetate and DCM (Tien, 1996). When the inoculum was pregrown on DCM a simultaneous utilisation of the two carbon sources occurred. These findings could easily be explained by the enzyme induction pattern: the DCM specific pathway was induced by the substrate (Tien, 1996). With an acetate grown inoculum a diauxic growth pattern was observed. This behaviour was explained as an inhibition by the carboxylic acid as frequently observed in pseudomonads (Harder & Dijkhuizen, 1976; Tien, 1996).
3.3.5 Maximum specific growth rates and growth yields in batch cultures with mixed substrates

Acinetobacter johnsonii 210A excreted large amounts of metabolites during growth on C₄-compounds. Therefore, batch cultures on butanol, isobutanol and malate are discussed in this section under “mixed substrates” (for growth on acetate and ethanol refer to sections 3.3.1 and 3.3.2). Generally, during mixed substrate utilisation the maximum specific growth rates were lower than during growth on single substrates. The growth rates found for acetate were 21% to 35% lower in mixtures of acetate and butanol (35%), acetate and ethanol (21%) and acetate and butyrate (30%). The effect was less pronounced in the case of ethanol (16%). Relatively slow growth on acetate might be explained by the fact, that although the inoculum had grown on acetate (D=0.1h⁻¹ or D=0.3h⁻¹), the cells were not optimally trained for fast growth. This assumption is strengthened by the result of another experiment from section 5, where cells had been taken from a continuous culture growing at 0.1h⁻¹ and 0.3h⁻¹ on acetate and transferred into batch medium; the maximum specific growth rates that were achieved were only 0.62h⁻¹ and 0.68h⁻¹.

Surprisingly, a higher growth rate on butyrate was observed in the second growth phase of a batch with butanol (0.36h⁻¹) than with butyrate as single substrate (0.25h⁻¹). There are two possible explanations for this finding but further experiments are needed to verify these assumptions. Firstly, it is possible that part of the energy, which had been generated during the oxidation of butanol to butyrate, might have been stored in the form of polyP and made use by the organism during growth on butyrate. Secondly, it should be considered that the inoculum used to determine the specific μₘₐₓ with butyrate as single substrate in batch culture had been pregrown on acetate. This fact could also have led to the reduced maximum specific growth rate.

A comparison of biomass yields shows that the values obtained for acetate as well as the other substrates are well in line with earlier studies (Tab. 3.4).
It is known that Acinetobacter johnsonii 210A contains a functioning membrane-bound glucose dehydrogenase (Bonting et al., 1993b; van Veen et al., 1993a; van Niel et al., 1999). The energy that is generated by the oxidation of glucose to gluconate can be used for phosphate uptake and/or for the formation of polyphosphate and biomass (van Veen et al., 1993a; van Niel et al., 1999). In a recent study Acinetobacter johnsonii 210A was grown in carbon-limited continuous culture at a dilution rate of 0.1h\(^{-1}\) with 10mM of acetate as substrate in the presence of 100mM glucose and 100nM of PQQ (van Niel et al., 1999). A maximum of 55mM of glucose was oxidised to gluconate: of the energy generated 83% went into biomass formation and 17% into polyphosphate formation. From these results the authors calculated that 15.1mM of glucose oxidised replaced 1mM of acetate dissimilated. This

Table 3.4:
Growth yields (\(Y_{Xc}\)) of *Acinetobacter johnsonii* 210A with different carbon substrates in batch and continuous culture. The experiments were performed at pH 7.0 unless otherwise stated.
confirms the results obtained in this work where no increase in biomass was observed when *Acinetobacter johnsonii* 210A was grown a dilution rate of 0.242h\(^{-1}\) with acetate in the presence of glucose and PQQ (0.77mMol glucose oxidised per mMol acetate utilised). The energy that *Acinetobacter johnsonii* 210A gains from the oxidation of glucose is very low compared to other *Acinetobacter* strains where higher energy yields from the oxidation of aldoses have been found (Müller & Babel, 1986; van Schie *et al.*, 1987). It remains an open question where the reducing equivalents from the oxidation of glucose are “lost” for the organism. Even more importantly, it has become obvious that the generation of energy by the oxidation of glucose is such an inefficient process that it is very unlikely to have a relevance for the organism at all.

3.3.6 Concluding remarks

Growth of *Acinetobacter johnsonii* 210A with short chain alcohols in batch culture showed complex utilisation patterns which still are not fully understood. During growth the corresponding carboxylic acids were excreted into the medium and utilised again. This behaviour is known from other bacteria. During batch growth on mixed carbon substrates acetate was always used preferentially. It would be interesting to feed the same mixtures of substrates in continuous culture to analyse the substrate utilisation of *Acinetobacter johnsonii* 210A under environmentally more relevant conditions.

\(\text{These observations are also supported from a theoretical point of view where one would expect 1Mol of acetate to yield 4Mol of ATP available for biomass production while the oxidation of 1Mol of glucose to gluconate yields 2Mol of ATP (assuming a P/O ratio of 2).}\)
SECTION 4

RESPONSE OF

ACINETOBACTER JOHNSONII 210A TO
CHANGING NUTRIENT AVAILABILITY IN
FED-BATCH AND CONTINUOUS CULTURE
Abstract

The response of *Acinetobacter johnsonii* 210A to changing concentrations of carbon, phosphorus and oxygen in the growth medium was examined in fed-batch and continuous culture. In shift experiments performed in continuous culture two different response patterns were observed when a carbon-limited continuous culture of *Acinetobacter johnsonii* 210A was supplied with phosphorus-free medium. When the initial carbon-limited steady-state cells contained polyP, the culture was transiently growing dual-(C/P)-limited as soon as phosphorus was depleted from the medium. The cells were able to utilise the stored phosphorus, however, a preferential degradation of polyP was not observed, its relative content was always 35±5wt% of total cellular phosphorus. No accumulation of PHB was found. A different response occurred when the initial carbon-limited steady steady-state culture contained less than 1wt% phosphorus. In this case *Acinetobacter johnsonii* 210A grew phosphorus-limited at a reduced specific growth rate and accumulated PHB up to 9.9% of cell dry weight. During this growth regime the INT-reducing capacity of the cells was 4-times lower than in the initial carbon-limited steady-state pointing to a reduced cell fitness. In transients from carbon to oxygen limitation in continuous culture it was clearly shown that growth stopped completely as soon as the culture grew oxygen-limited and the carbon source acetate accumulated but no PHB was formed. When oxygen was supplied again the cells resumed growth without lag. No influence of temperature on the response pattern was observed between 20°C and 30°C.

Keywords: *Acinetobacter johnsonii*, polyphosphate, poly-(R)-3-hydroxybutyrate, nutrient limitation, chemostat, unlimited growth, INT activity
4.1 Introduction

Microbial life in wastewater treatment plants is dominated by highly dynamic growth conditions. This is especially true for the feed regime of nutrients. Under such conditions micro-organisms can balance their growth by accumulating storage polymers while substrate is present and using them for growth when these nutrients are depleted (van Loosdrecht et al., 1997a). The cycling between accumulation and utilisation of storage polymers is likely to occur in many wastewater treatment systems, however, only in a few processes has their exact role been investigated (van Loosdrecht et al., 1997a). The most prominent example is that of enhanced biological phosphorus removal (EBPR). In this process the accumulation and utilisation of poly-(R)-3-hydroxybutyrate (PHB), glycogen, and polyP is driven by the cycling between anaerobic and aerobic growth conditions (Smolders et al., 1995). Typical cycling times in this process are in the range of a few hours and bacteria are far from reaching constant growth conditions. Moreover, it has been pointed out by van Loosdrecht and co-workers, that studies on polymer accumulation in pure cultures have often been conducted under conditions where a particular nutrient is limiting growth (van Loosdrecht et al., 1997a). In activated sludge, however, nutritional conditions are complex. Based on literature data and their own experiments with pure cultures of *Thiosphaera pantotropha* van Loosdrecht and co-workers have postulated that polymer formation is also occurring under non-growth-limiting conditions when the organisms are exposed to changing substrate fluxes (van Loosdrecht et al., 1997a). When they added a pulse of acetate to a acetate-limited continuous culture of *Thiosphaera pantotropha*, the bacteria took up the substrate at a high rate but did not increase their growth rate accordingly. The surplus of acetate was converted to PHB. When the external substrate was depleted the organism started to degrade the PHB (van Loosdrecht et al., 1997a).

Strictly aerobic *Acinetobacter* strains have been proposed to play a major role in EBPR and polymer accumulation is proposed to be a driving force in this
process (van Loosdrecht et al., 1997a). Therefore, many studies of polymer accumulation in *Acinetobacter* sp. have been conducted (for an overview see Fixter & Sherwani, 1991). Most of these studies have focused on polymer accumulation under nutrient-limited conditions, particularly for the accumulation of PHB (Vierkant et al., 1990; Rees et al., 1993; Woods et al., 1993; Weltin, 1996). So far, in *Acinetobacter johnsonii* 210A accumulation of PHB has always been associated with phosphorus limitation (van Groenestijn et al., 1989; Bonting et al., 1992a). High polyP contents on the contrary have been found during carbon-limited growth in continuous culture as well as during unrestricted batch growth, i.e., the exponential growth phase (Deinema et al., 1980; Beacham et al., 1992; Bonting et al., 1992a; Tandoi et al., 1998). This is a common feature in many *Acinetobacter* sp. which have been reported to be capable of “luxury uptake” of phosphorus (this term is used to describe the uptake of phosphorus in excess of that needed for growth). Under such conditions the phosphorus taken up by the cells is partly used for the synthesis of cellular constituents such as RNA, DNA or phospholipids and the excess is stored as polyP. In *Acinetobacter johnsonii* 210A this “luxury uptake” in batch culture led to a cellular phosphorus content of up to 5.8wt% P (van Groenestijn et al., 1989).

In this work the response of *Acinetobacter johnsonii* 210A to changing nutrient availability was investigated in continuous and fed-batch culture. The nutrients to which particular attention was given were carbon, phosphorus and oxygen, the latter being of great importance in the EBPR process, although it can not be considered a “nutrient” in a strict sense.

### 4.2 Results

In a first set of experiments, the cells were cultivated in a chemostat under carbon limitation until steady-state was reached, then shifted to phosphorus or oxygen limitation and back to carbon limitation again. In the case of carbon to phosphorus limitation the shifts were accomplished by supplying a continuous
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culture with growth media of different $C_0/P_0$ ratios. The response of *Acinetobacter johnsonii* 210A was examined in dependence of the nutritional state of the start-up culture. Parameters of interest were on one hand the residual nutrients carbon, oxygen, and phosphorus, the biomass formed, and on the other hand the cellular components PHB, polyP, and the cellular P content. Additionally, the INT reduction capacity of the cells was measured to assess the viability of the culture. In the carbon and oxygen limitation experiments the cells were supplied with carbon-limited minimal medium until steady-state was reached and then oxygen limitation was superimposed on the culture by switching the air supply to nitrogen gas. In these experiments the parameters measured were residual carbon and oxygen, as well as biomass and PHB. In a second set of experiments, batch cultures were used to examine the maximum accumulation of PHB under nongrowing conditions. These batch cultures were phosphorus-terminated. Thus, at the end of the exponential growth phase, all other nutrients, including acetate as the sole source of carbon and energy, were still present in excess. To these phosphorus-terminated batch cultures acetate was added in regular intervals (fed-batch) until biomass increase stopped. The disappearance of orthoP, the formation of biomass, and the accumulation of PHB was followed.

4.2.1 Transient from carbon to phosphorus limitation

Transient experiments from carbon limitation to phosphorus limitation and back to carbon limitation were performed at a growth rate of 0.4h^{-1}. From the several shifts made, two representative examples are presented here (Fig. 4.1 & 4.2). After starting up the continuous culture two different carbon-limited steady-states were observed with exactly the same growth medium. In both steady-states *Acinetobacter johnsonii* 210A was truly carbon-limited with a residual acetate concentration below 1mg L^{-1} C and the biomass concentration approximately 1g L^{-1} CDW. However, the steady-states differed with respect to the residual phosphorus concentration: the first type was characterised by a residual phosphorus concentration of 42.1mg L^{-1} P (Fig. 4.1). In the second
steady-state obtained, (Fig. 4.2) over 90% of the added phosphorus (95.5mg L\(^{-1}\) P) remained in the culture supernatant. These steady-states were reproducible and served as the starting points for two different types of transients. *Acinetobacter johnsonii* 210A was grown in carbon-limited, high phosphate mineral medium (100mg L\(^{-1}\) phosphorus, \(C_o=1000mg\ L^{-1}\) acetate-C) until steady-state was reached with respect to biomass and nutrient concentrations as well as the cellular components polyP and PHB. The transient was started by exchanging the medium supply for a phosphate-free medium (time zero), containing at the most 0.25mg L\(^{-1}\) P, while leaving all other medium components unchanged. After this shift, the residual phosphorus in the fermenter was used up and the biomass started to decrease. High phosphate medium was again supplied either when the optical density had fallen below \(OD_{546nm}=1\) (Fig. 4.2) or after 3.55h (Fig. 4.1). Finally, the carbon-limited steady-state was analyzed after 6.5 volume changes. During the whole experiment the concentration of acetate, orthoP-P, and the optical density at 546nm were measured at regular intervals. Biomass samples were taken less frequently because of the limited amount of total sample volume available and whenever they were taken only single measurements were made. For the same reason, the phosphorus content of the biomass was not determined directly but only estimated from the total phosphorus utilised. The cellular polymers PHB and polyP as well as the cells' capability to reduce the electron acceptor INT were determined at regular intervals.

The first transient was characterised by a wide dual-(C/P)-nutrient-limited growth zone which occurred after single carbon limitation. In the second type of transient however, no dual-(C/P)-nutrient-limited growth was observed and a sudden transition between carbon and phosphorus limitation occurred. In addition to these two types of transients, a third type was observed of which the response pattern was in between these two extremes. In the third transient the carbon-limited steady-state at the beginning of the experiment was characterised by a residual orthoP-P concentration of 63.5mg L\(^{-1}\) P (not shown).
Only one measurement indicated dual-nutrient-limited growth before the culture became clearly phosphorus-limited (not shown).

The pattern of the first type of transient is shown in Figure 4.1. At time zero when the phosphate-free medium was supplied to the fermenter, the initial phosphorus concentration in the culture supernatant (42.1 mg L\(^{-1}\) P) started to decrease straight away and fell below the detection limit (<0.07 mg L\(^{-1}\) P) within 5h. The residual DOC concentration never exceeded 40 mg L\(^{-1}\) during the whole experiment indicating that the culture was always carbon-limited (Fig. 4.1a). This was further confirmed by measuring the residual acetate concentration at 4 different times of the transient, namely at time 0, after 2, 3, and 25.3 hours, and it was always below 1.3 mg C L\(^{-1}\) (data not shown). After 5h phosphorus as well as carbon had been used up to completion and were not detectable anymore in the culture supernatant. The border between single carbon-limitation and dual-(C/P)-nutrient-limitation was at a carbon to phosphorus ratio of 98 g g\(^{-1}\). This ratio was calculated by dividing the inflowing acetate concentration, 1000 mg L\(^{-1}\) C, by the total amount of phosphorus present in the fermenter at this point in time (10.2 mg L\(^{-1}\) P). The zone of dual nutrient limitation was followed to a C\(_0\)/P\(_0\) ratio of 333 g g\(^{-1}\) (8.5h). At this point high phosphate medium was again supplied. During the whole experiment a steady decrease in OD\(_{546nm}\) was observed (Fig. 4.1a). The biomass was decreasing accordingly but showed some variations in the measurements (Fig. 4.1b). After the shift back to the C-limited medium the resulting carbon-limited steady-state was characterised by a significantly lower biomass but a similar residual phosphorus concentration compared to the starting conditions of the experiment. An effective growth rate of 0.36 h\(^{-1}\) was calculated when considering the decrease of the biomass in the fermenter to be a result of growth of the culture (\(\mu\)) and wash-out due to dilution (0.4 h\(^{-1}\)).
Figure 4.1:
Transient growth of *Ac. johnsonii* 210A in continuous culture at \(D=0.4\text{h}^{-1}\). Shift from C-limited steady-state to P limitation and back to C limitation. The C-limited culture was characterised by a high P-content. a) The residual acetate- and P-concentrations indicate dual-(C/P)-limited growth between the time of 4.95h and 8.5h after initiating the shift; b) observed disappearance of orthoP-P and calculated wash-out at \(D=0.4\text{h}^{-1}\); c) total cellular phosphorus content, cellular polyP content and PHB content of cells; d) cellular phosphorus in polyP fraction error bars represent \(\pm 5\%\).
Figure 4.1b shows the calculated wash-out curve for phosphorus (i.e. considering no cellular uptake of phosphorus occurred). The comparison between data and the theoretical wash-out indicated that during the first 3 hours of the experiment the phosphorus concentration in the culture supernatant was slightly higher than expected. Thereafter this changed and the measured values were lower than the ones calculated for wash-out. The high phosphorus concentration during the first 3 hours can only be explained by taking into account an excretion of phosphorus by the bacteria. Such a source could be the cellular polyP or RNA, which would suggest that the cells were not only growing on the intracellularly stored phosphorus but, on top of that, were releasing some of it into the medium.

The cellular composition of the bacteria during the experiment is depicted in Figure 4.1c. At the start, i.e. during carbon-limited steady-state, the cells had a total phosphorus content of 4wt%. Upon exchanging the medium the cellular phosphorus content started to decrease and reached its lowest value of 0.23wt% at 8 hours. The second carbon-limited steady-state at the end of the experiment was characterised by a high cellular phosphorus content of 5.5wt%. The PHB content of the cells remained low during the whole shift, did not change much and never exceeded 1wt% (Fig. 4.1c). PolyP made up at least 35% of the total cellular phosphorus during the whole experiment (Fig. 4.1d). At one point during dual-(C/P)-nutrient-limited growth it even accounted for as much as 58% of the total cellular phosphorus (Fig. 4.1d). At the end of the experiment, in the second carbon-limited steady-state, polyP had decreased to less than 30% of the total cellular phosphorus.

The typical pattern of the second type of transients is depicted in Figure 4.2. At time zero the feed was changed to a phosphate-free medium. Immediately after exchanging the medium the residual phosphorus in the culture supernatant started to decrease and became undetectable after 5h (Fig. 4.2a).
Figure 4.2:
Transient growth of *Acinetobacter johnsonii* 210A in continuous culture at $D=0.4\text{h}^{-1}$. Shift from C-limited steady-state to P limitation and back to C limitation. The C-limited culture was characterised by a low cellular phosphorus content. a) residual acetate and phosphorus concentrations indicate that no dual-(C/P)-limited growth occurred; b) phosphorus and PHB content of cells, comparison between observed disappearance of PHB and washout at $D=0.4\text{h}^{-1}$; c) INT activity.
In all fed-batch experiments Tris-buffered minimal medium with acetate as sole carbon and energy source was used. The inoculum had been precultured in the same medium supplied with 15mg L\(^{-1}\) P and had been transferred into fresh identical medium twice in order to train the cells for growth in the synthetic buffer. The growth behaviour in this Tris-buffered mineral medium turned out to differ from that in phosphate-buffered medium in two respects: the maximum growth rate at 30°C was only 0.25h\(^{-1}\) and the elemental growth yield for acetate-C was low at 0.5g g\(^{-1}\) (data from diploma thesis of Grab, 1996). The fed-batch cultures were started with initial phosphorus concentrations between 5.19 and 19.78mg L\(^{-1}\) P (Tab. 4.1). After 6h, 1 day, 2 days, and 3 days, the culture was amended with acetate at a concentration of 1g L\(^{-1}\) C (Fig. 4.3). To ensure that no nitrogen limitation would occur additional nitrogen was supplied to the culture on the first day to a concentration of 260mg L\(^{-1}\) N.

In contrast to the experiments described in the first part of this chapter, the cells were not always growing exponentially in these fed-batch cultures. Unrestricted exponential growth was only possible before the depletion of phosphorus in the medium. Thereafter, a slower increase of OD was observed.

The course of one fed-batch experiment is depicted in Figure 4.3a. Within one day after start-up the cells grew to an OD\(_{546\text{nm}}\) of 3 (1100mg L\(^{-1}\) CDW) using up all the phosphorus in the medium. After this point the optical density still increased and reached its maximum after 2 days. Due to the limited total sample volume no biomass sample was taken at this point (but since the OD\(_{546\text{nm}}\) was always closely related to the biomass concentration an increase in biomass concentration can be assumed). On the third day the biomass and OD\(_{546\text{nm}}\) had already started to decrease.
As soon as phosphorus was not detectable anymore the biomass in the fermenter washed out rapidly and acetate was appearing in the culture supernatant (Fig. 4.2a). The effective growth rate between 5h and 12.25h, which was calculated as the difference between the dilution rate (0.4h⁻¹) and the wash-out rate (0.24h⁻¹), was constant at 0.16h⁻¹. After 12.25h when the optical density of the culture had fallen below OD₅₄₆nm=1 high phosphate medium was again supplied. Immediately upon addition, phosphorus appeared in the culture supernatant and exponential growth was observed. The $\mu_{\text{max}}$ of this exponential growth was 0.68h⁻¹ (wash-out considered). After 10.1h of batch growth acetate was used to completion. The biomass of the culture reached a higher value than under steady-state conditions due to growth on the previously accumulated acetate as well as on the acetate in the feed. During phosphorus limitation PHB was formed in the cells, the maximum content of 9.9wt% was reached after 4h of phosphorus-limited growth (Fig. 4.2b). Then the PHB content of the cells decreased again during unlimited batch growth. Comparison of the rate of decrease with the wash-out curve for PHB reveals that the PHB content of the culture followed the wash-out curve suggesting that the cells had stopped all PHB formation (Fig. 4.2b).

INT activity was followed throughout the experiment. This test was used to assess the overall viability of the cells during the transient from carbon to phosphorus limitation. A decrease of activity of a factor of 2 to 4 was found under phosphorus limitation pointing to a reduced cell fitness under these growth conditions. These results are consistent with other observations in phosphorus-limited continuous cultures of *Acinetobacter johnsonii* 210A as described in section 5 of this thesis.

### 4.2.2 Fed-batch cultures

Fed-batch experiments were done to determine the maximum capacity for PHB accumulation in *Acinetobacter johnsonii* 210A under distinctly phosphorus-limited growth conditions.
The PHB content showed a slight increase throughout the whole experiment, i.e., before and after the exhaustion of phosphorus. At the end of the fed-batch experiment the maximum content was 6.5±0.91 wt%. Surprisingly, PHB was found already at the start of the experiment. This means that it was present in the preculture which was a carbon terminated batch culture. The cellular carbon and nitrogen contents remained nearly constant during the whole experiment which was reflected in a stable C/N (wt%/wt%) ratio (Fig. 4.3b).
The results of the 4 fed-batch experiments show that the biomass formed as well as the PHB content of the cells finally reached depended on the initial phosphorus concentration (Tab. 4.1). As expected, with higher initial phosphorus concentrations more biomass was produced. However, the phosphorus content in the final biomass varied between 0.42% for the lowest initial orthoP-P concentration and 0.69% for the highest initial concentration. For PHB a negative correlation was observed: in the fed-batch with the highest initial phosphorus concentration almost no PHB was accumulated whereas a low P-concentration led to the accumulation of 9.9wt% PHB.

<table>
<thead>
<tr>
<th>Initial orthoP-P concentration [mg L⁻¹ P]</th>
<th>Final biomass [mg L⁻¹]</th>
<th>PHB content of cells [wt%]</th>
<th>P content of cells [wt%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.19</td>
<td>1240</td>
<td>9.9</td>
<td>0.42</td>
</tr>
<tr>
<td>6.0*</td>
<td>1200*</td>
<td>4.85*</td>
<td>0.50*</td>
</tr>
<tr>
<td>10.64</td>
<td>1880</td>
<td>2.2</td>
<td>0.56</td>
</tr>
<tr>
<td>19.78</td>
<td>2860</td>
<td>0.6</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Table 4.1: Initial orthoP-P concentration, final biomass and cellular PHB and P content in fed-batch cultures of Acinetobacter johnsonii 210A. * data from diploma thesis of Grab, 1996.

4.2.3 Transient from carbon to oxygen limitation

Since cycling between aerobic and anaerobic conditions is known to stimulate EBPR, the response of a carbon-limited continuous culture of Acinetobacter johnsonii 210A to oxygen limitation was investigated. To assess the influence of temperature on the behaviour of Acinetobacter johnsonii 210A the experiment was performed at 20°C and 30°C.

At time zero the air supply to a carbon-limited steady-state culture was turned off and the culture was continuously flushed with nitrogen (Fig. 4.4). The oxygen concentration immediately decreased and became undetectable by the oxygen probe within less than two minutes (Fig. 4.4). As soon as nitrogen
gas was supplied to the fermenter the culture started to wash out and acetate was washed in. Both, the decreasing biomass concentration and the acetate concentration followed the wash-out and wash-in curve, respectively (assuming no growth). Phosphorus was always present in excess (data not shown). After 8.0h of anaerobiosis the aeration was turned on again. Immediately, the cells started to take up the accumulated acetate, indicating that they had not lost viability during the 8h of anaerobiosis.

Figure 4.4:
Response of a carbon-limited continuous culture (D=0.3h⁻¹) of Acinetobacter johnsonii 210A to oxygen limitation at 30°C and 20°C.
The PHB content of the cells did not increase under anaerobic growth conditions. However, in both transients, after anaerobiosis the PHB content of the cells decreased to undetectable amounts as aeration was turned on again (Fig. 4.4).

4.3 Discussion

4.3.1 Polymer accumulation under transient growth conditions

Accumulation of polymeric reserve materials is a common response to changing nutrient availability in the biological world. The ability of microorganisms to store nutrients intracellularly when they are available in excess has been shown to lead to an advantage during starvation periods. Today, a wealth of knowledge exists about the conditions under which these reserve materials are built up and utilised again. However, the dynamics of incorporation and degradation of storage polymers are still poorly understood. This is especially true for EBPR, a highly dynamic process in which polyP, PHB, and glycogen play an imminent role.

The transient experiments in this work were performed with a wastewater treatment plant in mind where changes in growth conditions occur within hours or less. In the case of carbon to phosphorus limitation the wash-out of phosphorus, which was equal to the hydraulic dilution rate (0.4h⁻¹), determined the rate of change. In the case of carbon to oxygen limitation the duration of the transient phase between the two limitations was governed by the oxygen uptake rate of the carbon-limited steady-state culture, resulting in a steep gradient with all oxygen consumed within less than 2 minutes. No comparisons to steady-state results may be made with respect to quantitative aspects of growth (Zinn, 1998).

PHB

In EBPR plants the cycling of the sludge between anaerobic and aerobic stages is central to their functioning and the ability to store PHB anaerobically
is thought to be one of the major characteristics of bacteria involved in this process. According to the current models of EBPR, polyP and glycogen serve as sources for energy and reducing power when PHB is accumulated anaerobically (Mino et al., 1987; Satoh et al., 1992; Kortstee et al., 1994; Maurer et al., 1997). The models also postulate that during the subsequent aerobic phase, which is characterised by a low external concentration of easily degradable carbon sources, PHB is degraded again and serves as an internal carbon source for growth. During this phase polyP is accumulated intracellularly and the glycogen stores are refilled. Clearly, the carbon and phosphorus metabolism are closely coupled.

In this work, *Acinetobacter johnsonii* 210A failed to accumulate PHB when it was shifted from carbon limitation to oxygen limitation in continuous culture. The results confirm what other authors have reported from a number of *Acinetobacter* strains, namely that PHB accumulation is not usually triggered by oxygen limitation (Deinema et al., 1985; Rees et al., 1993; Woods et al., 1993; Tandoi et al., 1998). Only Weltin and co-workers found PHB accumulation in *O₂*-limited batch cultures by *Acinetobacter* strains isolated from activated sludge (Weltin, 1996). However, when they examined the behaviour of the isolates in a more realistic system, i.e. in an alternating aerobic/anaerobic continuous culture, no PHB was found. Moreover, the results from this study are consistent with earlier reports from *Acinetobacter johnsonii* 210A where no PHB was found in anaerobic batch cultures (Deinema et al., 1985). The results also agree with the currently accepted models of EBPR insofar as it has been postulated that PHB is only accumulated anaerobically in the presence of glycogen. However, glycogen has never been detected in *Acinetobacter johnsonii* 210A.

P limitation has been identified as the main trigger for PHB accumulation in *Acinetobacter johnsonii* 210A (van Groenestijn et al., 1989). In this study, PHB accumulation was also followed in phosphorus terminated fed-batch cultures as well as in transient experiments from carbon to phosphorus limitation in
continuous culture. The precultures of the fed-batch experiments gave indications that P limitation may not be necessary to stimulate PHB accumulation: because the cells contained PHB although they had not been phosphorus-limited at any point in time. However, Tris-buffered medium was used to grow the cells and a direct influence of the medium on the accumulation capacity for PHB was not tested. Shifting Acinetobacter johnsonii 210A from a carbon-limited steady-state to phosphorus limitation and back to carbon limitation led to the transient accumulation of PHB. However, PHB accumulation clearly depended on the cellular phosphorus content during initial carbon-limited steady-state. An accumulation of PHB did only occur when the initial steady-state biomass had a low phosphorus content and clear P limitation was observed during the shift. These results confirm those obtained in steady-state experiments, where PHB accumulation was confined to phosphorus-limited growth (see section 5 of thesis). On the contrary, when the initial carbon limited steady-state was characterised by a high cellular phosphorus content the shift to phosphorus free medium did not lead to single-(P)-limited growth. These cells did not show clear phosphorus limitation but were growing dual-(C/P)-limited.

**Polyphosphate**

Bacteria that accumulate polyphosphate may utilise it as internal phosphorus- and/or energy sources under specific growth conditions (Kulaev & Vagabov, 1983; Kornberg, 1995). In Acinetobacter johnsonii 210A polyphosphate has been shown to fulfill two roles: it serves as an internal energy source under aerobic as well as anaerobic conditions (van Groenestijn, 1988; Streichan & Schön, 1991). Further, it was shown that polyP can act as a phosphorus reserve when Acinetobacter johnsonii 210A was grown in phosphorus-free medium (van Groenestijn & Deinema, 1985). Also under transient growth conditions we found indications that cells which were deprived of an external phosphorus source had the capability to use their internal phosphorus as a P source for growth. Thereby, these cells were able to
maintain a growth rate of $0.36h^{-1}$ while the specific growth rate of cells with a lower P content dropped to $0.16h^{-1}$ in an identical experiment. However, the cells did not preferentially use polyP as a phosphorus source. This finding is in line with the results reported by van Groenestijn and co-workers who observed polyP degradation only under conditions when energy generation in the cell was not possible, inhibited or uncoupled (van Groenestijn, 1988).

4.3.2 Transients and physiological states

The different outcomes of these shift experiments can be explained considering the fact that a culture which is subjected to changing growth conditions, develops in different directions when the starting point in itself i.e. its "physiological state" is different (Malek, 1976). According to Malek, any physiological state of micro-organisms is a result of external conditions and at the same time the starting point for potential changes under the influence of new conditions (Malek, 1976). Thus, the outcome of the transient experiments was always dependent on the starting culture as well as the changing conditions. Clearly, the initial carbon-limited steady-state cultures differed greatly with respect to their phosphorus content. As far as we can judge from our results this seems to be an important variable for the subsequent development of the culture.

4.3.3 Viability during transient growth conditions

*Acinetobacter johnsonii* 210A is a strictly aerobic organism. Although it can maintain a relatively high ATP content during short periods of anaerobiosis it cannot increase its biomass (van Groenestijn, 1988). In this work it was shown that an aerobically growing continuous culture did not loose viability during nearly 8 hours of anaerobiosis. The cells immediately started to take up acetate when oxygen was again supplied to the fermenter. Most likely, the cells contained polyP which phosphorylated adenylates, pyridine nucleotides and other organic compounds (Kulaev & Vagabov, 1983).
Usually, phosphorus limitation induces enormous stress in microbial cells. From marine micro-organisms it is known that phosphorus starved cells are highly susceptible to secondary stress factors such as temperature shifts or UV-radiation (Nyström et al., 1992). In our carbon to phosphorus transients the cells showed different resistance to phosphorus limitation stress depending on their initial cellular phosphorus content. Clearly, cells which contained polyphosphate showed a higher specific growth rate when phosphorus became growth-limiting ($\mu=0.36\text{h}^{-1}$) than the cells with a low phosphorus content ($0.16\text{h}^{-1}$). In the latter, the respiratory activity was followed during the experiment by measurement of the INT reducing capacity. A clear decrease of INT activity during phosphorus-limited growth was observed. Nevertheless, the culture was immediately growing at a high growth rate after phosphorus had been supplied again. This led to the conclusion that cell fitness was completely restored after a short time.
SECTION 5

NUTRIENT-LIMITED GROWTH OF
ACINETOBACTER JOHNSONII 210A IN
CONTINUOUS CULTURE,
WITH SPECIAL REFERENCE TO LIMITATION BY
CARBON, NITROGEN, OXYGEN, AND/OR
PHOSPHORUS
Abstract

*Acinetobacter johnsonii* 210A was grown in chemostat culture at dilution rates between 0.07h⁻¹ and 0.585h⁻¹ under carbon, nitrogen, phosphorus and/or oxygen limitation. In individual experiments performed at a constant dilution rate the medium feed ratio of either C₆H₁₀, C₆H₁₂O₆ or C₆H₁₂O₆/P₅ was changed stepwise and after each change the culture was allowed to reach steady-state before samples were taken. The response of the cells to nutrient limitation was studied with respect to the elemental growth yields, the cellular C, N, and P content and the accumulation of the reserve material poly-(R)-3-hydroxybutyrate (PHB). At the dilution rates tested the elemental growth yields varied little between cells grown under C, N, or O₂ limitation. Neither during single- nor dual-nutrient limitation by C, N, and/or O₂ the cells accumulated PHB. As a consequence, the extension of the dual-nutrient-limited growth regimes was rather narrow. In contrast, during P-limited growth the cells accumulated up to 35% of their cell dry weight as PHB and a dual-(C/P)-limited growth zone was observed where the residual concentrations of both, carbon and phosphate in the culture were below the detection limit. The cellular phosphorus content was only 0.54% during P-limited growth and increased to 3.4% during C-limited growth. This change in cellular phosphorus was also reflected in the RNA content which increased from 1% of cell dry weight under severe phosphorus limitation to 10.4% under carbon limitation. Clearly, dual-(C/P)-nutrient-limited growth was a result of the flexibility of *Acinetobacter johnsonii* 210A to change its cellular phosphorus content.

Key words: *Acinetobacter*, chemostat, nutrient limitation, growth yields, carbon, nitrogen, phosphate, poly-β-hydroxybutyrate, RNA content
5.1 Introduction

In wastewater treatment the removal of phosphorus is one of the most important tasks in order to prevent the eutrophication of the aquatic environment. Usually phosphorus is removed by chemical precipitation. However, nowadays also an alternative method, based on a biological process, has become available. This biological process, called “enhanced biological phosphorus removal” (EBPR), was discovered in the late 1960’s and since then has received increasing attention, both from wastewater engineers and microbiologists. However, its potential has not yet been fully explored mainly because of the restricted knowledge about many biological processes underlying EBPR.

Today, the design and operation of EBPR plants still largely relies on the experience from well functioning plants. The cycling of activated sludge between anaerobic and aerobic growth conditions leads to the establishment of a specialised microbial population that drives this process. It has become evident that the polymers glycogen, PHA (poly(R)-3-hydroxyalkanoate), and polyP (poly-phosphate) play a key role. During the anaerobic phase carbon is taken up from the bulk liquid and is stored as PHA. During the subsequent aerobic phase PHA is utilised again for growth and the build-up polyP and glycogen (Kortstee et al., 1994; van Loosdrecht et al., 1997a). Several metabolic models have been put forward which explain the biochemical processes involved in EBPR (for an overview see Wentzel et al., 1991). The biochemical models are mostly based on the physiology of Acinetobacter species because bacteria of this genus have repeatedly been isolated from sewage sludge of EBPR plants (Wentzel et al., 1991; van Loosdrecht et al., 1997b). One of the organisms that has been extensively studied is Acinetobacter johnsonii 210A. This Gram-negative bacterium was isolated from a wastewater treatment plant exhibiting enhanced biological phosphorus removal in 1980 and it is able to intracellularly accumulate polyP and PHB (Deinema et al., 1980; Deinema et al., 1985; van Groenestijn et al., 1989; Bonting et al., 1992a). However, still today
the role of Acinetobacter species in biological phosphorus removal is not clear (van Loosdrecht et al., 1997b). Many attempts have been made to isolate bacteria which are directly responsible for the process but so far none of them was successful. Therefore, Acinetobacter is still considered the best model organism for studying the fundamental biological processes that lead to EBPR.

PHA, glycogen and polyphosphate are typical examples of bacterial storage polymers. These storage polymers may have different functions in the cell. They can either supply the organism with energy, serve as a source of a particular element, or both. Glycogen or PHA can be used as a carbon/energy source, whereas polyP can supply the cell with energy and/or phosphorus (Dawes & Senior, 1973; Kulaev, 1979; Kulaev & Vagabov, 1983; Fixter & Sherwani, 1991; Towner et al., 1991). Generally, the accumulation of storage polymers such as PHA, glycogen or polyP is associated with nutrient availability, i.e. the limitation of one and the excess of another nutrient in the bacterial growth environment. Polymer accumulation has been well investigated in nitrogen-limited batch cultures where excess carbon is channelled into the reserve polymer as soon as the nitrogen source is used up (Wanner & Egli, 1990). However, in the process of EBPR as well as in pure cultures of Acinetobacter johnsonii 210A the dynamics and interactions of the reserve polymers and their dependence on nutrient availability are still only marginally understood (Jenkins & Tandoi, 1991).
It is well established that the best experimental approach to study microbial physiology in the laboratory is the use of continuous cultures with defined mineral media (Pirt, 1975; Tempest & Neijssel, 1976; Veldkamp, 1976; Harder & Dijkhuizen, 1983). In such systems the growth rate is controlled and biomass production is limited by the availability and amount of a single nutrient. This allows to cultivate and investigate the cells under clearly defined reproducible conditions at different rates of growth without changing the composition of the medium. Research has been carried out to elucidate the cellular composition of bacteria dependent on the nature of the growth-limiting nutrient as well as the growth rate (Herbert, 1976; Pirt, 1975). However in natural ecosystems, microorganisms encounter a situation far more complex: substrate and nutrient concentrations are usually very low. Simultaneous utilisation of homologous substrates, i.e. compounds that serve the same physiological function, is the rule rather than the exception (for a review see Egli, 1995). Also in the case of non-homologous nutrients it is probably an erroneous assumption that one specific nutrient governs growth but probably the different nutrients interact in a presently largely unknown fashion (Veldkamp & Jannasch, 1972; Paerl, 1982).

Both, the development of continuous culturing techniques and the growing awareness about the complex nutritional environment of micro-organisms in

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3 In the literature the term “nutrient-limited growth” is generally used to describe two different aspects of microbial growth. Firstly, it is used to describe the control of the growth rate of microorganisms by the limited availability of a certain nutrient. Often Monod-type equations are used to relate the growth rate to a limiting nutrient concentration (Monod, 1942; Kovarova-Kovar & Egli, 1998). The second usage of the expression “nutrient limitation” is a stoichiometric one: here the amount of biomass that can be produced is related to the supply of a bacterial culture with a certain amount of a specific nutrient (Liebig’s law). This relationship is characterised by the yield coefficient ($Y_{XS}$). Cellular growth yields for most nutrients are known (Herbert, 1961; Herbert, 1976; Pirt, 1975; Linton & Stephenson, 1978). In this chapter the term “nutrient-limited growth” is used only in the sense of stoichiometric limitation.
natural ecosystems has led to a number of laboratory studies on stoichiometric limitation of biomass production by combinations of different nutrients (Egli & Quayle, 1984; Egli & Quayle, 1986; Gräzer-Lampart et al., 1986; Pengerud et al., 1987; Minkevich et al., 1988; Duchars & Attwood, 1989; Rutgers et al., 1990). As a result it has become evident that additionally to clearly single-nutrient-limited growth - biomass production can also be limited by more than one nutrient at a time.

For this work *Acinetobacter johnsonii* 210A was chosen as a model organism to study growth in continuous culture under carbon, nitrogen, oxygen, and phosphorus limitation. Combinations of these non-homologous nutrients which are assumed to be important in the process of enhanced biological phosphorus removal were also examined. The main focus of this work was to define the growth conditions under which *Acinetobacter johnsonii* 210A accumulates the reserve polymers PHB and polyP and to examine the response of the organism to limitation by single and combinations of nutrients.

### 5.2 Results

#### 5.2.1 Carbon- and nitrogen-limited growth

*Acinetobacter johnsonii* 210A was cultivated in continuous culture at dilution rates of 0.07h\(^{-1}\), 0.185h\(^{-1}\), 0.35h\(^{-1}\), and 0.585h\(^{-1}\). The C\(_o\)/N\(_o\) ratio of the inflowing medium was varied between 2.5g g\(^{-1}\) and 20g g\(^{-1}\) by stepwise increasing C\(_o\) while leaving N\(_o\) constant at 0.2g L\(^{-1}\) N. At all dilution rates three different growth regimes were observed as a result of changing C\(_o\)/N\(_o\) ratios (Fig. 5.1a, b).

At low C\(_o\)/N\(_o\) ratios growth was carbon-limited and ammonium was present in the culture supernatant. At high C\(_o\)/N\(_o\) ratios acetate was not fully consumed and accumulated in the bioreactor. In between these two clearly single-nutrient-limited growth regimes a small range of C\(_o\)/N\(_o\) ratios was found, where both nitrogen and carbon were utilised to completion. At all the dilution rates tested
the extension of the dual-nutrient-limited growth zone was narrow and the
results are shown in Figure 5.1 for the dilution rates of 0.35h⁻¹ and 0.07h⁻¹. The
carbon and nitrogen content of the cells remained constant over the whole
range of Cₒ/Nₒ ratios (Fig. 5.1c, Tab. 5.1) and the cells were not able to
accumulate PHB at any of the Cₒ/Nₒ ratios tested (Tab. 5.1). As a consequence
the carbon and nitrogen growth yield coefficients were nearly identical for
carbon- and nitrogen-limited growth. The most pronounced difference between
the two yield coefficients was found at the lowest growth rate resulting in the
most extended dual-nutrient-limited growth zone.

Figure 5.2 shows the extension of the dual-nutrient-limited growth zone at
the four different dilution rates tested.

The extension as well as the location was dependent on growth rate. Dual-
nutrient-limited growth extended over a wider range of Cₒ/Nₒ ratios at lower
dilution rates and the boundaries between single- and dual-nutrient-limited
growth shifted to higher Cₒ/Nₒ ratios as the growth rate decreased (Fig. 5.2).
<table>
<thead>
<tr>
<th>Observed parameter</th>
<th>Abbreviation [unit]</th>
<th>Dilution rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.07h⁻¹</td>
</tr>
<tr>
<td>Observed borders of dual-nutrient-limited growth ¹</td>
<td>C₀/N₀ [g g⁻¹]</td>
<td>13.0</td>
</tr>
<tr>
<td>Calculated borders of dual-nutrient-limited growth ²</td>
<td>C₀/N₀(calc) [g g⁻¹]</td>
<td>13.0</td>
</tr>
<tr>
<td>Carbon growth yield coefficient</td>
<td>Yₓ/C [g g⁻¹]</td>
<td>0.62±0.1</td>
</tr>
<tr>
<td>Nitrogen growth yield coefficient</td>
<td>Yₓ/N [g g⁻¹]</td>
<td>8.05±1.18</td>
</tr>
</tbody>
</table>

Table 5.1:
Culture parameters, elemental growth yields, elemental composition and PHB content during growth of *Acinetobacter johnsonii* 210A in continuous culture with acetate and ammonium under either carbon- or nitrogen-limited conditions, as a function of the dilution rate. ¹obtained from linear regression of residual nutrient concentrations versus the C₀/N₀ ratio of the medium at residual nutrient concentrations equal to zero; ²values calculated from experimentally found yield coefficients for carbon and nitrogen (details in Egli and Quayle, 1986); n.d. = not determined.
<table>
<thead>
<tr>
<th>Observed parameter</th>
<th>Unit</th>
<th>0.07h⁻¹</th>
<th>0.185h⁻¹</th>
<th>0.35h⁻¹</th>
<th>0.585h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular PHB content</td>
<td>wt%</td>
<td>0.7⁽³⁾</td>
<td>1.0⁽³⁾</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cellular C content</td>
<td>wt%</td>
<td>42.6±1.3</td>
<td>42.6±1.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cellular N content</td>
<td>wt%</td>
<td>11.7±0.8</td>
<td>11.7±0.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 5.1: contd.

⁽³⁾ single value only

The borders of dual-nutrient-limited growth at \( \mu_{\text{max}} \) (0.81h⁻¹) were 8.0, calculated with mean values taken from the experimental results at \( D=0.585h^{-1} \): \( Y_{XC}=1.05 \text{ g} \) and \( Y_{XN}=8.35 \).
Figure 5.1:
Growth of *Acinetobacter johnsonii* 210A at dilution rates of 0.35h\(^{-1}\) and 0.07h\(^{-1}\) in continuous culture with media of different carbon to nitrogen ratios. Acetate and ammonium were used as the sole sources of carbon and nitrogen, respectively. To obtain different C\(_{\nu}/N_0\) ratios in the medium feed, the concentration of acetate was varied whereas that of ammonium was kept constant. a) & b) culture parameters; c) elemental cell composition. The extension of the dual-nutrient-limited growth zone was calculated from the slopes of the respective residual nutrients and is indicated by the shaded area.
Section 5 99 Nutrient-limited Growth

![Diagram showing growth rate (μmax) vs. carbon to nitrogen ratio in medium feed (g/g)]

(*•*) experimentally determined values
(a) values calculated according to Egli and Quayle, 1986

**Figure 5.2:**
Extension of the dual-(carbon/nitrogen)-limited growth zone (marked grey) as a function of growth rate for a culture of *Acinetobacter johnsonii* 210A cultivated in a chemostat with increasing ratios of acetate : ammonium in the supplied growth medium. The boundaries (black dots) have been obtained from the slopes of either residual nitrogen (lower boundary) or carbon (upper boundary) vs. the C0/N0 ratio in the medium feed. For comparison the values calculated from cellular growth yields for carbon and nitrogen are also shown.

### 5.2.2 Carbon- and oxygen-limited growth

To test the influence of the ratio of carbon to oxygen supplied on the cellular composition and physiology, *Acinetobacter johnsonii* 210A was grown at a dilution rate of 0.35h⁻¹ with a constant aeration rate of 0.535vvm (250mL N₂ min⁻¹ and 285mL of air min⁻¹) and increasing concentrations of carbon in the medium feed. The dilution rate of 0.35h⁻¹ was chosen because stable steady-state cultures could be established easily at this growth rate and the biomass production was maximal.
Up to a concentration of 1 g L\(^{-1}\) of C (C\(_{\text{c}}\)/\(O_2\)=0.075 g g\(^{-1}\)) all carbon was used up by the cells and the biomass increased accordingly (Fig. 5.3a). At higher C\(_{\text{c}}\)/\(O_2\) ratios oxygen was limiting growth and acetate appeared in the culture medium. The transition zone between single carbon limitation and oxygen-limited growth was very narrow, only between C\(_{\text{c}}\)/\(O_2\) ratios of 0.081 g g\(^{-1}\) and 0.105 g g\(^{-1}\) both nutrients were consumed to completion. No significant amounts of PHB were accumulated during the whole experiment (Fig. 5.3b) and \(^{31}\)P-NMR analysis revealed that the cells did also not store significant amounts of polyP, neither during carbon- nor during oxygen-limited growth (results not shown).

**Figure 5.3:**
Growth of *Acinetobacter johnsonii* 210A at different C\(_{\text{c}}\)/\(O_2\) ratios in continuous culture at a dilution rate of 0.35 h\(^{-1}\). The C\(_{\text{c}}\)/\(O_2\) ratios on the x-axis were obtained by dividing the carbon input from the medium (g C per hour) by the oxygen input through aeration (g O\(_2\) per hour). a) culture parameters b) PHB content and residual DOC concentration in the culture medium. The range of the dual-nutrient-limited growth zone was calculated from the slopes of the residual concentration of the two nutrients.
Furthermore, DOC analysis of the culture supernatant showed that no side-products were excreted during the whole experiment (Fig. 5.3b). Oxygen limitation was verified by enhancing the rate of air supply upon which an immediate increase of OD$_{540nm}$ of the culture was observed.

5.2.3 Carbon- and phosphorus-limited growth

In analogy to the previously reported experiments with varying C$_0$/N$_0$ and C$_0$/O$_2$ concentrations *Acinetobacter johnsonii* 210A was grown with media of different C$_0$/P$_0$ ratios. In the medium feed of a continuous culture the C$_0$/P$_0$ ratio was decreased from 1083 g g$^{-1}$ (P$_0$=0.97 mg L$^{-1}$ of P) to 23 g g$^{-1}$ (P$_0$=33.1 mg L$^{-1}$ of P) by increasing P$_0$ while leaving C$_0$ constant at 1 g L$^{-1}$ of C. The experiment was also performed at dilution rates of 0.25 h$^{-1}$, 0.35 h$^{-1}$ and 0.585 h$^{-1}$ in order to establish the influence of the growth rate on the extension of a possible dual-(C/P)-nutrient-limited growth zone. However, only the experiments at growth rates of 0.1 h$^{-1}$ and 0.185 h$^{-1}$ were successful. Attempts to establish dual-(C/P)-nutrient-limited or P-limited growth at µ>0.185 h$^{-1}$ always resulted in wash-out of the culture. Both, at growth rates of 0.1 h$^{-1}$ and 0.185 h$^{-1}$ clear dual-(C/P)-nutrient-limited growth zones were found and the results of the experiment at µ=0.1 h$^{-1}$ are depicted in Figure 5.4a & b.

The experiment was started with a C$_0$/P$_0$ ratio of 1083 g g$^{-1}$ in the medium feed. With this growth medium a clearly P-limited steady-state was established. As more phosphate was added to the medium the C$_0$/P$_0$ ratios decreased. The P-limited growth regime extended down to a C$_0$/P$_0$ ratio of 196 g g$^{-1}$. Dual-(C/P)-limited growth was observed between a C$_0$/P$_0$ ratio of 196 g g$^{-1}$ and 50 g g$^{-1}$. When the phosphorus concentration in the medium feed was increased further (i.e. at C$_0$/P$_0$ ratios < 50 g g$^{-1}$) the culture was growing C-limited (Fig. 5.4a).

The biomass concentration in the chemostat was approximately proportional to the inflowing phosphorus between a C$_0$/P$_0$ ratio of 1083 g g$^{-1}$ and 325 g g$^{-1}$. A further increase in P$_0$ did not result in significantly more biomass being produced (Fig. 5.4a).
Growth of *Acinetobacter johnsonii* 210A at a dilution rate of 0.1 h⁻¹ in a chemostat with 1000 mg L⁻¹ acetate-carbon and increasing concentrations of phosphorus in the medium feed (i.e. the experiment was started with medium of high C₀/P₀ ratios which are shown at the right end of the x-axis). The dual-nutrient-limited growth zone is marked in grey. a) culture parameters, b) phosphorus and RNA content of biomass. The range of the dual-nutrient-limited growth zone was calculated from the slopes of the respective residual nutrients.
During phosphorus-limited growth up to 35% of the cell dry weight consisted of PHB. Surprisingly, as soon as carbon was limiting growth, i.e. during dual (C/P) nutrient limitation and carbon limitation, no PHB was found in the cells (Fig. 5.4b). As expected the cellular phosphorus content varied markedly during the experiment. It was constantly low at 0.5-0.9wt% when the cells grew phosphorus-limited. With decreasing C₀/P₀ ratios in the medium feed the cells passed to dual-(C/P)-nutrient-limited growth and the phosphorus content of the cells increased. It reached a maximum of 3.5wt% under clearly carbon-limited growth conditions (Fig. 5.4b).

In Table 5.2 culture parameters and cellular composition for both growth rates 0.1h⁻¹ and 0.185h⁻¹ are presented under C-limited and P-limited conditions. The carbon growth yield coefficient Yₓc was 0.71g g⁻¹ at a growth rate of 0.1h⁻¹ and 0.85g g⁻¹ at 0.185h⁻¹. This difference can be attributed to the influence of the maintenance energy requirement at the lower growth rate.

The intracellular allocation of phosphorus under carbon- and phosphorus-limited growth conditions was also investigated. Phosphorus and RNA content were directly measured while the polyP content was indirectly estimated (Fig. 5.5, Tab. 5.2). Estimation of the distribution of phosphorus in different cell components was mainly based on literature data which have shown that the major phosphorus containing components of microbial cells are nucleic acids, phospholipids and cell wall constituents.
Table 5.2:
Culture parameters and cellular composition during growth of *Acinetobacter johnsonii* 210A in continuous culture with acetate and orthophosphate under either carbon- or phosphorus-limited conditions. Mean values represent steady-state measurements from 2-4 different C$_{0}$/P$_{0}$ ratios; 1) obtained from linear regression of residual nutrient concentrations versus the C$_{0}$/P$_{0}$ ratio of the medium at residual nutrient concentrations equal to zero; 2) only one value; 3) calculated as a difference between total cellular phosphorus and the phosphorus contained in major cellular components (DNA being constant 0.5wt% P, phospholipids being constant 0.5wt% P, RNA being variable); 4) % of PHB free biomass.

<table>
<thead>
<tr>
<th>parameter</th>
<th>abbr.</th>
<th>unit</th>
<th>dilution rate</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1h$^{-1}$</td>
<td>0.185h$^{-1}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>observed borders of dual-nutrient-limited</td>
<td>C$<em>{0}$/P$</em>{0}$</td>
<td>g g$^{-1}$</td>
<td>51.2</td>
<td>195.1</td>
<td>52</td>
<td>224.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>growth 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>calculated borders of dual-nutrient-limited</td>
<td>C$<em>{0}$/P$</em>{0}$</td>
<td>g g$^{-1}$</td>
<td>42.1</td>
<td>216.3</td>
<td>47.3</td>
<td>125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>growth (calc)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbon growth yield coefficient</td>
<td>Y$_{XC}$</td>
<td>g g$^{-1}$</td>
<td>0.71±0.1</td>
<td>0.80±0.2</td>
<td>0.85±0.1</td>
<td>0.97±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phosphorus growth yield coefficient</td>
<td>Y$_{XP}$</td>
<td>g g$^{-1}$</td>
<td>29.9±1.3</td>
<td>173±39.5</td>
<td>40.2±2.1</td>
<td>121.2±19.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular PHB content</td>
<td>wt%</td>
<td></td>
<td>0.14±0.16</td>
<td>32.1±3.2</td>
<td>0.55 3)</td>
<td>27.6±4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular RNA content</td>
<td>wt%</td>
<td></td>
<td>10.4±1.9</td>
<td>1.17±0.23</td>
<td>n.d.</td>
<td>2.19±0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular polyP content</td>
<td>wt%</td>
<td></td>
<td>1.23 3)</td>
<td>-</td>
<td>0.85 2)</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular C content</td>
<td>wt%</td>
<td></td>
<td>(n.d.)</td>
<td>(n.d.)</td>
<td>(n.d.)</td>
<td>(n.d.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cellular P content 4)</td>
<td>wt%</td>
<td></td>
<td>3.35±0.15</td>
<td>0.90±0.29</td>
<td>2.46±0.16</td>
<td>1.14±0.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

"Chironomus dilutus" — a genus of flies belonging to the midge family Chironomidae.
Literature data demonstrate that the DNA content of cells is roughly constant over the growth rates examined (5wt%) (Neidhardt et al., 1990). Therefore, the phosphorus contained in DNA was set to 0.5% of cell dry weight. Phosphorus allocated in the cell envelope i.e. mostly in phospholipids amounts roughly to 0.5% of total cellular dry weight (Neidhardt & Umbarger, 1996).

Figure 5.5:
Allocation of phosphorus in *Acinetobacter johnsonii* 210A during carbon-and phosphorus-limited growth in continuous culture at $D=0.1\,h^{-1}$ and $0.185\,h^{-1}$. All values are taken from Table 5.2 and are given in % of cellular dry weight. The P content of both, RNA and DNA, is 10wt%. The DNA and phospholipid-P were considered to be constant, whereas the content of polyP and RNA were assumed to vary.

Under P-limited growth conditions the RNA content was always below 2.5wt% and most phosphorus was allocated to DNA (Fig. 5.5, Tab. 5.2). The RNA/DNA ratio increased as the $C_o/P_o$ ratios in the medium feed became lower, i.e. as more phosphorus was supplied to the culture. During carbon-limited
growth the RNA content was highest at 10.4wt%. As a comparison the RNA content was also measured in a batch culture: Cells growing exponentially at \( \mu = 0.35h^{-1} \) in mineral medium with acetate had an RNA content of 10.8wt% (results not shown). 

When *Acinetobacter johnsonii* 210A was grown under phosphorus limitation its cellular RNA content was very low and the culture repeatedly washed out of the chemostat at growth rates \( \mu > 0.185h^{-1} \) (see above). To assess the culture’s overall fitness under different growth conditions the maximum oxygen uptake rate and \( \mu_{\text{excess}} \) in batch culture were determined for cells taken from a C- or P-limited chemostat operated at different dilution rates (Tab. 5.3).

<table>
<thead>
<tr>
<th>dilution rate ([h^{-1}])</th>
<th>( \mu_{\text{excess batch}} ) ([h^{-1}])</th>
<th>maximum oxygen uptake rate ([\text{mmolO}_2/\text{min}^{*}\text{gCDW}])</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.46</td>
<td>0.52</td>
</tr>
<tr>
<td>0.1</td>
<td>0.59</td>
<td>0.62</td>
</tr>
<tr>
<td>0.11</td>
<td>0.36(^2)</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.126</td>
<td>0.51</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.15</td>
<td>0.37(^2)/0.55</td>
<td>0.64</td>
</tr>
<tr>
<td>0.3</td>
<td>wash out(^3)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

**Table 5.3:**

\( \mu_{\text{excess}} \) and maximum specific oxygen uptake rates of cells pregrown in either carbon- or phosphorus-limited continuous culture. In row 1 the dilution rate at which the cells were cultivated is listed. For determination of \( \mu_{\text{excess}} \) the cells were transferred into complete batch medium with acetate as sole source of carbon and energy. Endogenous uptake rate was determined in complete chemostat medium without carbon source and was subtracted from the maximum specific rate which was obtained upon the addition of acetate. \(^1\)maximum specific oxygen uptake rate measured in phosphate buffer; \(^2\)values obtained with cultures growing P-limited for over 15 generations; \(^3\)no P-limited steady-state culture could be established at a dilution rate of 0.3h\(^{-1}\); n.d.: not determined.
The cells exhibited significantly lower maximum growth rates and maximum specific oxygen uptake rates when grown in a P-limited chemostat as compared to carbon-limited cells. This was the case for all dilution rates tested. The lowest \( \mu_{\text{excess}} \) values were found in cells grown under phosphorus limitation for over 15 generations. The maximum specific oxygen uptake rate was determined in phosphate buffer as well as complete chemostat medium. The rates obtained in complete medium were always higher by a factor of at least 2.

### 5.2.4 Elemental growth yields for carbon

A comparison of the elemental growth yields for carbon determined under different nutrient limitations is given in Figure 5.6.

![Graph showing elemental growth yields for carbon](image)

**Figure 5.6:** Elemental growth yields for carbon at different growth rates in the chemostat under P-, C-, N-, and O\(_2\)-limited growth conditions. The data for C-limited growth is compiled from 3 different experiments (Source: Tab. 5.1, Tab. 5.2, and Fig. 5.3).

The data confirms the tendency to lower growth yields for carbon with decreasing growth rates. The lowest value (measured at a growth rate of 0.07h\(^{-1}\)) was merely 60% of the maximally found values. This trend was virtually
independent of the nature of the growth-limiting nutrient. Surprisingly however the values measured under carbon limitation were lower than under P limitation (Fig. 5.6).

5.3 Discussion

5.3.1 Accumulation of reserve polymers in Acinetobacter during single- and dual-nutrient-limited growth

Reserve polymers have different functions in bacterial cells, they can act as energy reserves or as sources of the respective elements or both. Reserve polymers must fulfill three requirements in order to be considered energy-storage compounds (Wilkinson, 1959). Firstly, they are accumulated during a growth regime when the cells are supplied with more energy than they need for growth and associated processes. Secondly, they are utilised when the supply of energy to the cells is insufficient for growth and division, or maintenance of viability. Thirdly, the cells gain readily utilisable energy from the breakdown of the compound. Bacterial polymers which meet all these criteria are glycogen, PHA, and PHB (Dawes & Senior, 1973). The role of polyP is not always that of storing energy but it may simply act as an intracellular source of phosphorus (Harold, 1966; Kulaev & Vagabov, 1983).

In this work the accumulation of PHB and polyP was investigated in *Acinetobacter johnsonii* 210A growing under carbon, nitrogen, oxygen, and phosphorus limitation and combinations thereof. Surprisingly, *Acinetobacter johnsonii* 210A was not able to accumulate detectable amounts of PHB neither under nitrogen nor under oxygen limitation. This is astonishing because nitrogen limitation is usually most favourable for the accumulation of PHB (Egli, 1995) whereas oxygen or phosphorus limitation are often less effective.

Single nutrient limitation in chemostat cultures of *Acinetobacter johnsonii* 210A led to the accumulation of PHB up to 35wt% only during distinctly phosphorus-limited growth. The PHB content was substantially higher than the previously reported 20wt% (Bonting et al., 1992a). The reason
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for this significant difference is not entirely clear, particularly because Bonting and co-workers cultivated the organism with butyrate, a substrate that is more reduced than acetate and, therefore, should be more favourable for PHB accumulation. Another reason might be the difference in the growth temperature (30°C used here vs. 20°C). However, the influence of both parameters on the accumulation of PHB in *Acinetobacter johnsonii* 210A has not yet been investigated (Bonting et al., 1992a). From the literature only one report is known where *Acinetobacter* was shown to accumulate PHB under oxygen limitation (Weltin, 1996), however, this author cultivated the bacterium in batch culture and also in this case the production of PHB was not linked to polyP hydrolysis. Comparing the results to other studies where the accumulation of PHB by *Acinetobacter* sp. had been investigated it becomes evident that several *Acinetobacter* strains isolated from activated sludge do accumulate PHB only under phosphorus or sulphur limitation but not under nitrogen limitation (Vierkant et al., 1990; Rees et al., 1993; Weltin, 1996). Obviously, also *Acinetobacter johnsonii* 210A belongs to this group of organisms.

The pattern of phosphorus accumulation in *Acinetobacter johnsonii* 210A was such that during C-limited and dual-(C/P)-limited growth an enhanced level of cellular phosphorus (up to 3.35wt%) was observed indicating the accumulation of polyP.

*Acinetobacter johnsonii* 210A is known to accumulate polyP under carbon, nitrogen and sulphur limitation and PHB under phosphorus limitation (van Groenestijn et al., 1989; Bonting et al., 1992a). In this work we could confirm the high PHB content under phosphorus limitation and also show that PHB accumulation was not possible under nitrogen nor oxygen limitation. The hydrolysis of polyP has been demonstrated to yield ATP in this organism (van Groenestijn et al., 1987). In contrast, the role of PHB has never been elucidated (Fixter & Sherwani, 1991). Although polyP can act as a source of readily utilisable energy in *Acinetobacter johnsonii* 210A the growth conditions under
which the polymer is accumulated are not typical for the storage of energy reserves as described by Wilkinson (Wilkinson, 1959). According to Wilkinson one of the prerequisites for energy reserve accumulation is energy "oversupply". However, *Acinetobacter johnsonii* 210A accumulates polyP in acetate-limited continuous culture (van Groenestijn *et al.*, 1987; van Groenestijn *et al.*, 1989) and consequently the question must be raised if such a continuous culture is carbon-or energy-limited. Van Groenestijn and co-workers speculated that energy limitation, as a possible forerunner of energy depletion, would somehow induce the accumulation of polyP (van Groenestijn *et al.*, 1989). According to Linton & Stephenson one would expect acetate as a growth substrate to be energy- and not carbon-limited (Linton & Stephenson, 1978). Energy limitation of a substrate in the sense of Linton & Stephenson simply means that the oxidation state of the average substrate carbon atom is higher than that of the produced biomass (Linton & Stephenson, 1978). Cells, however, as a consequence of this, may channel more substrate into carbon dioxide production balancing their carbon and energy needs. An indication that the state of reduction of a substrate carbon is only partly determining whether growth with it is carbon- or energy-limited was presented by Neijssel and co-workers (Neijssel & Tempest, 1976). These authors found that glucose which is generally considered to be a energy-limited growth substrate may in fact result in carbon-limited growth (Neijssel & Tempest, 1976). They grew *Klebsiella aerogenes* NCTC 418 in glucose- and gluconate-limited aerobic chemostat cultures. Comparing the oxygen consumption rate per biomass produced for growth with the two substrates it turned out to be higher for glucose over a wide range of dilution rates. Since glucose is the more reduced of the two substrates they concluded that there must be "surplus" energy produced from glucose. The authors speculated that part of this energy was probably used by the cells for their maintenance requirements (Neijssel & Tempest, 1976).
5.3.2 Elemental growth yields during single-nutrient-limited growth

The elemental growth yields for carbon, nitrogen, and phosphorus in *Acinetobacter johnsonii* 210A were determined for growth under carbon, nitrogen, and phosphorus limitation, respectively (see Fig. 5.2, Tab. 5.2 & 5.3). For growth with acetate a maximum growth yield of 1.05 g g\(^{-1}\) was found under carbon and nitrogen limitation which is well in accordance with the values described in the literature (Linton & Stephenson, 1978). Somewhat unexpected, the elemental growth yield for carbon was clearly dependent on the growth rate. \(Y_{XC}\) was reduced already at growth rates below 0.35 h\(^{-1}\), independently of the growth-limiting nutrient. For carbon limitation it was only 60% of the maximum value found at \(\mu=0.585\text{ h}^{-1}\). This can be attributed to the increased importance of the maintenance energy requirement of the cells at low growth rates. The same effect has been observed with other bacteria, although often less pronounced i.e. constant growth yields down to much lower growth rates (Pirt, 1975). Earlier studies by van Groenestijn and co-workers have also reported reduced carbon growth yields for *Acinetobacter johnsonii* 210A at low growth rates (van Groenestijn et al., 1989). At \(\mu=0.05\text{ h}^{-1}\) they found a yield of 0.69 g g\(^{-1}\) which is in good agreement with the value of 0.62 g g\(^{-1}\) found in this report. However, clearly lower \(Y_{XC}\) values were found only at growth rates below \(\mu=0.1\text{ h}^{-1}\) whereas in the present study the growth yields at \(\mu=0.2\text{ h}^{-1}\) were already lowered. These results agree more with findings from *Pseudomonas oleovorans* which also showed decreased carbon yields at growth rates below 0.2 h\(^{-1}\) as reported by Durner (Durner, 1998).

The elemental growth yield for carbon under different nutrient limitations was similar for all limitations tested. In the case of nitrogen-limited growth this was because *Acinetobacter johnsonii* 210A was not able to accumulate PHB and hence the elemental growth yields were nearly identical for nitrogen and carbon limitation. Surprisingly, at a growth rate of 0.185 h\(^{-1}\) the highest carbon growth yield was not found under carbon limitation (0.85 g g\(^{-1}\)) but under phosphorus limitation (0.97 g g\(^{-1}\)). Normally, the biomass yield of a certain
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substrate should be highest (i.e. most efficient growth) when this element is limiting biomass production. During phosphorus-limited growth with acetate the cells accumulated PHB. This reserve material had to be reduced from the level of acetate at the expense of reducing equivalents. Therefore, the cells should have had a higher proportion of the carbon channelled into dissimilation, and as a consequence, should exhibit a lower carbon growth yield. Why such an increased dissimilation was not observed remains a matter of speculation. A change in the phosphorylation efficiency or a reduced requirement for maintenance energy could explain the results. From *Paracoccus denitrificans* it is known that sulphate and iron limitation change the efficiency of oxidative phosphorylation in continuous culture, mainly due to their presence in the iron-sulphur centres involved in energy coupling (Meijer *et al.*, 1977). As phosphorus is not a key component of the oxidative phosphorylation chain it is unlikely to influence the oxidative phosphorylation to a similarly strong extent. The maintenance requirement was calculated from Fig. 5.6 and was found to be similar for all growth regimes tested. It was 0.052gC*(gCDW*h⁻¹) for carbon-and phosphorus-limited growth and 0.068gC*(gCDW*h⁻¹) for nitrogen-limited growth, respectively. Most likely, the differences found were due to experimental errors which could be as high as 20% (i.e. no statistical difference). But more data would be needed to further clarify this question.

The elemental growth yield for phosphorus differed significantly between carbon limitation, under which it was between 30 and 40g g⁻¹, and phosphorus limitation under which it was between 120 and 170g g⁻¹. The variation among the data within the respective limitations can be explained by the difficulties to determine phosphorus in the μg L⁻¹ range - due to for example precipitation of phosphorus components - which can affect the determination of the elemental growth yield considerably.
5.3.3 Dual-nutrient-limited growth of Acinetobacter johnsonii 210A

Dual-nutrient-limited growth has usually been associated with a micro-organism’s ability to change the elemental growth yields as a function of different nutrient limitations. Such changes, for example, occur when an organism excretes metabolites, accumulates storage polymers or changes its growth efficiency as is common under certain nutrient limitations. Most data on dual-nutrient-limited growth have been collected with the nutrients carbon and nitrogen. In general, when growing micro-organisms in continuous culture with media of different C₀/N₀ ratios three distinctive physiological states can be observed: carbon limitation at low C₀/N₀ ratios, nitrogen limitation at high C₀/N₀ ratios, and in between a transient zone which is characterised by the complete utilisation of both nutrients, carbon and nitrogen (Egli & Quayle, 1986; Gräter-Lampart et al., 1986). The same pattern of behaviour has been shown for several different combinations of non-homologous nutrients (for an overview see Egli, 1995).

In this work Acinetobacter johnsonii 210A grew dual-nutrient-limited under carbon and nitrogen, carbon and phosphorus, as well as carbon and oxygen limitation. The extension of the dual-nutrient-limited growth regimes was, however, different. Although no PHB was accumulated by Acinetobacter johnsonii 210A when growing at different carbon:nitrogen or carbon:oxygen ratios in the medium feed dual-nutrient-limited growth was observed. This must have been due to minor changes in the allocation of the nutrients in the cells, a change in the growth efficiency or a difference in maintenance requirements. Similar behaviour was also observed in Pseudomonas oleovorans growing in continuous culture with different ratios of citrate and ammonium: although the organism was not able to accumulate PHA a small dual-nutrient-limited growth zone was observed (Durner, 1998). In contrast, the observed dual-nutrient-limited growth in the case of the combination of carbon to phosphorus limitation was due to a clear increase in cellular phosphorus content. Much to our surprise PHB was not accumulated during dual-(C/P)-limited growth as
would have been expected from results of dual-(C/N)-limited growth obtained with many other organisms, such as Pseudomonas oleovorans (Zinn, 1998), Hyphomicrobium ZV620 (Gräzer-Lampart et al., 1986), and methylotrophic yeast (Egli & Quayle, 1986). Dual (C/P) limitation was observed at both dilution rates tested between C₀/P₀ of 51g g⁻¹ and 195g g⁻¹. During dual-(C/P)-limited growth the cellular phosphorus content increased from 0.5 - 1wt% up to 2.5 - 3.6wt%. Interestingly, the P content under both, C and P limitation was very low; lower than the values obtained in an earlier study by Bonting and co-workers who found 1.5wt% under P limitation and 6wt% under C limitation at a growth rate of 0.1h⁻¹ (Bonting et al., 1992a). Only for Candida utilis even lower P contents have been reported (Lucca et al., 1991). This eucaryotic organism contained only 0.3wt% P when grown in a phosphorus-limited continuous culture at a growth rate of 0.1h⁻¹. Attempts to establish a stable P-limited culture of Acinetobacter johnsonii 210A at higher growth rates than 0.185h⁻¹ in order to test the dependency of the dual-nutrient-limited growth zone on the growth rate failed due to wash-out of the culture. This wash-out must have been somehow related to phosphorus limitation as further discussed in the next section.

In the literature it is generally emphasised that the capability of microorganisms to change the cellular composition increases with decreasing growth rate (Herbert, 1976). As a result the extent of accumulation of reserve materials such as PHA, glycogen or polyP is more pronounced at low growth rates. Based on the available data a concept for dual-nutrient-limited growth has been developed (Egli & Schmidt, 1988). Different growth regimes with respect to the limiting nutrient can be clearly defined and predicted on the basis of cellular yield coefficients determined under single-nutrient-limited growth conditions. The concept has been shown to describe well the different growth regimes when shifting a continuous culture from carbon to nitrogen limitation (Egli, 1991). However, reliable data for other combinations of nutrients are still scarce.

The extent of dual nutrient limitation was calculated with the help of elemental growth yields for respective elements according to Egli and Quayle.
Section 5 Nutrient-limited Growth

(1986) and compared to the experimentally found boundaries for dual-nutrient-limited growth. For growth at different $C_0/N_0$ ratios the calculated values differed significantly from those determined experimentally as is depicted in Figure 5.2. Hence, the accurate determination of the elemental growth yields is essential in order to make precise predictions of nutrient-limited zones within the concept of Egli & Quayle (Egli & Quayle, 1986). This was difficult for *Acinetobacter johnsonii* 210A for the reasons outlined in the last section. Despite these limitations, some general conclusions about growth limitation zones can be deduced from the results obtained. Firstly, the dual-($C/N$)-nutrient-limited growth zones extended over a wider range of $C_0/N_0$ ratios at low growth rates since the capability of changing the cellular composition is greater at low growth rates (Egli, 1995). This also holds true when no PHB is formed as in the case of *Acinetobacter johnsonii* 210A although to a lesser extent. Secondly, the boundaries between single-nutrient-limited and dual-nutrient-limited growth shifted to higher $C_0/N_0$ ratios as the growth rate increased. This is due to decreasing growth yields at $\mu<0.35 \text{h}^{-1}$ which reflect the increasing importance of the maintenance energy coefficient at low growth rates. This finding has important implications for the design of growth media: a growth medium with $C_0/N_0$ ratios between 8 and 12 may lead to carbon- or nitrogen-limited growth depending on the growth rate. Only media with a $C_0/N_0$ ratio of less than 7 ensure C-limited growth at all growth rates while for N-limited growth a medium with a $C_0/N_0$ ratio $>16$ is needed. In the case of dual ($C/P$) limitation a comparison between the experimentally found values and the calculated borders also reveals differences. At $D=0.1 \text{h}^{-1}$ the observed and calculated borders were well in agreement but at $D=0.185 \text{h}^{-1}$ there is a discrepancy in the position of the higher border. As already stated in the last paragraph, the accuracy of phosphorus determination plays an important role and directly influences the results. Thus, for a more accurate determination of the dual-($C/P$)-limited growth regime one solution would be to work with higher overall nutrient concentrations.
5.3.4 Physiology of Acinetobacter johnsonii 210A during phosphorus-limited growth

Although in theory it should be possible to grow the cells near $\mu_{\text{max}}$ in a chemostat under any nutrient limitation, Acinetobacter johnsonii 210A repeatedly washed out at growth rates above 0.185h\(^{-1}\). This finding is in accordance with a report by Lucca and co-workers who grew Candida utilis under phosphorus limitation in continuous culture and found that the cells washed out at dilution rates between 0.2h\(^{-1}\) and 0.3h\(^{-1}\) whereas the usual growth rate in a balanced batch medium is 0.4 - 0.6h\(^{-1}\) (Callicri et al., 1984; Lucca et al., 1991). These results must be viewed in the light of phosphorus allocation in bacterial cells: the cell’s main need for phosphorus is to build nucleic acids (Tempest, 1969). It is well established that the nucleic acid content increases with growth rate (Neidhardt et al., 1990). This increase largely reflects the increased rRNA content of cells which accounts for over 80wt% of total RNA in E. coli (Neidhardt et al., 1990), whereas the other major P containing nucleic acid fraction, namely DNA, remains almost constant over the whole range of growth rates (Neidhardt et al., 1990). In this work, the RNA content of Acinetobacter johnsonii 210A dropped to less than 1.5wt% under phosphorus limitation compared to over 10wt% under carbon limitation. Comparable results have been reported from Candida utilis (Lucca et al., 1991). When it was grown in continuous culture under phosphorus limitation the RNA content was clearly lower (3.6wt%) than under carbon limitation (9.4wt%). Interestingly, in Candida utilis the protein to RNA ratio was much higher in phosphorus-limited cells indicating that the cells were able to maintain their overall protein concentration with an increased protein production rate (Lucca et al., 1991). These observations are in contrast to studies with other Gram-negative bacteria which showed that the cellular RNA content was not so strongly affected by phosphorus limitation (Tempest, 1969; Alton & Koch, 1974). The RNA content of a phosphate-limited chemostat culture of E. coli was 8.2wt% ($\mu=0.09h^{-1}$) that of Aerobacter aerogenes, 5.7wt% ($\mu=0.1h^{-1}$).
In the present work, in oxygen uptake experiments were performed to test the immediate reaction of the cells to a surplus of nutrients and in batch experiments the response over several hours after relieve from phosphorus limitation was followed. Both, the maximum specific oxygen uptake rate as well as $\mu_{\text{excess}}$ were clearly reduced compared to carbon-limited pregrown cells. This reduced cell fitness was even more pronounced at higher growth rates pointing to an effect of phosphorus limitation on the protein synthesising capacity of *Acinetobacter johnsonii* 210A. Therefore, it could well be that under severe phosphorus limitation the cells did not have enough RNA to increase their protein production rate to a level high enough for faster growth, that the time required for adaptation was longer than the observation period or that the shift-up was performed too fast. These results were clearly in contrast to the study of Alton and Koch where they found that P-limited *E. coli* cells were maintaining an "unused protein synthetic capacity" which was immediately functioning after nutritional shift-up in this case the transfer into complex growth medium (Alton & Koch, 1974). However, phosphorus limitation not only affects the protein synthesising machinery but also the synthesis of cell walls where a minor part of the cellular phosphorus is allocated (Cooney & Wang, 1976b; Herbert, 1976; Harder & Dijkhuizen, 1983). In *Pseudomonas fluorescens* growing under phosphate limitation the phospholipids of the cell envelope were completely replaced by lipids lacking phosphorus (Minnikin & Abdolrahimzadeh, 1974). No further investigations were undertaken as to the physiological consequences of this change. Nothing is known about the influence of phosphorus limitation on the cell envelope of *Acinetobacter johnsonii* 210A. However, it seems likely that the reduced cell fitness observed in phosphorus-limited cultures of *Acinetobacter johnsonii* 210A originates from a combination of effects on the nucleic acids as well as the cell envelope. In summary the information presently available points to an interesting problem that occurs during P limitation that has so far been neglected but has important implication for growth, metabolic flexibility and survival of cells under different nutritional regimes (see also Holmquist & Kjelleberg, 1993).
5.3.5 Concluding remarks

In the literature it has been postulated that the COD/P ratio of the inflowing waste water is of imminent importance in the functioning of EBPR plants since it affects the P-removal efficiency as well as the composition of the microbial population (Randall et al., 1992). But so far nutrient availability has not obtained much attention in the whole modelling of EBPR processes. *Acinetobacter johnsonii* 210A, an organism extensively studied in the context of EBPR, is able to respond to different carbon : phosphorus ratios in the medium feed. The accumulation of PHB was observed under P limitation and changes in P content under dual (C/P) as well as under C limitation. It may well be that other micro-organisms which play a role in EBPR respond to changing nutrient concentrations in a similar manner as shown here for *Acinetobacter johnsonii* 210A. Therefore, the question of nutrient availability in waste water, especially of carbon and phosphorus, and its significance for the EBPR process needs to be readdressed. It has also become obvious that *Acinetobacter johnsonii* 210A is rather inflexible when it comes to carbon and/or oxygen limitation. However, oxygen limitation is an indispensable feature in the process of EBPR. Therefore, results from pure culture studies of *Acinetobacter* must be met with caution when conclusions are drawn for processes in EBPR plants, as has been stated numerous times by other authors (Jenkins & Tandoi, 1991; van Loosdrecht et al., 1997a). Nevertheless *Acinetobacter johnsonii* 210A could be a suitable model to study the response of heterotrophic bacteria to phosphorus limitation in general due to its fast growth, easy culturability in the laboratory, and also its very pronounced reaction towards P limitation.
SECTION 6

GENERAL CONCLUSIONS & OUTLOOK
A common feature of many micro-organisms when they grow in an environment short of a specific nutrient is a pronounced change of metabolism which often exhibits itself in the excretion of products or accumulation of storage polymers. Some of these specific responses to nutrient limitations have been taken advantage of in biotechnological processes such as the production of biodegradable polymers such as PHA’s or antibiotics (Calam, 1986; Byrom, 1987). Also in biological waste water treatment polymer accumulation by micro-organisms is an important feature but it is generally only poorly understood in these systems (van Loosdrecht et al., 1997a). This is especially true for EBPR where dynamic growth conditions lead to the establishment of a specialised microbial population which is able to accumulate carbon and phosphorus intracellularly (van Loosdrecht et al., 1997c).

A waste water treatment plant is a complex ecosystem where microbes grow in the presence of a variety of different carbon-and energy sources most of them being present only at low concentrations. The main substrates for microbes in EBPR are short chain fatty acids which are released by fermenting organisms in the anaerobic stage. However, only recently it has been pointed out that more research is needed to elucidate the role and influence of other carbon sources present in the sludge, such as sugars or amino acids (Mino et al., 1998).

In this work the influence of varying nutrient availability and the presence of mixtures of substrates on the growth behaviour of Acinetobacter johnsonii 210A was investigated.

**Growth of Acinetobacter johnsonii 210A with single and mixed substrates**

Growth of Acinetobacter johnsonii 210A with single and mixed substrates pretty much followed well known growth patterns. When growing with short chain alcohols an excretion of the oxidation product, namely the fatty acid was observed. It would be interesting to see how the utilisation of these substrates
was accomplished in continuous culture and also how the growth yields compare to those obtained with the single substrates.

In this work an acetate-limited continuous culture was supplied with additional energy in the form of glucose and PQQ. No increase in biomass production could be observed in our experimental set-up. In a study by van Niel and co-workers a much higher glucose oxidation rate was used and a small increase in biomass formation was found (van Niel et al., 1999). Clearly, the energy yield from the oxidation of glucose is very low in *Acinetobacter johnsonii* 210A although the energy generated with glucose and PQQ was shown to be useful for ATP generation and transport processes (van Veen et al., 1993b). This also fits into the picture of the whole *Acinetobacter* genus, for which the significance of the aldose dehydrogenase is far from clear.

**Response of *Acinetobacter johnsonii* 210A to nutrient limitation**

In *Acinetobacter johnsonii* 210A neither nitrogen nor oxygen limitation triggered the accumulation of PHB as is often observed in micro-organisms. However, *Acinetobacter johnsonii* 210A was confirmed to be extremely versatile when responding to a changing phosphorus supply, i.e. a changing $C_0/P_0$ ratio in the medium feed. P limitation not only led to the accumulation of PHB but also to a drastic decrease in the cellular RNA content (1.2% of cell dry weight). Concentrations of PHB as high as reported in this study have never before been found in *Acinetobacter* species (Vierkant et al., 1990; Bonting et al., 1992a). Generally, it can be concluded that even though *Acinetobacter johnsonii* 210A is probably not directly involved in EBPR, the organism is well equipped to cope with changing $C_0/P_0$ ratios as they appear in EBPR plants. Moreover, *Acinetobacter johnsonii* 210A is a good model organism to study the influence of P limitation on the metabolism of Gram-negative bacteria. Many features of the phosphorus metabolism have already been investigated with the main focus being on the biochemistry of processes possibly involved in EBPR.
The results of this work have extended this knowledge further to a field which has so far been largely neglected, namely that of severe phosphorus limitation.

**Dual-nutrient-limited growth of *Acinetobacter johnsonii 210A***

*Acinetobacter johnsonii 210A* grew dual-nutrient-limited with carbon and nitrogen, carbon and oxygen, and carbon and phosphorus, respectively. During dual-(C/P)-limited growth *Acinetobacter johnsonii 210A* varied its phosphorus content by a factor of 3 and consequently the dual-nutrient-limited growth zone covered a wide range of $C_0/P_0$ ratios. In contrast, the dual-nutrient-limited growth regime with carbon and nitrogen, and carbon and oxygen, respectively, was small. This was expected since the growth yields for the elements were nearly constant under carbon, nitrogen, and oxygen limitation. Our results strengthen the argument that dual-nutrient-limited growth is a general phenomenon, occurring whenever micro-organisms are grown under conditions of substrate limitation in media of different ratios of two nutrients.

For the optimal functioning of EBPR the composition of the inflowing waste water is of prime importance. As a rule of thumb it has been stated that phosphorus elimination is most effective when the COD/P ratio of the inflowing waste water is higher than 40:1 and it was found that at ratios lower than 30:1 the elimination of P was significantly reduced (Randall *et al.*, 1992). What is the growth behaviour of *Acinetobacter johnsonii 210A* at these $C_0/P_0$ ratios? Clearly, at $C_0/P_0$ ratios below 50 growth in pure culture was C-limited while at ratios above ca. 50g g$^{-1}$ either dual-(C/P)-limited growth or P-limited growth occurred. PHB was only accumulated when the $C_0/P_0$ ratios were higher than 200g g$^{-1}$. These results indicate that *Acinetobacter johnsonii 210A* is very unlikely to accumulate PHB under growth conditions as they are encountered in activated sludge.
Transient versus steady-state growth conditions

The response of *Acinetobacter johnsonii* 210A to varying ratios of $C_\theta/P_0$ and $C_\theta/O_2$ in the medium feed was compared under steady-state and transient growth conditions. The result was a consistent pattern of polymer accumulation. Clearly, the trigger for polymer accumulation was the growth-limiting nutrient and not the experimental set-up, i.e. the different growth conditions.
SECTION 7

LITERATURE


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