

**Studies of cell release and biomass activity of alginate
immobilized lactic acid bacteria**

by

Geir Klinkenberg

A thesis submitted in partial fulfillment of the requirements for the academic title

Doktor Ingeniør

Department of Biotechnology, Faculty of Natural Sciences and Technology,

Norwegian University of Science and Technology

Trondheim, June 2002

ACKNOWLEDGEMENTS

The work presented in this thesis was carried out at the Department of Biotechnology at the Norwegian University of Science and Technology, in close cooperation with the Group for Industrial Biotechnology at SINTEF Applied Chemistry. The work was financed by the Research Council of Norway, as a part of the project nr. 110682/420.

I would like to thank Professor David William Levine, for supervising the work and for his always positive and friendly attitude. I would also like to express my gratitude to Dr. ing. Nils Dyrset. His support and dedication throughout this study has been of invaluable importance and is highly appreciated. Kathinka Q. Lystad is also thanked for her helpfulness and positive attitude during our cooperation - this was highly appreciated.

Furthermore, I would like to thank all my colleagues at Industrial Biotechnology at SINTEF Applied Chemistry for their friendliness and helpful attitude during these years. They are to be thanked for their keen interest, for valuable discussions and for letting me be a part of their exciting work climate. During these years I have also appreciated the company and support of my fellow doctoral students at the Department of Biotechnology.

Last, but not least, I would like to thank my family and Lisbeth for endless support and patience during this study.

SUMMARY

The scope of the work presented in this thesis has been the study of growth, cell release and metabolite production of gel-entrapped viable lactic acid bacteria. As these fermentation systems are mainly aimed at food applications, immobilization methods based on alginate and chitosan were chosen due to the biocompatibility of these materials. In these studies, ways to measure, characterize and influence the activity and release of the immobilized lactic acid bacteria were investigated.

The use of sequential coatings with chitosan (C) and alginate (A) on alginate beads immobilizing *Lactococcus lactis* ssp. *lactis* NCMIB 6681 was investigated. The effects of these coatings on biomass activity and cell release were evaluated in batch and continuous systems. In this work, an argument is made for the use of an experimental system based on continuous fermentation at a controlled pH and a high dilution rate for these types of studies. When examined in this system, chitosan coating alone seemed to reduce the ratio of cell release to lactate production in the early stages of fermentation, while sequential coatings with chitosan and alginate (CAC) showed significant reductions in this ratio throughout the whole 48 hour test period.

Under normal growth conditions, diffusional limitations direct most of the biomass growth in beads immobilizing lactic acid bacteria to the outermost parts of the beads. Therefore, a study was conducted in order to investigate if biomass distributions, and thereby cell release, could be affected through changes of the growth conditions. A change in operating pH from 6.5 to 9.25 initially reduced the ratios of rates of cell release to lactate production by almost a factor of 10^5 . Compared to fermentations at pH 6.5, growth at pH 9.25 also increased the final internal bead biomass concentration by a factor of 5 and increased the final rate of lactate production by 25%. After 48 hours, the ratio of the rates of cell release to lactate production was still 10 times lower than in fermentations at pH of 6.5. These data illustrate that diffusional limitations and corresponding pH-gradients can be exploited in affecting the distribution of immobilized growing cells and their concomitant release.

The use of sequential coatings with chitosan and alginate was investigated as a way to a reduced cell release during immobilized bacteriocin production. Production of the bacteriocins enterocins A and B by immobilized *E. faecium* CTC492 in uncoated and CAC-coated alginate beads was evaluated in batch and continuous fermentations. In batch fermentations with *E. faecium* CTC492 immobilized in CAC-coated beads a substantial amount of the maximal bacteriocin activity in traditional free-cell fermentation was obtained,

combined with a more than 3 log unit reduction of the amount of free biomass in the product. Longer-term studies of bead performance were performed using continuous fermentations at high dilution rates. These experiments showed that the use of CAC-coatings reduced the ratio of rates of bacteriocin production to cell release significantly the first 20 hours of fermentation. In these studies, the presence of large pH gradients within beads containing immobilized *E. faecium* CTC492 during continuous fermentation were demonstrated using a microelectrode. As expected, the bacteriocin production and fermentation characteristics of immobilized *E. faecium* CTC492 seemed to be significantly influenced by the presence of these local pH-gradients.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	i
SUMMARY	ii
LIST OF APPENDIX PAPERS.....	v
1 INTRODUCTION.....	1
1.1 Immobilization of whole cells.....	1
1.2 Lactic acid bacteria	2
1.3 Types of cell immobilization	3
1.3.1 Immobilization by attachment to surfaces	4
1.3.2 Immobilization by entrapment within porous matrixes	4
1.3.3 Immobilization by containment behind a barrier	5
1.3.4 Immobilization by self aggregation	5
1.3.5 The choice of immobilization method.....	6
1.4 The immobilization materials	6
1.4.1 Alginate gel.....	6
1.4.2 Chitosan.....	8
1.5 The behavior of immobilized lactic acid bacteria.....	10
1.5.1 Growth of cells within gel matrixes.....	10
1.5.2 Transport of substrates and waste products.....	11
1.5.3 Fermentations with immobilized lactic acid bacteria	16
1.5.4 Cell release in fermentations with immobilized lactic acid bacteria	17
2 RESULTS AND DISCUSSION	19
2.1 Measuring cell release in a system with chitosan and alginate coated beads.	19
2.2 pH controlled biomass distribution and cell release from Ca-alginate beads.....	20
2.3 Immobilized production of Enterocin A and B.....	22
2.4 Final remarks.....	23
2.4.1 Measurement of cell release by continuous fermentation	23
2.4.2 Growth and release of immobilized cells	24
2.4.3 Suggestions for future work.....	26
3 REFERENCES.....	29

LIST OF APPENDIX PAPERS

- I. Klinkenberg, G., Lystad, K.Q., Levine, D.W and Dyrset, N. (2001) Cell release of alginate immobilized *L. lactis* ssp. *lactis* immobilized in chitosan and alginate coated beads. *Journal of Dairy Science*, 84, 1118-1127.
- II. Klinkenberg, G., Lystad, K.Q., Levine, D.W. and Dyrset, N. (2001) pH controlled cell release and biomass distribution of alginate immobilized *Lactococcus lactis* ssp. *lactis*. *Journal of Applied Microbiology*. 91, 705-714.
- III. Klinkenberg, G., Levine, D.W. and Dyrset, N. Enterocin production and cell release by *Enterococcus faecium* CTC 492 immobilized in uncoated and chitosan/alginate/chitosan coated alginate beads. Submitted to *Journal of Applied Microbiology*

1 INTRODUCTION

1.1 IMMOBILIZATION OF WHOLE CELLS

Whole cell immobilization can be defined as the physical confinement or localization of intact cells to a certain defined region of space with the preservation of some desired activity (Karel *et al.* 1995). This region usually consists of three components: the cells, the support material, and the solution that fills the rest of the interstitial space. The terms "the immobilized cell system" and "the immobilized cell aggregate" are used for this region (Willaert and Baron 1996). However, the support material together with the interstitial solution is also often referred to as "the immobilization matrix". Similarly, the term "micro-environment" is often used for the environment within or close to the immobilization matrix. This term is used because the environment within or close to the immobilization matrix can be significantly different from the environment in the surroundings.

The use of immobilized cells is often considered to be a new technology. In fact, mankind has used immobilized cells since ancient times. Kefir production is an example of an ancient process based on immobilized cells (Champagne *et al.* 1994). In this process, Kefir grains, which are an insoluble mixture of yeast and lactic acid bacteria, are inoculated into fresh milk. The cells immobilized within the grains initiate a fermentation process, which converts the fresh milk to Kefir. The Kefir grains can be recovered after fermentation and can be used to initiate further fermentations.

The ancient Kefir process highlights some of the main advantages of immobilizing cells. In fact, the use of immobilized cells has some significant advantages compared to free cell processes. Industrial benefits to immobilization include enhanced fermentation productivity and cell stability and lower cost of downstream processing (Groboillot *et al.* 1994). Moreover, immobilization facilitates cell separation, enables high cell concentrations within reactor systems, allows continuous fermentors to be operated beyond the nominal washout flow rate, and may protect against contamination (Atkinson and Mavituna 1987).

Immobilization of cells is used for a variety of reasons in modern biotechnology. The applications range from immobilization in order to exploit catalytic activities of single enzymes or sequences of enzymes within nonviable cells, to the use of immobilization as a way to provide a highly concentrated source of viable cells for production purposes.

The aim of this work has been to study the phenomena that control growth, cell release and metabolite production by entrapped viable lactic acid bacteria – and to apply the gained knowledge in order to affect the performance of the immobilized system.

1.2 LACTIC ACID BACTERIA

Lactic acid bacteria have attracted considerable interest due to the essential role these bacteria play in food processing. The most well known applications of lactic acid bacteria are the processes, which transform fresh milk into fermented products such as cheese, yogurt and kefir. Aside from their fundamental roles in the formation of the characteristic organoleptic and rheological properties of fermented food products, lactic acid bacteria are important through their ability to inhibit unwanted bacteria, thus increasing the shelf life of the products. Lactic acid bacteria inhibit other bacteria mainly through their ability to produce organic acids (mainly lactic acid), thereby lowering the pH. However, lactic acid bacteria can also produce other inhibitory compounds, such as bacteriocins (Champagne *et al.* 1994). Bacteriocins are biologically active peptides and proteins with antimicrobial activity against other Gram-positive bacteria, including food-spoilage and/or pathogenic strains (De Vuyst and Vandamme 1994).

An unequivocal definition of the term lactic acid bacteria does not exist (Axelsson 1998). However, *typical* lactic acid bacteria are Gram-positive, nonsporing, catalase-negative, devoid of cytochromes, of non-aerobic habitat but aerotolerant, fastidious, acid-tolerant, and strictly fermentative with lactic acid as the major end product during sugar fermentation (Axelsson 1998). Lactic acid bacteria are generally associated with habitats rich in nutrients, such as various food products (milk, meat beverages, vegetables), but some are also members of the normal flora of mouth, intestine and vagina of mammals. The classification of Lactic acid bacteria has been subject to some controversy in the recent years, mostly due to the recent developments in genetic tools for phylogenetic characterization. However, according to Axelsson (1998) lactic acid bacteria can be classified into the following genera: *Aerococcus*, *Alloiococcus*, *Dolosigranulum*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weisiella*. In food applications, species within *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* are most common.

In this study, *Lactococcus lactis* ssp. *lactis* (Strain NCDO 6681) has been selected as a model organism in experiments with immobilized lactic acid bacteria. In addition, *Enterococcus faecium* CTC 492 is used in order to produce the bacteriocins Enterocin A and Enterocin B from immobilized cells. Strains of *L. lactis* ssp. *lactis* are involved in a number of dairy fermentations, especially in the manufacture of various cheeses and fermented milks (Johnson and Steel 1997).

1.3 TYPES OF CELL IMMOBILIZATION

The successful application of an immobilized cell system relies on the proper choice of the components of the system. A variety of immobilization methods exist, and the choice of immobilization method is directed by the type of application and possible operational conditions. Karel *et al.* (1985), Buck (1986), Groboillot *et al.* (1994) and Willaert and Baron (1996) have all reviewed methods for cell immobilization. The following introduction is based on these review articles.

Immobilization methods can be classified into four categories based on the physical mechanism of cell confinement and the nature of the support material. These categories are "attachment to a surface", "entrapment within a porous matrix", "containment behind a barrier" and "self aggregation".

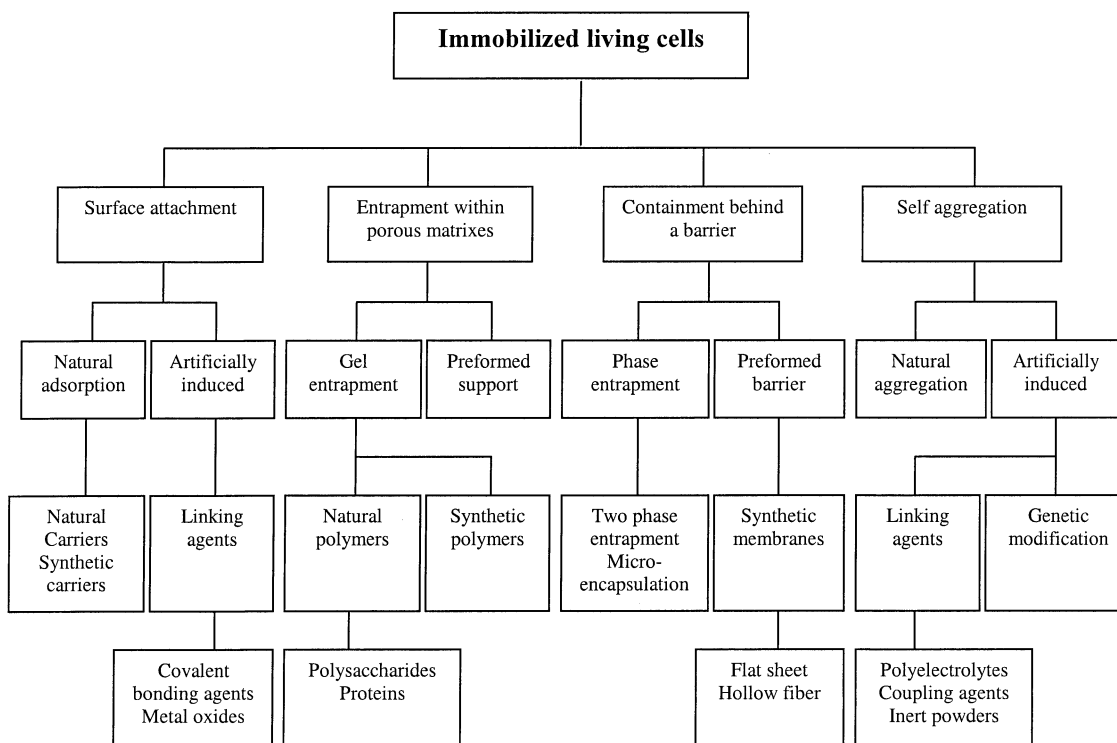


Figure 1: Classification of immobilized cell systems into four classes. (Willaert and Baron 1996)

1.3.1 Immobilization by attachment to surfaces

Cells can be immobilized onto the surface of a carrier matrix. This immobilization may be due to adsorption or covalent bonding. The surface attachment can be artificially induced using linking agents (metal oxides or covalent bonding agents) or can occur naturally by adsorption on various carriers. When adsorption techniques are used, the cells are initially attracted to the support by electrostatic interactions (van der Waals forces, ionic forces, and hydrogen bonds) (Willaert and Baron 1996). If cell growth occurs, a biofilm may form on the support matrix. Porous carriers are often used in order to increase the cell loading of the system. This type of immobilization exploits the natural preferences of some cells to adhere to, and grow on, surfaces. In some cases immobilization is performed simply by providing a carrier with a suitable surface to the cells. Therefore, this type of immobilization can be simple and the immobilization conditions are gentle. The weakly bonded biomass is a disadvantage in some applications as the biomass may easily be detached from the carrier. In some applications, a covalent attachment of the biomass can circumvent this problem. The cells may, however, be damaged during the attachment, as the cell wall is covalently linked to the support material. Covalent attachment of cells is therefore often not a preferred technique for viable cell immobilization, but is in common use if enzyme activities with cells are being exploited.

1.3.2 Immobilization by entrapment within porous matrixes

It is common to distinguish between two different categories of entrapment techniques: gel entrapment and entrapment in a preformed support. In the first case the cells are immobilized by a gel network, which is formed around the cells during the immobilization process. In the second case, the cells are allowed to move into a preformed matrix. The latter form of entrapment is not discussed further in this introduction.

Immobilization by gel entrapment might be further divided into entrapment within natural or synthetic polymers. Gel entrapment within gels of natural polymers is a very mild technique and damage to living cells can be minimized. Due to the mild conditions during immobilization and the ease of handling, gel entrapment within natural polymers has become very popular. The most popular natural polymers are alginate and carrageen (Willaert and Baron 1996). Most of the synthetic polymers used for immobilization are covalently cross-linked in order to produce a gel network. The cross linking process often involves reactive agents, and a significant loss of viability is not uncommon due to the toxic effects of these

agents (Willaert and Baron 1996). However, in some cases, the viability-loss can be regenerated through growth of the immobilized organism(s).

Immobilization of cells within gel beads is typically performed in a two-step procedure involving dispersion and hardening. Dispersion is usually performed by extrusion or emulsification. (Groboillot *et al.* 1994). Hardening is performed by cooling or by addition of a cross-linking agent such as Ca-ions or K-ions. The most common immobilization procedures result in small gel beads ranging from 1 to 5 mm in diameter (Willaert and Baron 1996).

1.3.3 Immobilization by containment behind a barrier

It is common to distinguish between containment behind a preformed barrier and containment behind barriers formed around the cell during immobilization. In the first case, cells are immobilized within preformed barriers such as hollow fiber systems or in membrane reactors. In the second type, barriers such as microcapsules or simply the liquid/liquid surface of two immiscible liquids are immobilizing the cells.

A microcapsule consists of a semipermeable thin membrane surrounding a liquid core. A capsule is similar to a microcapsule, except for a larger diameter (microcapsule: a few microns to 1mm, capsule: 0.5 to 4 mm) (Groboillot *et al.* 1994). The terms microcapsule and capsule is, however, also often used for coated beads with a gelled core. Microencapsulation is often used in the pharmaceutical or medical field for special applications such as drug and enzyme immobilization (Groboillot *et al.* 1994). There has been a significant interest in the use of microencapsulated transplant tissue, especially in the use of microencapsulated islets of Langerhans cells as a cure for diabetes (Lim and Sun 1980, Soon-Shiong *et al.* 1992, Soon-Shiong *et al.* 1994). Similarly, there has been a significant interest in microencapsulation of mammalian and insect tissue cells for *in vitro* production of high-value products, such as antibodies, growth factors and interferon (Willaert and Baron 1996). However, only a few reports exist on use of this type of technology for immobilization of lactic acid bacteria (Groboillot *et al.* 1993, Groboillot *et al.* 1994, Larisch *et al.* 1994).

1.3.4 Immobilization by self aggregation

Natural aggregates are formed by a variety of cells. The most common are among fungi, algal, plant and animal cells (Willaert and Baron 1996). Artificial flocculation agents can be used in order to enhance flocculation of cells that do not flocculate naturally.

1.3.5 The choice of immobilization method

As seen from the discussion above a variety of immobilization methods exist. The choice of immobilization method is largely governed by the project goals and the nature of the cells, substrate and waste products. Bucke (1986) has summarized important criteria that must be fulfilled by an immobilization method in order to find a wide industrial acceptance:

- It must be safe
- It must be simple
- It must give a long-term immobilization
- It must give a high activity
- It must be cheap.

The most common types of immobilization are adsorption to surfaces, natural flocculation and entrapment within natural polymers such as Ca-alginate and κ -carrageen. These methods are the ones that affect the immobilized biomass the least and are in most cases in compliance with the nature of the immobilized cells.

This study has focused on immobilized lactic acid bacteria, which is used in food fermentations. Due to its biocompatibility, ease of handling, and approval as a food additive, alginate alone or in combination with chitosan has been chosen as the preferred immobilization matrix in this study.

1.4 THE IMMOBILIZATION MATERIALS

1.4.1 Alginate gel

Alginate is a polysaccharide whose occurrence in nature is mainly limited to marine brown algae (Painter 1983). In brown algae, alginate is located in the intracellular matrix as a gel in combination with sodium, calcium, magnesium, strontium and barium. Its main function is skeletal, giving both strength and flexibility to the algal tissue (Strand *et al.* 2000).

Alginate is a heteropolymer consisting of unbranched binary copolymers of 1→4 linked β -D-mannuronic acid (M) and its C-5 epimer α -L-guluronic acid (G). The uronic acids are arranged block-wise in chains as M-blocks and G-blocks, or in blocks with alternating sequence. The chains have widely varying chain lengths. Alginates have a varying composition and sequential structure, depending on the source organism and tissue. These factors, combined with the average molecular weight and concentration, affect important

functional properties of alginate solutions, such as gel-strength and viscosity (Smidsrød and Skjåk-Bræk 1990).

The most important functional property of alginate in cell immobilization is of course its ability to form ionotropic gels. Alginate gels are formed as the G-blocks interact with divalent cations, such as barium, strontium and calcium in a highly cooperative manner. The sizes of the cooperative units are reported to be more than 20 monomers (Smidsrød and Skjåk-Bræk 1990). The diaxially linked G-residues form cavities which function as binding sites for ions, and sequences of such sites form bonds to similar sequences in other polymer chains, thus forming the gel network (Smidsrød and Skjåk-Bræk 1990).

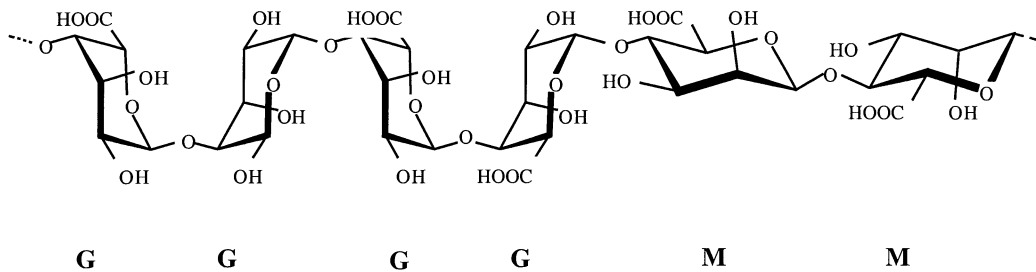


Figure 2: The molecular structure of alginate. 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). (Source: Strand *et al.* 2000)

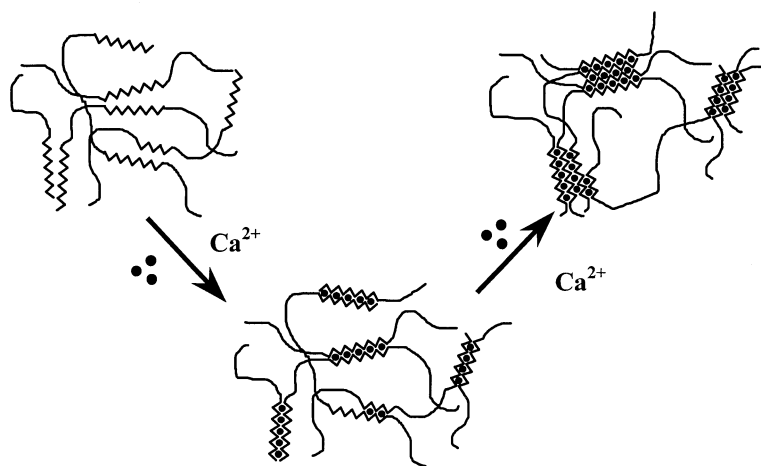


Figure 3: Cooperative binding of Ca^{2+} to G-blocks of different chains gives a gel according to the "egg-box" model. By adding more Ca^{2+} to the system, more G-blocks add to the junction zones, thereby increasing the porosity of the gel. (Source: Strand *et al.* 2000)

The length of the G-blocks and the fraction of G in the alginate is the most important structural feature contributing to gel formation (Martinsen *et al.* 1989, Smidsrød 1968, Skjåk-Bræk *et al.* 1986). However, studies with enzyme-modified alginates have shown that an increase of the amount of flexible segments around the junction zones through an introduction of MG-blocks results in increased syneresis and gel strength (Draget *et al.* 2000).

The algae *Laminaria hyperborea* contains alginate with a high content of G-units organized in long G-blocks. This type of alginate, which is used in this study, is characterized by high gel strength, low shrinkage during gelation, good stability towards swelling by Na⁺ ions, and a high porosity (Martinsen *et al.* 1990).

Immobilization of cells within alginate gel beads is simple and gentle. Cells and alginate polymer are mixed and dripped into a solution containing the gelling ion. Ca²⁺ and Br²⁺ are the most common gelling ions, but in principle any multivalent ion can be used (Strand *et al.* 2000). In this study, only Ca²⁺ has been used, although the use of Br²⁺ as gelling ion causes higher gel strengths (Strand *et al.* 2000). Calcium was preferred due to concern about possible health and regulatory problems with the use of Br²⁺, especially in food systems.

The distribution of the alginate polymer within the beads is influenced by the kinetics of gel-formation. Inhomogeneous alginate beads have a high polymer concentration at the surface, and decreasing concentrations against the core. According to Skjåk-Bræk *et al.* (1989) this inhomogeneity seems to be governed by the relative rate of diffusion of the sodium alginate molecules towards an inward moving gel zone. The coupled diffusion of sodium and alginate seems to result in a much faster diffusion rate than the self-diffusion rate of the macromolecule. When gels are formed in presence of non-gelling cations such as sodium or magnesium, the coupled diffusion of macromolecules and counterions are impaired, and homogeneous gels are formed (Strand *et al.* 2000).

1.4.2 Chitosan

Chitosan is an unbranched polysaccharide composed of β -(1 \rightarrow 4)-linked D-glucosamine and 2-acetamido-2-deoxy-D-glucosamine. Chitosan is produced commercially from chitin, which occurs in the exoskeleton of crustaceans. In chitin, only 2-acetamido-2-deoxy-D-glucosamine occurs. In order to produce chitosan, chitin is deacetylated under strong alkaline conditions. Chitosan is characterized by average molecular weights (M_n, M_w) and the degree of deacetylation (F_A), which is a measure of the amount of remaining acetyl groups. These groups are randomly or almost randomly distributed along the chain. (Ottøy *et al.* 1996).

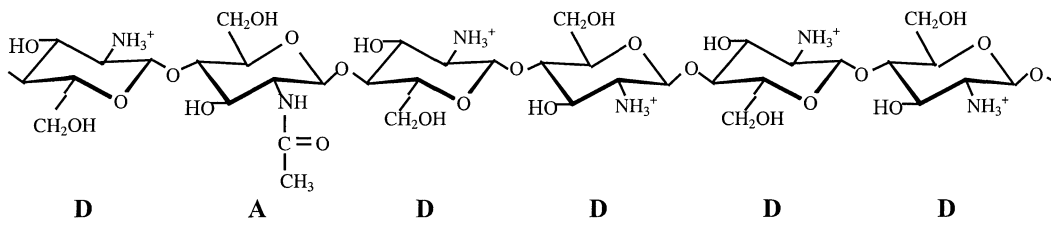


Figure 4: The molecular structure of chitosan. β -(1 \rightarrow 4)-linked D-glucosamide (A) and 2-acetamido-2-deoxy-D-glucosamide (D). (Source: Gåserød *et al.* 1998a)

The monomers in the chain are linked through β -(1 \rightarrow 4) diequatorial linkages as in cellulose and in the M-blocks of alginate. This type of linkage imposes high chain stiffness and causes an extended molecular conformation, leading to viscous solutions. The remaining acetyl groups contribute to additional chain stiffness, probably through intermolecular hydrogen bonding (Wang *et al.* 1991, Antonsen *et al.* 1993).

Chitosan has a pK_a value of 6.2-7.0 (Rinodau and Domard 1989), and becomes water soluble in acidic solution. The solubility of chitosan is, however, influenced by the degree of deacetylation (F_A). When the pH of an acidic solution of chitosan is increased, a low acetylated chitosan ($F_A = 0.01$) starts to precipitate at pH 5.5, while a highly acetylated chitosan is still fully soluble at pH 8.0 (Vårum *et al.* 1994). The positive charges on the amino groups in acid conditions (a polycation), makes chitosan different from most polysaccharides, which are neutral or negatively charged. Polyelectrolyte complexes are formed between chitosan and alginate due to the opposite charges of the molecules.

There exist two different approaches used in order to produce beads of alginate and chitosan. Gåserød *et al.* (1998a) referred to these procedures as *the one-stage procedure* and *the two-stage procedure*. In the one-stage procedure, a solution of alginate is dropped directly into a solution containing the polycation (Huguet *et al.* 1996). In this case, a capsule membrane is formed almost instantaneously around the alginate drop. The core can be gelled by transferring the capsules to another solution containing gelling ions (Gåserød *et al.* 1998a). In the two-stage procedure, preformed alginate beads are allowed to react with chitosan in a chitosan solution.

When quantitatively studying the interaction of chitosan and alginate in microcapsules, Gåserød *et al.* (1998b) found that the amount of chitosan was 100 times less in microcapsules made by the one stage procedure. In a different study, Gåserød *et al.* (1998c) investigated the mechanical properties of different chitosan alginate microcapsules. The stability of alginate-chitosan capsules was shown to depend on the amount of chitosan bound to the capsules. Beads made by the one stage procedure (Chitosan Mn = 16000, $F_A = 0.11$) were reported to be significantly weaker than beads made by the two-stage procedure. With two stage beads, the use of homogeneous alginate beads, addition of CaCl_2 in the chitosan solution, and the use of low molecular weight chitosan (Mn = 18000) enhanced the mechanical strength. Later, Bartkowiak and Hunkeler (1999a, 1999b, 2000) have proposed a one-stage procedure using oligo-chitosan (short-chained chitosan) in order to produce strong microcapsules. In this work, strong, stable capsules are generated with a maximum strength at a chitosan molar mass of 2000-3000 (10-15 oligomers).

1.5 THE BEHAVIOR OF IMMOBILIZED LACTIC ACID BACTERIA

1.5.1 Growth of cells within gel matrixes

Microorganisms immobilized in gels like alginate are surrounded by a gel-network that strongly limits their movement. The pore size of 2% alginate gels ranges from 5 nm to 200 nm (Smidsrød and Skjåk-Bræk 1990). This is well below the size of most microorganisms, which in most cases have a diameter of more than 1 μm . Several authors have studied growth of microorganisms within gel-networks (Larreta Grade *et al.* 1981, Dhulster *et al.* 1984, Gòdia *et al.* 1987, Willaert and Baron 1993). Willaert and Baron (1993) studied growth of yeast cells immobilized in 2% alginate gel by online microscopy. Initially, the cells were evenly distributed within the gel network. As the cells divided, the mechanical stress generated by the growing immobilized cells pushed the gel network away, and colonies containing densely packed cells were formed within the gel. The cells are packed closely within the colonies by the resistance of the gel-network to the mechanical stress imposed by the growing cells. Willaert and Baron (1993) reported a cell volume fraction of 0.73 within the colonies. This value was compared to the volume fraction obtained with hexagonal close packing of spheres, which gives a volume fraction of 0.74.

The mechanical stress generated by growing immobilized cells can be significant. Stewart and Robinson (1989) have reported stresses of up to 3 atm exerted by entrapped *Escherichia coli*. In view of this substantial stress, it is hardly surprising that growing microorganisms can rupture gels and hollow fiber membranes (Stewart and Robinson 1989).

As a growing colony expands, it may eventually reach the surface of the gel-bead. This may lead to an eruption in which all or parts of the colony is released to the surrounding medium (Hüsken *et al.* 1996). This eruption leaves a cavity in the gel. Once the cavities have released all or parts of the content, they may close again. As a consequence, new cycles of growth and release may occur in the cavities (Sodini *et al.* 1997). This mechanism may explain the occurrence of cross-contamination of beads initially immobilizing different strains. Lacroix *et al.* (1996) observed that beads initially containing 1 of 4 strains of lactic acid bacteria were cross-contaminated when beads containing all strains were used together in continuous fermentations of milk. This was explained by a mechanism in which a small amount of the surrounding medium had been entrapped within the gel as the cavities closed. If so, recolonization of the cavities would occur as a result of the activity of the original bead culture and the entrapped contaminating culture. The final strain composition within the beads is thus a result of the competition between the enclosed strains. The outcome of this competition is a result of the response of the entrapped strains on the local environment within the beads.

1.5.2 Transport of substrates and waste products

In fermentations with entrapped growing microorganisms, several authors have reported that the bead size influences significantly the fermentation kinetics (Yabannawar and Wang 1990, Audet *et al.* 1989, Cachon *et al.* 1995a). Cachon *et al.* (1995a) reported that a reduction in bead size from 1.3 mm to 0.75 caused an increased productivity, a doubling of the immobilized biomass, and a 50% increase in growth yield in fermentations with immobilized *L. lactis* ssp. *lactis* bv. *diacetylactis*. Similarly, several authors have reported that a heterogeneous distribution of the biomass appears during fermentation (Prevost and Divies *et al.* 1988, Arnould and Lacroix 1991, Cachon *et al.* 1993). It has been reported that 95% of the biomass was localized in the outermost 300 µm of beads containing *L. lactis* ssp. *lactis* bv. *diacetylactis* fermented continuously for 50 hours (Cachon *et al.* 1993). Obviously, these phenomena appear as a consequence of uneven growth conditions within the beads. The main causes of the uneven growth conditions are limitations in the transport of substrates and waste products to and from the immobilized bacteria. This transport occurs mainly through diffusion.

Basic diffusion theory:

Molecular diffusion can be defined as the transfer or movement of individual molecules through a space by means of the random, individual movement of the molecules. Concentration gradients are the driving force of molecular diffusion and the net transport of molecules by diffusion is from high- to low-concentration regions. Fick's first law describes the molecular diffusion flux of a component A in the ω -direction in a mixture of the components A and B:

$$J_A = -D_{AB} \cdot \frac{\partial C_A}{\partial \omega}$$

Where J_A is the rate of the molecular diffusion ($\text{kmol/m}^2\text{s}$), D_{AB} is the diffusion coefficient of A in a mixture of A and B (m^2/s), and $\partial C_A/\partial \omega$ is the concentration gradient of A in the ω -direction ($(\text{kmol/m}^3)/\text{m}$).

All mass transfer processes have an initial period of time with unsteady state conditions. This means that the concentration at a certain point varies with time, until a steady state is reached. The unsteady-state molecular diffusion in one direction through a fluid is given by:

$$\frac{\partial C_A}{\partial t} = D_{AB} \cdot \frac{\partial^2 C_A}{\partial \omega^2}$$

Which is known as Fick's second law and relates concentration C_A to a position ω at time t .

Diffusion in systems with gel immobilized cells

In fermentations with gel-immobilized microorganisms, substrates are consumed and waste products are formed within the immobilization matrix. As a consequence, these solutes must be transported between the bulk liquid phase and the microorganisms within the beads. The total activity of the immobilized system is dependent on both the transport capability and the metabolic activity within the beads. If the potential of microbial consumption/production of solutes rises above a certain level, the transport process becomes the rate-limiting step. The transport processes occur through the action of three processes: distribution, dispersion and diffusion. The two first processes occur through fluid movements. Transport by distribution

and dispersion is, however, limited to the sizes of the smallest eddies which are formed in the fluid under the current mixing conditions. Beyond this distance, transport processes occur through diffusion. In systems with gel-immobilized microorganisms, several transport processes occur through diffusion.

The solutes are transported to and from the surface of the beads by diffusion. This transport is normally denoted external mass transfer or film diffusion. If the external mass transfer is limiting, the concentration close to the surface of the beads will be significantly lower than the bulk-phase concentration. The bead diameter, viscosity, and the stirring of the fermentation broth are important parameters affecting the external resistance to mass transfer.

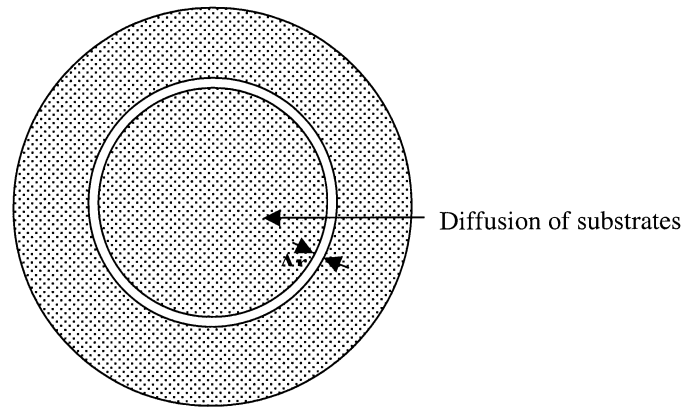
Within gels, diffusion is usually the only mechanism of solute transport. The macromolecules, which form the gel network, are normally present in dilute concentrations in most gels. Therefore, gels can in most cases be looked upon as “porous” semisolid materials where the “pores” or open spaces in the gel structure are filled with water (Øyaas 1995). As a consequence, the diffusion rates of small solutes are somewhat lower in gels, compared to in aqueous solution. The presence of cells within the gel-matrix causes a further reduction of the diffusivity of small molecules in the system. Compared to free cell systems, the presence of the immobilization matrix increases the path length of solute transport by diffusion in gel-immobilized systems. Another consequence of the gel-matrix is that uneven equilibrium distributions of solutes between the gel phase and the liquid phase might occur. Øyaas *et al.* (1995) reported a thermodynamic equilibrium constant of 1.18 of lactic acid in 2% alginate gel. A similar uneven equilibrium distribution of lactose was not observed by Øyaas *et al.* (1995).

The impact of diffusional limitations on the performance of gel immobilized cells

The transport phenomena related to diffusion of substrates and waste products have been intensely studied. In a classical paper, Engasser and Horwarth (1973) were the first to use mathematical models as an approach to study kinetics of heterogeneous enzyme systems. A material balance was used in order to produce a differential equation representing diffusion and reaction in a spherical biocatalyst. In this mass balance, Michaelis Menten kinetics was combined with Fick's first law and applied to a thin spherical shell with a thickness of Δr within a spherical biocatalyst.

Later, mathematical simulations have been used to study mass transfer phenomena in fermentations with immobilized lactic acid bacteria. Yabannavar and Wang (1990) used mathematical simulations to examine the influence of substrate and product transport in

extractive lactic acid fermentations with *Lactobacillus delbrueckii* immobilized in κ -carrageen. Cachon *et al.* (1995a) used mathematical simulations and practical experiments in order to study growth and mass transfer of *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* immobilized in alginate beads. In the later study the model included both external mass transfer, a model of the kinetic behavior of the organism, effects of cell loading on solute diffusion and cell release.



$$D_{Ae} \left(\frac{d^2 C_A}{dr^2} r^2 + 2r \frac{dC_A}{dr} \right) - r_A r^2 = 0$$

Figure 5: Diffusion and reaction in a spherical biocatalyst. The mass balance across a spherical shell with thickness Δr located a distance r from the surface. The local reaction rate within the shell is r_A

Both models indicate that a heterogeneous distribution of the biomass will evolve during fermentation. This heterogeneous biomass distribution does not, however, evolve due to a limitation in the growth substrate, but due to diffusional limitations of the lactate efflux. This limitation results in an accumulation of lactate/lactic acid and a concomitant acidification of the local environment (Yabannavar and Wang 1990, Cachon *et al.* 1995a). The model published by Cachon *et al.* (1995b) calculates a pH of 4.4 within the central parts of beads at 70 h of continuous fermentation. At this pH, the growth of *L. lactis* ssp. *lactis* bv. *diacetylactis* is severely inhibited, most likely due to inhibition by nondissociated lactic acid (Cachon *et al.* 1994). Kinetic studies performed by Gätje and Gottschalk (1991) showed that

pH acts indirectly on the activity of lactic acid bacteria by changing the proportion of nondissociated lactic acid. Nondissociated lactic acid represents 22% of the total lactic acid content at pH 4.4. The nondissociated form of lactic acid diffuses through the cell membrane and dissociates within the cells, thus interfering with the pH-gradient across the cell membrane (Kashket *et al.* 1987). Due to this, the cells are forced to use energy on maintenance of the pH gradient. This energy loss reduces the cellular growth, as the energy lost would otherwise have been used for cell growth.

Several studies have confirmed the presence of large pH gradients within beads immobilizing lactic acid bacteria. Masson *et al.* (1994) were the first to use microelectrodes in order to measure the local pH within beads containing immobilized lactic acid bacteria. Cachon *et al.* (1997) used a similar technique in order to compare experimental results from fermentations with immobilized *L. lactis* ssp. *lactis* bv. *diacetylactis* to mathematical simulations of the immobilized system. In both these cases, a sharp pH gradient was measured within the beads.

Previously, several authors have studied the performance of gel-immobilized lactic acid bacteria in continuous culture (Prevost *et al.* 1985, Prevost and Divies 1988, Audet *et al.* 1988 and 1991, Arnauld *et al.* 1992). Continuous culture systems are well suited for studies of immobilized cells, as control of the external environment is easily facilitated in these systems. In the reported studies, an initial phase with increasing activity was followed by a pseudo steady state activity. Both the simulations published by Cachon *et al.* (1995,1997) and the experimental data indicates that during the pseudo steady state biomass growth within the beads is balanced by cell release to the fermentation broth. In the pseudo steady state, the heterogeneous biomass distribution within the beads has been established and most of the biomass is located close to the surface of the beads. In the work reported by Cachon *et al.* (1997), the effects of a varying dilution rate on the pH gradient, biomass and activity distributions within the beads as well as cell release were examined with mathematical modeling and experimental measurements. The biomass within the beads, the release of cells to the fermentation broth and the pH gradient within the beads were all affected by the dilution rate. A reduction in the dilution rate led to lactate accumulation in the fermentation broth, and increased the amount of undissociated lactic acid within the beads. This reduced the amount of active biomass within the center of the beads and decreased the pH-gradient within the beads.

1.5.3 Fermentations with immobilized lactic acid bacteria

The use of immobilized lactic acid bacteria has been proposed for a variety of applications, mainly associated with the dairy industry. The main areas are in raw milk treatments, production of diary starters, yogurt production, cheese manufacture and cream fermentation (Champagne *et al.* 1994).

In raw milk treatment, the use of immobilized lactic acid bacteria has been proposed in order to inhibit psychrotropic bacteria. This application is based on the lactoperoxidase system of milk, which can be activated by selected strains of lactic acid bacteria through production of H₂O₂ (Champagne 1990). Similarly, the use of immobilized lactic acid bacteria has been proposed in order to acidify raw milk prior to ultrafiltration in cheese production (Kim *et al.* 1985). Preacidification of milk prior to ultrafiltration modifies permeate fluxes and reduces the calcium content of the resulting caseins (Champagne *et al.* 1994).

Several authors have proposed to use immobilized lactic acid bacteria for continuous inoculation. In continuous fermentations with gel-immobilized lactic acid bacteria, a steady state occurs in which biomass growth within the immobilization matrix is balanced with a release of cells into the fermentation broth. As a consequence, a continuous prefermentation step might be combined with inoculation of a subsequent batch fermentation. This combination has been shown to reduce the total time of fermentation necessary in yogurt and fresh cheese production (Prévost and Divies 1988a, Prévost and Divies 1987).

Immobilized lactic acid bacteria have been proposed for several applications within cheese manufacture. In addition to prefermentation and starter production, the use of immobilized lactic acid bacteria has been proposed for in-situ bacteriocin production. Due to a simplified cell recovery, Zezza *et al.* (1993) investigated the use of nisin producing *L. lactis* ssp. *lactis* NZ1 immobilized in calcium alginate beads in order to inhibit *Clostridium tyrobutyricum* in cheese manufacture.

Furthermore, the use of immobilized lactic acid bacteria has been shown to protect against bacteriophages (Champagne *et al.* 1988) and to reduce problems with contamination (Champagne *et al.* 1989). Immobilization has also been proposed in order to enhance the stability of freeze-dried starter cultures (Champagne *et al.* 1994), and as a way to produce concentrated cultures of lactic acid bacteria (Morin *et al.* 1992, Champagne *et al.* 1993). Alginate immobilized lactic acid bacteria have also been used in a continuous process for treatment of dairy wastewater, producing a feed supplement product (Dyrset *et al.* 1998).

1.5.4 Cell release in fermentations with immobilized lactic acid bacteria

In applications such as prefermentation, continuous inoculation and yogurt production, a high production of free cells is desirable. In other applications, the main product is a metabolic product, and not the producing cells. A high content of the producing organism in the product might even be undesirable. Production of fermented cream is one such application. The use of fermented cream is desirable in cottage cheese making and butter manufacture, but a high content lactic acid bacteria in the product is undesirable, since cells released into the product may result in continued acidification during storage (Champagne and Côte 1987). Other examples of applications where a high content of the production organism in the product is undesirable are inhibition of psychotropic bacteria, acidification of raw milk, and *in situ* bacteriocin production. In some applications, problems with release of free cells can be circumvented through the use of nongrowing immobilized microorganisms. This is, however, not possible in applications where growth associated products, such as extracellular polysaccharides or bacteriocins, are produced. The use of nongrowing cells is also difficult in many traditional food fermentations such as raw milk fermentations.

The use of gel-immobilized lactic acid bacteria has been shown to reduce the amount of free cells in fermentation. Champagne and Côte (1987) investigated the use of alginate immobilized mesophilic lactic acid bacteria in order to produce a fermented cream with a lower amount of free cells, compared to cream fermented in a traditional free cell process. Although the use of immobilized lactic acid bacteria resulted in a lower amount of free cells in the fermented cream, there was still a significant release of free cells. As described in the previous chapter, a significant release of free cells is normal in fermentations with growing gel-immobilized microorganisms. Due to this, a significant amount of effort has been focused on reducing the amount of cell release in immobilized fermentations during the recent years. The first work on reducing cell release was done with immobilized yeast used in sparkling wine manufacture. In traditional sparkling wine fermentations, a second fermentation stage is performed in order to produce dissolved CO₂. In the Champagne process, this fermentation is done in the bottles. However, the following removal of the yeast from the bottles is an extremely labor-intensive process. Gòdia *et al.* (1991) demonstrated a greatly simplified process in which beads with alginate immobilized yeast coated with a cell free alginate layer was used in order to produce the necessary CO₂ in the bottles. The presence of the cell free layer on the beads lowered the amount of cells released to the wine during the fermentation. Later, several strategies to a reduced cell release have been investigated. Prevost and Divies (1992) used alginate coated alginate beads immobilizing lactic acid bacteria in order to

produce a fermented cream with a low content of free cells in batch fermentations. Champagne *et al.* (1992) rinsed alginate beads containing immobilized lactic acid bacteria with distilled water, ethanol, $\text{Al}(\text{NO}_2)$ or hot CaCl_2 solutions before fermentation in order to kill bacteria near the bead surface. Another approach exploited by Champagne *et al.* (1992) was the application of multiple coats of poly L-lysine and alginate, which reduced the final concentration of free cells by a factor of 10 in sequential batch fermentations of milk. Yet another approach to a reduced cell release was demonstrated by Zhou *et al.* (1998), who investigated the use of chitosan in reducing cell release. Zhou *et al.* (1998) reported that the final number of free cells was reduced in repeated 2-hour batch-fermentations of milk with chitosan coated alginate beads, compared to fermentations with uncoated beads.

Batch fermentations are perhaps the most realistic experimental design in order to investigate whether a process can be performed with a lower concomitant cell release. However, in all cases above a concomitant reduction of the bead activity was reported along with the lower cell release which prologs the time of fermentation. The final concentration of free cells in batch fermentation is not only a function of release of cells from the beads, but also a function of growth of free cells. As a consequence, a different experimental approach might be in more useful order to get additional information on the relationship between cell release and the activity of the immobilized biomass.

2 RESULTS AND DISCUSSION

As seen in the literature review in the previous chapter, a significant amount of scientific effort has been focused on studies of immobilized lactic acid bacteria. The literature shows that a significant release of free cells normally occurs from growing gel-entrapped microorganisms. Therefore, some scientific effort has been focused on the mechanisms of cell release and on ways to influence on the amount of cells release during operation.

The aims of this work have been to study growth, cell release and metabolite production of entrapped viable lactic acid bacteria in fermentation systems mainly relevant for food applications. Therefore, immobilization methods based on alginate and chitosan have been chosen due to the biocompatibility of these materials. With these systems, we wanted to further investigate ways to measure, characterize and influence activity and release of immobilized lactic acid bacteria. Immobilization methods and materials as well as changes in growth environment and process configuration have been investigated in order to affect the performance of the immobilized systems. The strains *Lactococcus lactis* ssp. *lactis* NCIMB 6681 and *Enterococcus faecium* CTC 492 have been selected as model organisms in these studies.

2.1 MEASURING CELL RELEASE IN A SYSTEM WITH CHITOSAN AND ALGINATE COATED BEADS.

Previously, several authors have proposed the use polycation coatings on gel beads in order to affect rates of cell release (Champagne *et al.* 1992, Larisch *et al.* 1994, Yoo *et al.* 1996, Zhoo *et al.* 1998), Zhoo *et al.* (1998) used chitosan coatings on alginate beads immobilizing lactic acid bacteria in order to reduce the final amount of free cells in batch fermentations. In the work presented in paper 1 we wanted to further study the use of chitosan in order to affect release of immobilized lactic acid bacteria during fermentation. However, in most of the previous studies, a reduction of cell release has led to a reduction of the activity of the beads. In batch systems, this reduction in activity prolongs the time of fermentation. Therefore, alternative experimental systems were examined in order to account for possible effects of this reduction in activity. Please refer to Appendix 1: *Cell release from alginate immobilized Lactococcus lactis* ssp. *lactis* in chitosan and alginate coated beads. (Paper 1.)

In this work, effects of chitosan and alginate coatings of alginate beads with entrapped *Lactococcus lactis* ssp. *lactis* were studied in batch and continuous fermentations. The use of chitosan coating reduced the final concentrations of free cells, the initial release of free cells

and the rate of lactate production in five consecutive batch fermentations of milk, fermented to a final pH of 4.7. In this study, differences in metabolic activity between uncoated alginate beads and chitosan-coated beads were taken into account by studying cell release in batch fermentations in which a final pH was used as a stop criterion.

The lower rates of lactate production seen with chitosan-coated beads prolonged the times of batch fermentation, thereby increasing the time available for cell release and growth of free cells. Furthermore, the final amount of free cells in these fermentations might be influenced by the varying operational pH, and by differences in the initial release of free cells. Therefore, an alternative experimental approach based on continuous fermentation at a controlled pH and a high dilution rate was used in order to study cell release without these disturbing effects. This experimental system facilitated direct measurements of rates of cell release during fermentation through the aid of simple mass balances. In addition, this system facilitated evaluation of bead treatments on the basis of the ratio of the rates of cell release to lactate production at any given time during the test period. Therefore, this method allowed differences in activity imposed by the bead treatments to be taken into account.

In order to estimate the effects of different bead coatings on cell release, alginate beads were coated with chitosan or alginate, or sequentially with chitosan/alginate, or chitosan/alginate/chitosan (CAC-coatings). Chitosan coating alone seemed to reduce rates of cell release only in the early stages of the fermentation, while sequential coatings with chitosan and alginate showed significant reductions throughout the whole 48-hour test period. Studies of the ratio of rates of cell release to lactate production showed that the observed reductions in rates of cell release were not simply a result of a decrease of the metabolic activity of the biomass within these beads.

2.2 PH CONTROLLED BIOMASS DISTRIBUTION AND CELL RELEASE FROM CA-ALGINATE BEADS

As seen from the literature review in chapter 1, a significant amount of scientific effort has been focused on the study of the processes that influence on the biomass distribution within the beads. Practical experiments and mathematical simulations performed by Cachon *et al.* (1995b, 1997) indicate that heterogeneous biomass distributions within gel beads immobilizing lactic acid bacteria appear due to diffusional limitations of lactate efflux, causing low pHs and high concentrations of lactic acid in the central parts of the beads. Therefore, a study was conducted in order investigate whether cell release and biomass distribution of alginate immobilized *Lactococcus lactis* ssp. *lactis* NCIMB 6681 could be affected through changes in the growth conditions. Please refer to Appendix 2: “pH controlled

cell release and biomass distribution of alginate immobilized Lactococcus lactis ssp. lactis” (Paper 2).

Biomass distributions within Ca-alginate beads immobilizing *L. lactis* ssp. *lactis* NCIMB 6681 and rates of lactic acid production of beads with different sizes were examined in the pseudo steady state of continuous fermentations. Both biomass distribution profiles and the observed influence of bead-size on volumetric rates of lactic acid production were consistent with the present hypotheses that biomass and metabolic activity are primarily located close to the surface. As the non-homogeneous distribution of biomass allegedly evolve due to low pH and high concentrations of nondissociated lactic acid, we examined whether a change in pH of the external medium would affect internal biomass distributions and the subsequent cell release. These experiments were based on the hypothesis that an increased pH in the growth medium might lead to unfavorable growth conditions in the growth medium and in the periphery of the beads. In this situation, growth may proceed in the interior of the beads as the cells are protected by the pH gradient caused by lactic acid production.

A change in operating pH from our reference pH of 6.5 to pH 9.25 initially reduced the ratio of the rates of cell release to lactate production by almost a factor of 10^5 . After 48 hours of operation, the ratio of rates of cell release to lactate production was still 10 times lower than in fermentations at pH of 6.5. Similarly, the changed conditions in the surrounding medium influenced the courses of the biomass concentration in the beads during the fermentations. The biomass concentration in the beads fermented at pHs of 6.5, 8.5, 8.75, and 9.00 reached steady state values within 24 hours of continuous fermentation. However, the biomass concentration in the beads fermented at a pH of 9.25 never reached a steady state concentration within the 48 hours of fermentation examined. Although the increased pH in the surrounding medium slowed the initial increase in internal biomass concentration, these beads reached an internal biomass concentration that was approximately 5 times the steady state biomass concentration observed for the other beads fermented at pHs of 6.5, 8.5, 8.75, and 9.0. Compared to fermentations at pH 6.5, growth at higher pH's also increased the rate of lactate production. In the fermentation operated at 9.25, the final rate of lactate production was 25% greater than observed at pH 6.5.

Measurements of the pH gradient within the beads showed larger pH gradients within beads fermented at higher pH values than in beads fermented at pH 6.5. A zone with a nearly stable high pH was seen in the outermost parts of beads fermented at pH 9.25. Such a zone was not seen in beads fermented at pH 9.0, or in beads fermented at lower pH values. Both visual inspections of the beads and a pH above the pH-tolerance of the organism indicated

that there was little biological activity within this zone. This illustrates that diffusional limitations and corresponding pH-gradients can be exploited in affecting the distribution of immobilized growing cells and their concomitant release.

2.3 IMMOBILIZED PRODUCTION OF ENTEROCIN A AND B

During the last years, there has been a significant focus on natural food preservatives, including bacteriocins from lactic acid bacteria. However, only a limited amount of work has been done on improving the fermentation technology and productivity of bacteriocin production (Bhugaloo-Vial *et al.* 1996). As mentioned in chapter 1, immobilizing the bacteriocin producing cells might be a powerful tool in bacteriocin production. Therefore, a study was performed in order to investigate growth and bacteriocin production by immobilized *Enterococcus faecium* CTC492. Furthermore, use of CAC-coatings on the beads was investigated and the resulting effects on bacteriocin production, cell release and biomass were measured. Please refer to appendix 3: "*Enterocin production and cell release by alginate immobilized Enterococcus faecium CTC 492 in uncoated and chitosan/alginate/chitosan coated beads*" (Paper 3).

In this study, growth and bacteriocin production by *E. faecium* CTC492 immobilized in uncoated and CAC-coated alginate beads was evaluated in batch and continuous fermentations. Maximal enterocin activities in batch fermentations with immobilized cells were lower than in fermentations with freely suspended cells. However, the reductions of the amount of free biomass in the product phase in the immobilized systems were far greater than the concomitant reductions in enterocin activity. At most, a more than 3 log unit reduction of the amount of free biomass was obtained with CAC-coated beads. The magnitude of these reductions was, however, strongly influenced by the number of cycles of bead use and somewhat less influenced by the operational pH.

Real time studies of bead performance were performed using our experimental method of continuous fermentations at high dilution rates. These experiments showed that the use of CAC-coatings reduced the rates of cell release significantly the first 27 hours of fermentation. Beyond this time, there were no significant differences in the rates of cell release of uncoated and CAC-coated beads. However, similar to lactate production in paper 1, the use of coatings on the beads reduced rates of enterocin production as well as cell release. Therefore, ratios of rates of enterocin production to cell release were considered to be a more powerful tool in accessing effects of bead coatings. Studies of these ratios showed that the use of CAC-coatings significantly increased the ratio of rates of enterocin production to cell release during

the first 20 hours of fermentation. These data demonstrate that the relationship between enterocin activity and biomass concentration in fermentations with immobilized *E. faecium* CTC492 can be altered through use of sequential coatings with alginate and chitosan – at least within a limited time of operation.

However, growth and bacteriocin production by free and immobilized *E. faecium* CTC492 was shown to depend strongly on fermentation pH. The presence of large pH gradients within Ca-alginate beads containing immobilized *E. faecium* CTC492 during continuous fermentation were demonstrated using direct measurements of the local pH. Bacteriocin production and fermentation characteristics of immobilized *E. faecium* CTC492 seemed to be significantly influenced by the presence of these pH-gradients. A pattern of glucose consumption seen in batch fermentations with immobilized cells indicated an additional pH shift imposed by the CAC-coating. The presence of such a pH shift was detected through direct measurements of the pH-gradients within these beads.

2.4 FINAL REMARKS

Over the last decades, a large amount of scientific effort has been focused on the immobilization of living cells. This effort has generated a large amount of scientific knowledge on the behavior of immobilized cell systems and their use. The main scope of the work presented in this thesis was to contribute to a further enlightenment of a few topics within this exciting research field.

2.4.1 Measurement of cell release by continuous fermentation

In this thesis, arguments are presented in favor of using continuous fermentations as an experimental method in studies of activity and cell release of lactic acid bacteria. Previously, several authors have studied biomass production and cell release in continuous fermentations with immobilized lactic acid bacteria. Audet *et al.* 1989 examined biomass production and rates of cell release of alginate immobilized *Streptococcus salivarius* subsp. *thermophilus* at dilution rates between 0.5 and 3h⁻¹. Arnauld *et al.* (1992) examined the effect of agitation rate on cell release and metabolism of *Lactobacillus casei* subsp. *casei*. However, in the present study continuous fermentations at high dilution rates have been used as an experimental system, evaluating the use of immobilization methods and bead treatments as ways to influence the performance of the immobilized system. This approach was used in paper 1 studying the ratio of rates of cell release to rate of lactate production of beads with different coatings, and in paper 3 studying the ratio of rates of bacteriocin production to rates of cell

release. In both these studies, this experimental system provided information not easily accessed by other experimental systems. The main advantages are that this experimental approach facilitates direct measures of rate of cell release and metabolic activity at all times during the test period. Therefore, this experimental approach allows validation of treatments based on a measure of release of biomass correlated to production of the desired fermentation product at all times during the test period.

2.4.2 Growth and release of immobilized cells

In this thesis, only systems with growing immobilized cells have been studied. In some applications, growth of the immobilized biomass is not mandatory. As growth of the immobilized biomass is a major cause of cell release, problems connected to cell release are expected to be significantly less in these systems. However, in many applications growth of the immobilized organism is required. Examples of such applications are production of growth-associated products such as bacteriocins and extracellular polysaccharides (EPS).

Immobilization can influence the relationships between yields of biomass and fermentation products obtained in the product phase in at least two ways. The presence of the immobilization matrix may cause concentration gradients and high local cell densities, which might influence the metabolic activities of the cell, thereby affecting the relationship between growth and metabolite production. The immobilization matrix can also act to retain the growing biomass, thereby allowing production of growth-associated fermentation products at a greatly reduced biomass release to the product phase. However, the high mechanical stresses generated by growing cells seems to cause an inevitable release of excess biomass to the surroundings as the immobilization matrix becomes fully colonized. The initial period of reduced cell release can, however, be substantially lengthened through careful selection of proper immobilization materials and growth conditions. In this thesis, this has been demonstrated by the use of sequential coatings of chitosan and alginate, which altered ratios of rates of cell release to lactate production in paper 1 and altered ratios of bacteriocin production to cell release in paper 3.

As described in the literature review in chapter 1, diffusional limitations influence the growth patterns of immobilized lactic acid bacteria within gel beads. Transport limitations cause pH and solute gradients that favor growth and activity in the periphery of the beads and limits growth within the gel beads under normal operational conditions. Therefore, a situation that might resemble biofilm growth on inert carrier particles seems to develop as the heterogeneous biomass distribution is established and the fermentation reaches a pseudo

steady state stage. This type of development seems to be typical for systems with gel-immobilized microorganisms operated under normal operation conditions. However, results presented in paper 2 show that changes in the operation conditions can influence significantly this development. These experiments demonstrated that it was possible to change the external pH of the medium so that growth is disfavored in the surrounding medium and in the periphery of the beads, while growth and lactate production continued in the interior of the beads. Thus, this situation changed the ratio of the rate of cell release to the rate of lactate production leading to a product stream containing fewer free cells lasting throughout the 46-hour test period. Requirements for rather special growth conditions may, however, exclude this approach to a reduced cell release in some food applications. However, other possibilities may open up due to awareness of diffusional gradients within immobilization matrixes.

One obvious such possibility is operation at extreme pHs. It has previously been reported that immobilized lactic bacteria can maintain high activities after short-time exposures to extreme pH values (Dyrset *et al.* 1998). The fact that gel-immobilization imposes significant changes in the local environment experienced by the immobilized biomass was demonstrated in paper 2 and 3 through the use of microelectrodes. Similarly, a significant shift in optimal pH for growth and lactate production of alginate immobilized *L. lactis* ssp. *lactis* was seen in the studies reported in paper 2. In fact, the rate of lactate production in the pseudo steady state phase was significantly higher at pH 9.0, compared to rates of lactate production seen at pH 6.5 – the pH optimum for freely suspended cells. This increase could not be explained only by a higher maintenance production of lactic acid, as rates of biomass production were also significantly higher at pH 9.0 than seen at pH 6.5. These results show that it may be possible to exploit diffusional limitations with the concomitant pH-gradients to apply immobilized fermentations to processes involving harsh conditions that provides a certain protective and stabilizing effect to the immobilized biomass, but effectively prevents growth of contaminating organisms.

Another exciting example of use of solute gradients has been reported by dos Santos *et al.* (1996). In that paper, the authors proposed a system for nitrogen removal in which double layer beads were used for coimmobilization of microbial cells. In their system, concentration gradients within double layer beads were exploited in order to selectively immobilize different microbial populations with complementary metabolic pathways that would otherwise compete for a common substrate if immobilized in mixed culture.

Results from paper 3 might also indicate that the localization of the active biomass as well as solute gradients influence the activity of the immobilized system. As discussed above,

significant shifts in pH tolerance and operational pH can be observed in fermentations with lactic acid bacteria immobilized in alginate beads. In the pseudo steady state phase of continuous fermentations with immobilized lactic acid bacteria, most of the active biomass is located close to the surface in uncoated beads. However, within CAC-coated beads, the biomass is initially localized beneath a layer of cell free CAC-coating. The presence of this initially cell free CAC-coating will thus affect the environment experienced by major parts of the immobilized biomass. The presence of such an additional pH shift within CAC-coated beads was indicated by microelectrode measurements and a pattern of glucose consumption for bacteriocin production observed in fermentations with free and immobilized *E. faecium* CTC492.

2.4.3 Suggestions for future work

The main focus of this work has been to study and influence the processes that controls growth, release and distribution of immobilized biomass within gel beads. The use of alternative growth conditions and immobilization methods has been demonstrated as ways to influence the performance of the immobilized system. In novel applications, the selection of appropriate operating conditions will be a powerful tool to affect important properties such as cell release and biomass activity. However, in fermentations applied for the manufacture of most traditional food products, strict demands for product composition and sensory attributes may limit the possibility to alter the growth conditions. The use of novel immobilization techniques and improvements of reactor design and process layout are probably the most likely options to affect the performance of the immobilized system in these applications.

In this work, lack of time has limited the amount of effort focused on the optimization of immobilization materials and methods. Nonetheless, exciting possibilities lay in further improvement of existing immobilization materials, and in the development of novel immobilization methods for lactic acid bacteria.

The use of liquid core microcapsules to immobilize growing lactic acid bacteria is one such interesting possibility. As described previously, diffusional limitations and the mechanical consequences of microbial growth within gels seems to direct most of the growth and activity to a highly populated zone located close to the surface of the beads. The use of membrane-confined microcapsules with a liquid core might avoid this development. Growth of cells in the liquid core of microcapsules will most likely not generate the mechanical stress that causes cell release through eruptions as seen in gel-beads. Likewise, heterogeneous biomass distributions cannot evolve due to the liquid conditions within the core. Immobilization of

lactic acid bacteria within microcapsules made by cross-linked chitosan membranes has been reported by Groboillot *et al.* (1993). The harsh conditions needed to cross-link chitosan, did however, cause a significant loss of viability, leading to an initially prolonged time of fermentation. Cell release from the microcapsules was not investigated by Groboillot *et al.* (1993), and the author is not aware of any other reported studies of cell release by lactic acid bacteria immobilized within similar cross-linked liquid core capsules. Our preliminary attempts to develop such microcapsules of cross-linked chitosan were not successful. Therefore, more research is needed in order to get conclusive results on the prospects of this technique for immobilization of lactic acid bacteria.

Various other methods of immobilization within liquid core capsules have been described (Willaert and Baron 1996). Förster *et al.* (1994) immobilized *Yarrowia lipolytica* in cellulose sulphate/poly(dimethyldiallylammonium chloride) polyelectrolyte complex capsules (CS/PD-MDAAC) in order to produce citric acid from glucose in a fermentation process. In this study, microencapsulation of *Yarrowia lipolytica* strongly reduced cell release. Yoo *et al.* (1996) has described a method for immobilization of *Lactobacillus casei* cells within liquid core alginate capsules for lactic acid production. These capsules are produced by dripping a mixture of xanthan gum and cells containing Ca-ions into an alginate solution. Yoo *et al.* (1996) reports a reduction in cell release and an increase of cell viability in batch fermentations with *Lactobacillus casei* immobilized in capsules, compared to traditional beads. Bartkowiak and Hunkeler (1999ab, 2000) have recently described a method for production of alginate-oligochitosan polyelectrolyte capsules. These beads can be manufactured under physiological conditions and consist of a strong membrane surrounding a liquid core. The author is, however, not aware of any published work studying fermentation properties, such as stability and cell release with these capsules.

Preliminary studies of the kinetics of cell release and biomass activity of the latter two types of capsules have been performed. In the case of uncoated and chitosan coated alginate capsules (made according to Yoo *et al.* (1996) and coated with chitosan according the description in paper 1) a reduction in the ratio of cell release to lactate production by immobilized *Lactococcus lactis* ssp. *lactis* was seen throughout the 50 hour test periods in our experimental system (described in paper 1), compared to uncoated beads. However, this reduction was less than the reduction seen with CAC-coated beads, especially in the early phases of the fermentations. In these experiments, insufficient mechanical strength and surface colonization seemed to be the major causes of cell release. In the case of alginate-oligochitosan capsules, insufficient mechanical strength, causing a major loss of capsules, was

observed during the 46-hour test period. In both these cases, further optimization of the immobilization materials and procedures might very well resolve these problems. Therefore, more research is needed in order to get conclusive results on the possibilities these exciting new techniques may bring forth.

3 REFERENCES

- Anthonsen, M.W., Vårum, K.M. and Smidsrød, O. (1993) Solution properties of chitosans: Conformation and chain stiffness of chitosans with different degrees of N-acetylation. *Carbohydrate Polymers* 22, 193-201.
- Arnaud, J.P. and Lacroix C. (1991) Diffusion of Lactose in κ -carrageen/locust bean gum gel beads with or without entrapped growing lactic acid bacteria. *Biotechnology and Bioengineering* 38, 1041-1049.
- Arnaud, J.P., Lacroix, C. and Choplin, L. (1992) Effect of agitation rate on cell release and metabolism during continuous fermentation with entrapped growing *Lactobacillus casei* subsp. *casei*. *Biotechnology Techniques* 6, 265-270.
- Atkinson, B. and Mavituna, F. (1997) Biochemical engineering and biotechnology handbook. 1st. edition, New York: Stockton Press.
- Audet, P., Paquin, C. and Lacroix, C. Immobilized growing lactic acid bacteria with κ -carrageen-locust bean gum gel. *Applied Microbiology and Biotechnology* 29, 11-18.
- Audet, P., Lacroix, C. and Paquin C. (1991) Continuous fermentation of a supplemented whey permeate medium with immobilized *Streptococcus salivarius* subsp. *thermophilus*. *International Dairy Journal* 1, 1-15.
- Audet, P., Paquin, C. and Lacroix C. (1989) Sugar utilization and acid production by free and entrapped cells of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* subsp. *lactis* in a whey permeate medium. *Applied and Environmental Microbiology* 55, 185-189.
- Axelson, L. (1998) Lactic acid bacteria: classification and physiology In *Lactic acid bacteria. Microbiology and functional aspects, Second edition* ed. Salminen, S. and von Wright, A. pp 1-72. New York: Marcel Dekker, inc.
- Bartkowiak, A. and Hunkeler, D. (1999a) New microcapsules based on oligoelectrolyte complexation. *Annals New York Academy of Sciences* 875, 36-45.
- Bartkowiak, A. and Hunkeler, D. (1999b) Alginate-oligochitosan microcapsules: A mechanistic study relating membrane and capsule properties to reaction conditions. *Chemistry of Materials* 11, 2486-2492.
- Bartkowiak, A. and Hunkeler, D. (2000) Alginate-oligochitosan microcapsules II: Control of mechanical resistance and permeability of the membrane. *Chemistry of Materials* 12, 206-212.
- Buck, C. (1998) Methods of immobilizing cells In *Process engineering aspects of immobilized cell systems* ed. Webb, C., Black, G.M and Atkinson, B. pp. 20-33. Rugby, Warwickshire: The Institution of Chemical Engineers.
- Cachon, R. and Divies, C. (1994) Generalized model of the effect of pH on lactate fermentation and citrate bioconversion in *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis*. *Applied Microbiology and Biotechnology* 41, 694-699.

- Cachon, R., and Divies, C. (1993) Localization of *Lactococcus lactis* ssp. *lactis* bv. *diactylactis* in alginate gel beads affects biomass density and synthesis of several enzymes involved in lactose and citrate metabolism. *Biotechnology Techniques* 7, 453-456.
- Cachon, R., Catté, M., Nommé, R., Prévost, H. and Divies, C. (1995a) Kinetic behavior of *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* immobilized in calcium alginate gel beads. *Process Biochemistry* 30, 503-510.
- Cachon, R., Lacroix, C. and Divies, C. (1997) Mass transfer analysis for immobilized cells of *Lactococcus lactis* ssp. (*lactis* biovar. *diacetylactis*)??? using both simulations and *in-situ* pH measurements. *Biotechnology Techniques* 11, 251-255.
- Cachon, R., Molin, P. and Divies C. (1995b) Modeling of Continuous ph-stat stirred tank reactor with *Lactococcus lactis* ssp. *lactis* bv. *diactylactis* immobilized in calcium alginate gel beads. *Biotechnology and Bioengineering* 47, 567 – 574.
- Champagne, C.P, Girard, F. and Morin, N. (1988) Bacteriophage development in an immobilized lactic acid system. *Biotechnology Letters* 10, 463-468.
- Champagne, C.P. (1990) Inhibition of psychrotropic bacteria in raw milk by immobilized lactic acid bacteria. *Biotechnology Letters* 12, 771-776.
- Champagne, C.P. and Clôté, C. (1987) Cream fermentation by immobilized lactic acid bacteria. *Biotechnology letters* 9, 329-332.
- Champagne, C.P., Bailargeon-Côte, C. and Gulet, J. (1989) Whey fermentation by immobilized cells of *propionibacterium shermanii*. *Journal of Applied Bacteriology* 66, 175-184.
- Champagne, C.P., Gaudy, C., Poncelet, D. and Neufeld R.J. (1992) *Lactococcus lactis* release from calcium alginate beads. *Applied and Environmental Microbiology* 58, 1429-1434.
- Champagne, C.P., Girard, F. and Rodrique, N. (1993) Production of concentrated suspensions of thermophilic lactic acid bacteria in calcium-alginate beads. *International Dairy Journal* 3, 257-275.
- Champagne, C.P., Lacroix, C. and Sodini-Gallot, I. (1994) Immobilized cell technologies for the dairy industry. *Critical Reviews in Biotechnology* 14, 109-134.
- De Vuyst, L. and Vandamme, E.J. (1994) Lactic acid bacteria and bacteriocins: Their practical importance. In *Bacteriocin of lactic acid bacteria* ed. De Vuyst, L., Vandamme, E.J. pp. 1-11. Glasgow: Blackie academic & professional
- Dhulster, P., Barbotin, J.P. and Thomas, D (1984) Culture and bioconversion use of plasmid harboring strain of immobilized *E. coli*. *Applied Microbiology and Biotechnology* 20, 87-93.
- Dos Santos, V.A.P.M, Tramper, J. and Wijffels, R.H. (1996) The magic bead concept: an integrated approach for nitrogen removal. *Applied Microbiology and Biotechnology* 45, 447-453.

- Draget, K.I., Strand, B., Hartmann, M., Valla, S., Smidsrød, O. and Skjåk-Bræk, G. (2000) Ionic acid gel formation of epimerised alginates: the effect of AlgE4. *International Journal of Biological Macromolecules* 27, 117-122.
- Dyrset, N., Selmer-Olsen, E., Havrevoll, Ø., Ratnaweera, H., Storrø, I. and Birkeland S.E. (1998) Feed supplement recovered from dairy wastewater by biological and chemical pretreatment. *Journal of Chemical Technology and Biotechnology* 73, 175-182.
- Engasser, J.M. and Horwarth, C. (1973) Effect of internal diffusion in heterogeneous enzyme systems: Evaluation of the true kinetic parameters and substrate diffusivity. *Journal of Theoretical Biology* 42, 137-155.
- Föster, M., Mansfield, J., Schellenberger, A. and Dautzenberg, H. (1994) Immobilization of citrate-producing *Yarrowia lipolytica* cell in polyelectrolyte complex capsules. *Enzyme and Microbial Technology* 16, 777-784.
- Gåserød, O. (1998) Microcapsules of alginate-chitosan: A study of capsule formation and functional properties. Dr.ing Thesis, Trondheim: Department of biotechnology, Norwegian University of Science and Technology.
- Gåserød, O., Sannes, A. and Skjåk-Bræk, G. (1998b) Microcapsules of alginate-chitosan – II. A study of capsule stability and permeability. In *Microcapsules of alginate-chitosan: A study of capsule formation and functional properties*. Dr.ing.-thesis ed. Gåserød, O. Trondheim: Department of biotechnology, Norwegian University of Science and Technology.
- Gåserød, O., Smidsrød, O. and Skjåk-Bræk, G. (1998b) Microcapsules of alginate-chitosan – I: A quantitative study of the interaction between alginate and chitosan. *Biomaterials* 19, 1815-1825.
- Gätje, G. and Gottschalk, G. (1991) Limitation of growth and lactic acid production in batch and continuous cultures of *Lactobacillus helveticus*. *Applied Microbiology and Biotechnology* 34, 446-449.
- Gòdia, F., Casas, C. and Solà, C. (1991). Application of immobilized yeast cells to sparkling wine fermentation. *Biotechnology Progress* 7, 468-470.
- Gòdia, F., Casas, C., Castellano, B. and Solà, C. (1987) Immobilized cells: behaviour of carrageen entrapped yeast during continuous ethanol fermentation. *Applied Microbiology and Biotechnology* 26, 342-346.
- Groboillot, A.F., Boadi, D.K., Poncelet, D. and Neufeld, R.J. (1994) Immobilization of cells for application in the food industry. *Critical Reviews in Biotechnology* 14, 75-108.
- Groboillot, A.F., Champagne, C.P., Darling, G.D., Poncelet, D. and Neufeld, R.J. (1993) Membrane formation by interfacial cross-linking of chitosan for microencapsulation of *Lactococcus lactis*. *Biotechnology and Bioengineering* 42,1157-1163.
- Huguet, M.L. and Dellacherie, E. (1996) Calcium-alginate beads coated with chitosan: Effect of the structure of encapsulated materials on their release. *Process Biochemistry* 31, 745-751.

- Hüsken, L.E., Tramper, J. and Wijffels, R.H. (1996) Growth and Eruption of gel-entrapped microcolonies. In *Immobilized Cells: Basics and Applications*. ed. Wijffels, R.H., Buitelaar, R.M., Bucke, C. and Tramper, J. pp. 336-340 Amsterdam: Elsevier Science publishers.
- Johnson, M.E. and Steel, J.L. (1996) Fermented dairy products In *Food microbiology Fundamentals and Frontiers*. ed. Doyle, M.P., Beuchat, L.R. and Montville, T.J. pp. 581-595 Washington: ASM Press.
- Karel, S.F., Libicki, S.B. and Robertson, C.A. (1985) The immobilization of whole cells: engineering principles. *Chemical Engineering Science* 40, 1321-1354.
- Kashket, E.R. (1987) Bioenergetics of lactate acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiology Reviews* 46, 233-244.
- Kim, H.S., Kamura, B.J. and Olson, N.F. (1985) Continuous acidification of milk before ultrafiltration by an immobilized cell bioreactor. I. Development of the bioreactor. *Milchwissenschaft*. 40, 645-652.
- Lacroix, C., Sodini, I. And Corrieu, G. (1996) Microbiological stability of and immobilized bioreactor with mixed lactic acid bacteria during continuous fermentation of milk. In *Immobilized Cells: Basics and Applications*. Eds. Wijffels, R.H., Buitelaar, R.M., Bucke, C. and Tramper, J. pp. 600-607 Amsterdam: Elsevier Science publishers.
- Larisch, C.B., Poncelet, D., Champagne, C.P. and Neufeld, R.J. (1994) Microencapsulation of *Lactococcus lactis* subsp. *cremoris*. *Journal of Microencapsulation* 11, 2, 189-195.
- Larreta Grade, V., Thomasset, B. and Barbotin, J.N. (1981) Electron microscope evidence of an immobilized living cell system. *Enzyme and Microbial Technology* 3, 216-128.
- Lim, F. and Sun, A.M. (1980) Microencapsulated islets as bioartificial endocrine pancreas. *Science* 210, 908-910.
- Martinsen, A. (1990) Alginates as immobilization materials – a study of some molecular and functional properties. Dr.ing Thesis, Trondheim: Institute of biotechnology, The Norwegian institute of technology.
- Martinsen, A., Skjåk-Bræk, G. and Smidsrød O. (1989) Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. *Biotechnology and Bioengineering* 33, 79-89.
- Masson, F., Lacroix, C., and Paquin, C. (1994) Direct measurements of pH profiles in gel beads immobilizing *lactobacillus helveticus* using a pH sensitive microelectrode. *Biotechnology Techniques* 8, 551-556.
- Morin, N., Bernier-Cardou, M. and Champagne, C.P. (1992) Production of concentrated *Lactococcus lactis* subsp. *cremoris* suspension in calcium alginate beads. *Applied and Environmental Microbiology* 58, 545-588.
- Ottøy, M.H., Vårum, K.M. and Smidsrød, O. (1996) Compositional heterogeneously deacetylated chitosans. *Carbohydrate Polymers* 29, 17-24.

- Painter, T.J. (1983) Algal polysaccharides. In *The polysaccharides* ed. Aspinall, G.O. pp. 196-286. London: Academic Press.
- Prevost, H. and Divies, C. (1988) Continuous pre-fermentation of milk by entrapped yogurt bacteria. I. Development of the process. *Milchwissenschaft*. 43, 620-625.
- Prévost, H. and Divies, C. (1992) Cream fermentations by a mixed culture of lactococci entrapped in two-layer calcium alginate gel beads. *Biotechnology Letters* 14, 583-588.
- Prevost, H. and Divies, C. (1997) Fresh fermented cheese production with continuous pre-fermented milk by a mixed culture of mesophilic lactic streptococci entrapped in Ca-alginate. *Biotechnology Letters* 9, 789-794.
- Prevost, H., Divies, C. and Rousseau, E. (1985) Continuous yogurt production with lactobacillus bulgaricus and streptococcus thermophilus entrapped in Ca-alginate. *Biotechnology Letters* 7, 247-252.
- Rinodau, M. and Domard, A. (1989) Solution properties of chitosan. In *Chitin and Chitosan* ed. Skjåk-Bræk, G., Anthonsen, T. and Sandford P. pp. 71-86 London: Elsevier Applied Science.
- Skjåk-Bræk, G. Smidsrød, O. and Larsen B. (1986) Tailoring of alginates by enzymatic modification in vitro. *International Journal of Biological Macromolecules*. 8, 330-336.
- Skjåk-Bræk, G., Grasdalen, H. and Smidsrød, O. (1986) Inhomogeneous polysaccharide ionic gels. *Carbohydrate Polymers* 10, 31-54.
- Smidsrød, O. (1968) Molecular basis for some physical properties of alginates in the gel state. *Journal of the Chemical Society, Faraday Transactions* 57, 263-273.
- Smidsrød, O. and Skjåk-Bræk, G. (1990) Alginate as immobilization matrix for cells. *Trends in Biotechnology* 8, 71-78.
- Sodini, I., Boquien, C.Y., Corrieu, G. and Lacroix C. (1997) Microbial dynamics of co- and separately entrapped mixed cultures of mesophilic lactic acid bacteria during the continuous prefermentation of milk. *Enzyme and Microbial Technology* 20, 381-388.
- Soon-Shiong, P., Feldman, E., Nelson, R., Komtebedde, J., Smidsrod, O., Skjåk-Bræk, G., Espevik, T., Heintz, R. and Lee, M. (1992) Successful reversal of spontaneous diabetes in dogs by intraperitoneal microencapsulated islets. *Transplantation* 54, 769-774.
- Soon-Shiong, P., Heintz, R.E., Merideth, N., Yao, Q.X., Yao, Z., Zheng, T., Murphy, M., Moloney, M.K., Schmehl, M., Harris, M., Mendez, R. and Sandford, P.A. (1994) Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. *Lancet* 343, 950-951.
- Stewart, P.S. and Robinson C.R. (1989) Microbial growth in a fixed volume: studies with entrapped *Escherichia coli*. *Applied Microbiology and Biotechnology* 30, 34-40.
- Strand, B.L., Mørch, Y.A. and Skjåk-Bræk, G. (2000) Alginate as immobilization matrix for cells. *Minerva Biotec* 12, 223-233.

- Vårum, K.M., Ottøy, M.H. and Smidsrød (1994) Water-solubility of partially N-acetylated chitosans as a function of pH: effect of chemical composition and depolymerisation. *Carbohydrate Polymers* 25, 65-70.
- Wang, W., Bo, S., Li, S. and Qin, W. (1991) Determination of the Mark-Houwink equation for chitosans with different degrees of acetylation. *International Journal of Biological Macromolecules* 13, 281-285.
- Willaert G.W. and Baron, G.V. (1996) Gel entrapment and micro-encapsulation: methods, applications and engineering principles. *Reviews in Chemical Engineering* 12, 2-205.
- Willaert, R. and Baron, G. (1993) Growth kinetics of gel-immobilized yeast studied by on-line microscopy. *Applied Microbiology and Biotechnology* 39, 347-352.
- Yabannavar, V. and Wang, D.I.C. (1991) Analysis of mass transfer for immobilized cells in an extractive lactic acid fermentation. *Biotechnology and Bioengineering* 38, 544-550.
- Yoo, I.K., Seong, G.H., Chang, H.N. and Park, J.K. (1996) Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production. *Enzyme and Microbial Technology* 19, 428-433.
- Zeza, N., Pasini, G., Lombardi, A., Mercenier, A., Spettoli, P., Zamorani, A. and Nuti, M.P. (1993) Production of a bacteriocin active on lactate fermenting clostridia by *Lactococcus lactis* subsp. *lactis* immobilized in coated alginate beads. *Journal of Dairy Research* 60, 581-591.
- Zhou, Y., Martins, E., Groboillot, A., Champagne, C.P. and Neufeld, R.J. (1998). Spectrophotometric quantification and control of cell release with chitosan coating. *Journal of Applied Microbiology*. 84, 342-348.
- Øyaas, J. (1995) Study of diffusion processes and lactic acid production by *Lactobacillus helveticus* immobilized in Ca-alginate gel. Dr. ing. thesis, Trondheim: Institute of biotechnology, The Norwegian Institute of Technology.
- Øyaas, J., Storrø, I., Lysberg, M., Svendsen, H. and Levine, D.W. (1995) The effective diffusion coefficient and the distribution constant for small molecules in calcium-alginate gel beads *Biotechnology and Bioengineering* 47, 492-500.

PAPER I

Paper I is not included due to copyright.

PAPER II

Paper II is not included due to copyright.

PAPER III

Enterocin production and cell release by *Enterococcus faecium* CTC 492 immobilized in uncoated and chitosan/alginate/chitosan coated alginate beads

**G. KLINKENBERG^{*1}, D. W. LEVINE^{*}
AND N. DYRSET[†]**

*** Department of Biotechnology, Norwegian University of Science and
Technology, N-7491 Trondheim**

† SINTEF Applied Chemistry, N-7465 Trondheim

Abbrev. running headline:

Bacteriocin production by alginate immobilized *E. faecium* CTC492

Key words:

immobilized, lactic acid bacteria, bacteriocin, pH, cell release

¹ Corresponding author:

Postal address: Department of Biotechnology
Norwegian University of Science and Technology
N-7491 Trondheim
Norway

E-mail: geirk@sintef.no

SUMMARY

Aims: To investigate growth and bacteriocin production by alginate immobilized *Enterococcus faecium* CTC492, and to investigate the effects of chitosan/alginate/chitosan (CAC) coatings on the relationship between bacteriocin production and cell release.

Methods and results: Bacteriocin production by immobilized *E. faecium* CTC492 in uncoated and CAC-coated alginate beads was evaluated in batch and continuous fermentations.

In batch fermentations using CAC-coated beads, 27% of the maximal bacteriocin activity in traditional free-cell fermentation was obtained, combined with a more than 3 log unit reduction of the amount of free biomass in the product. Continuous fermentations at high dilution rates showed that the use of CAC-coatings reduced the ratio of rates of bacteriocin production to cell release significantly the first 20 hours of fermentation. Measurements of internal pH within the beads revealed large pH-gradients, which seemed to significantly influence the bacteriocin production and fermentation characteristics of immobilized *E. faecium* CTC492.

Conclusions: The bacteriocins enterocin A and B can be produced at higher productivity with fewer cells in the product by the use of alginate immobilized cells. The use of CAC-coatings can, for a limited time, further reduce the release of free cells.

Significance and Impact of the study: The possibility to alter the product composition with regard to enterocin activity and biomass concentration, combined with an improved productivity, might be used in order to produce enterocins more efficiently in novel applications.

INTRODUCTION:

Bacteriocins from lactic acid bacteria are biologically active peptides and proteins with antimicrobial activity against other Gram-positive bacteria, including food-spoilage and/or pathogenic strains (De Vuyst and Vandamme 1994). The antimicrobial activity is usually confined to strains closely related to the producer strain, but a number of bacteriocins from lactic acid bacteria have fairly broad inhibitory spectra (Jack *et al.* 1995). Furthermore, many bacteriocins are heat stable, making them applicable to food processing in combination with heat treatment (De Vuyst and Vandamme 1994). These compounds are especially interesting, as lactic acid bacteria have been used in food preservation and food fermentations for millennia. Recently, much research effort has been focused on the potential of bacteriocins as natural food preservatives. However, only a few bacteriocins are produced commercially. Among these is nisin, the most well known, which is currently licensed as a food additive in more than 40 countries (Delves-Broughton 1990).

In order to make these products commercially attractive, large-scale production needs to be economically viable. Batch fermentations with freely suspended cells are the most common approach to bacteriocin production. However, batch fermentation might be unsuitable for certain industrial applications (De Vuyst and Vandamme 1991). Only a limited amount of effort has been focused on modification of reactor design or fermentation technology in order to increase the productivity of bacteriocin production (Bhugaloo-Vial *et al.* 1996). Fermentation with immobilized cells offers a number of advantages over free cell fermentations, including simplified cell recovery, increased productivity, and the possibility of using high dilution rates in continuous fermentations (Groboillot *et al.* 1994). A popular method for immobilization is entrapment in gel materials – especially in seaweed gel-materials such as carrageen or alginate (Willaert and Baron 1996). A few reports exist on bacteriocin production with lactic acid bacteria immobilized in calcium alginate. Scannell *et al.* (2000) investigated continuous production of lactacin 3147 and nisin using cells immobilized in double-layer calcium alginate. Bhugaloo-Vial *et al.* (1997) studied continuous production of divercin and Wan *et al.* (1995) studied continuous production of brevicin, nisin and pediocin from calcium alginate-immobilized bacteria. Zezza *et al.* (1993) investigated the use of *L. lactis* ssp. *lactis* NZ1 immobilized in double layer calcium alginate beads for production of nisin.

Due to a simplified cell recovery, the use of immobilized lactic acid bacteria has been proposed for in-situ production of bacteriocins in target foods (Zezza *et al.* 1993). However, in fermentations with gel entrapped lactic acid bacteria there is normally a considerable release

of free cells from the immobilization matrix. Thus, some effort has been concentrated on controlling and monitoring rates of cell release in fermentations with immobilized lactic acid bacteria (Champagne *et al.* 1992, 1994, Zhou *et al.* 1998, Klinkenberg *et al.* 2001a). The mechanism behind cell release is related to growth of bacteria within the gel beads. When growth of gel-immobilized bacteria occurs, the gel-network is pushed away and colonies containing densely packed bacteria are formed (Willaert and Baron 1993). As the colony expands, it may reach the surface of the gel-bead. This leads to an eruption of the colony in which the content of the colony is released to the surrounding medium (Hüsken *et al.* 1996). Cell release is enhanced by uneven growth conditions within the beads. Since the bacteria are immobilized in gel materials, substrates and waste products must be transported to and from the bacteria by diffusion. Due to these processes, high biomass concentrations tend to build up close to the surface (Cachon *et al.* 1993, 1995a, 1995b, 1997, Klinkenberg *et al.* 2001b).

In this work, bacteriocin production with immobilized *Enterococcus faecium* CTC 492 is studied. *E. faecium* CTC 492 produces the bacteriocins enterocin A and B. These bacteriocins are heat stable and have broad inhibitory spectra, which include food-borne and pathogenic species like *Listeria monocytogenes*, *Clostridium tyrobutyricum* and *Staphylococcus aureus* (Casaus *et al.* 1997). The aim of this work was to study the potential of immobilized enterocin production, and to investigate the use of sequential coatings with chitosan and alginate as an approach to influence the amounts of bacteriocin and free cells produced. The local environment within the beads is examined since it is expected that the mode of immobilization will influence the local environment experienced by the immobilized microorganism. The use of chitosan coating on alginate beads containing immobilized lactic acid bacteria has previously been shown to reduce rates of cell release (Zhou *et al.* 1998, Klinkenberg *et al.* 2001a). In this work, both batch and continuous production of bacteriocin with chitosan and alginate coated alginate beads as immobilization matrix is examined. Previously, we have developed a system based on continuous fermentation in order to quantify rates of cell release from immobilized lactic acid bacteria without contributions from growth of free cells (Klinkenberg *et al.* 2001a). In this work, this system is used to quantify the rates of cell release and rates of bacteriocin production, and to study the courses of these rates on a longer-term basis.

MATERIALS AND METHODS

Materials

The following chemicals were used: MRS-broth, agar, yeast extract and peptone (Oxoid Unipath LDT, Basingstoke, UK); $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, K_2HPO_4 , NaH_2PO_4 , gelatin and sodium citrate (Riedel de Haën, Seelze, Germany); glucose monohydrate (Norsk Medisinaldepot, Oslo, Norway); NaCl (Kebo A/S, Oslo, Norway); perchloric acid (Merck, Darmstäd, Germany); sodium alginate (PROTANAL LF 10/60) and chitosan (CL 110) (FMC Biopolymer A/S, Drammen, Norway); chromatography standards of lactic acid and H_2SO_4 used as eluent for the high performance liquid chromatography (HPLC) analysis (Sigma Chemical Company, St. Louis, MO, USA).

Organisms

Enterococcus faecium CTC 492 was obtained from Dr. Trine Nilsen, Norwegian Food Research Institute, and has previously been described by Nilsen *et al.* (1998), Casaus *et al.* (1996), and Aymerich *et al.* (1996). The organism used as indicator organism in the bacteriocin analysis was *Lactobacillus sake* NCDO 2714 (National Collection of Dairy Organisms (later incorporated into National Collection of Food Bacteria), Aberdeen, Scotland). This strain is sensitive to both enterocin A and B (Casaus *et al.* 1997). Stock cultures of both strains were maintained at -80°C in MRS broth containing 15% glycerol. The revival of *Enterococcus faecium* CTC 492 was carried out in MRS broth at 30°C for 12 h.

Culture media

The following culture media were used: Fermentation medium: A modified MRS broth (mMRS) with similar composition to MRS broth, but lacking phosphate and citrate, and supplied with 0,74 g/l CaCl_2 , was used as fermentation medium. The glucose concentration of this mMRS-broth was 25 g/l. Plate count agar: MRS broth added 1.5 % (w/v) agar. Dilution buffer: 8.5 g l^{-1} NaCl, 0.3 g l^{-1} K_2HPO_4 , 0.60 g l^{-1} NaH_2PO_4 and 0.10 g l^{-1} gelatin. The pH was adjusted to 6.5 prior to autoclaving (121°C , 25 min.).

Cell Immobilization

E. faecium CTC 492 cells for immobilization were produced in MRS broth at a controlled temperature of 30°C for approximately 12 h. The cell production was conducted in a 2.5 l working volume fermentor (Applicon, Schiedam, the Netherlands) at an agitation speed of

250 rpm, with a pH maintained at 6.5 using a pH-controller with an automatic addition of 3M NaOH. The fermenters were inoculated with 2% (v/v) active culture in MRS broth.

Cells were harvested aseptically by centrifugation at 8,400 g for 20 minutes at 4°C. The pellets were suspended in dilution buffer to a concentration of 50 g cells l⁻¹. Resuspended cells were mixed with 2.5 % (w/v) sterile sodium alginate solution and sterile distilled water in order to reach a final cell concentration of 5 g_{d.w.} cells l⁻¹ and a final alginate concentration of 2 % (w/v), which was used in all experiments.

The mixture of alginate and cells was added drop-wise through pipette tips with an inner diameter of 0.5 mm into a sterile solution of sodium chloride (0.2 M) and calcium chloride (0.05 M). In order to achieve aseptic operation the beads were made in a 2.5 l working volume fermentor (Applicon, Schiedam, the Netherlands) at a stirring rate of 50 rpm using a single pitch-blade impeller. Sodium chloride was used in the gelling solution in order to ensure a homogeneous polysaccharide concentration throughout the beads (Skjåk-Bræk *et al.* 1999). To ensure complete gelling, the beads were stirred at least 40 min in this solution. The entire immobilization procedure was performed at ambient temperatures.

Bead coating was performed as described elsewhere (Klinkenberg *et al.* 2001a).

Fermentations

The growth rates of non-immobilized *E. faecium* CTC 492 in batch fermentations with mMRS at different pH values were determined at a controlled temperature of 30°. The fermentations were conducted in a 2.5 l working volume fermentor (Applicon, Schiedam, the Netherlands) at an agitation speed of 250 rpm, with pH maintained at 4.5 to 7.5 using a pH-controller with an automatic addition of 3M NaOH. The fermenters were inoculated with 2% (v/v) active culture in MRS broth.

Batch and continuous fermentations with immobilized *E. faecium* CTC 492 in alginate beads were carried out with 100 ml beads in reactors with a total working volume of 500 ml (modified Celstir, Wheaton, Millville, N.J.) operated at an agitation rate of 300 rpm, and at a controlled temperature of 30°C. mMRS was used as feed medium in the continuous fermentations at dilution rates of 1.3 h⁻¹ and 3 h⁻¹. The pH was maintained at 6.0, 6.3, 6.7, 7.0, and 7.5 using a pH-controller with an automatic addition of 1 M NaOH. The temperature, pH, outflow of each reactor and addition of NaOH were continuously registered by computer.

Determination of cell concentrations

Cell densities for determination of growth rates of freely suspended cells in batch culture were monitored by measuring the optical density of the culture at 660 nm (1 cm path length, UV visible spectrophotometer UV-160; Shimadzu, Kyoto, Japan). Samples were diluted in distilled water to give a final optical density of less than 0.4. Distilled water was used as a blank.

Samples for determination of free cell concentrations in fermentations with immobilized *E. faecium* were serially diluted with dilution buffer. Plate counts were conducted in triplicate at each dilution on MRS broth supplemented with 1.5% (w/v) agar. Plates were incubated at 30°C for 2 days. The results were calculated according to guidelines given by Lille *et al.* (1999), and are reported as cfu ml⁻¹. Tests were performed according to guidelines given by Lille *et al.* (1999) to assure a sufficient reproducibility (standard deviation within 8% - 30% of the results in test trials).

Samples of uncoated alginate beads with immobilized *E. faecium* for determination of internal bead cell density were separated from the fermentation broth and liquefied in a sterile 1% solution of sodium citrate (pH 6.0). Dilution and plate counts were conducted as described above.

Determination of bacteriocin activity

Samples for determination of bacteriocin activity were centrifuged at 13,500 g for 10 min and incubated at 100°C for 10 min. in order to obtain a sterile cell free supernatant. Bacteriocin activity was quantified by a microtiter assay as described by Holo *et al.* (1991). Each Nucleon 96 microwell plate (Nunc A/S, Roskilde, Denmark) was divided into wells containing sterile MRS broth (blank), wells containing MRS broth inoculated with the reference organism (growth reference), and wells with MRS broth containing sample fractions at twofold dilutions inoculated with the indicator organism. Each well contained 200 µl medium, and the reference organism was inoculated at a level equal to a 10⁴ dilution of the stock culture. The microtiter plates were incubated at 30°C until an optical density of 0,33±0,03 was reached in the reference wells, and the optical density at 630 nm was measured with a SPECTRA max PLUS³⁸⁴ microplate spectrophotometer (Molecular Devices, Sunnyvale, Ca). The activity of the individual samples was measured as the dilution corresponding to 50% growth inhibition by a computer regression of the optical density results to the corresponding dilution factors. In order to make the assays comparable, each result was compared to the result of a standard

sample analyzed on each microwell plate. The bacteriocin activity is expressed as arbitrary units (AU/ml) and calculated as % activity of the standard sample. Both standards and samples were analyzed in duplicate on each plate.

Measurement of pH-gradients within alginate beads.

Measurements of pH within alginate beads were performed using a microelectrode as described elsewhere (Klinkenberg *et al.* 2001b).

Determination of lactic acid and glucose concentrations

Determination of lactic acid and glucose concentrations were performed as described elsewhere (Klinkenberg *et al.* 2001b).

RESULTS

Growth and bacteriocin production in modified MRS-medium

It has previously been reported that the local environment within alginate beads immobilizing lactic acid bacteria can be significantly different from bulk fluid surrounding the beads, especially with regard to pH. Therefore, the influence of pH on growth and bacteriocin production of freely suspended *Enterococcus faecium* CTC 492 in modified MRS-broth was investigated. Modified MRS-broth was inoculated with an overnight culture of *E. faecium* CTC 492 (2 % (v/v)), and fermented batch-wise at pH-values ranging from pH 4.5 to 7.5. Biomass concentration, glucose consumption and bacteriocin production were monitored throughout the fermentations. Figure 1 illustrates the maximum bacteriocin activity and the maximum specific growth rate observed at each fermentation pH.

In all fermentations, a short period of exponential growth was observed. The growth rate in the exponential phase increased with increasing pH until pH 6.7, the pH optimum for growth. The highest bacteriocin activity was reached in fermentations operated at pH 6.0. At this pH, a maximum bacteriocin activity was seen after 16 hours of fermentation, at a biomass concentration of 1.1×10^{10} cfu/ml (95% conf. limit: $<9.0 \times 10^9, 1.3 \times 10^{10}>$). In all fermentations operated at pH 6.0 and higher, the observed bacteriocin activity reached a peak value, followed by a significant decline. This peak in bacteriocin activity was observed even though glucose was still present in the fermentation broth at concentrations above 14 mM. The peak value seemed to coincide with a significant drop in the growth rate of the organism. However, in fermentations operated at pH 5.7 and below, the highest bacteriocin activity coincided with depletion of glucose in the fermentation broth.

Immobilized production of enterocin A and B.

Batch fermentations with immobilized E. faecium CTC 492.

The potential of using alginate immobilized *E. faecium* CTC 492 for production of the bacteriocins enterocin A and B was investigated. Sequential batch fermentations of mMRS were performed in a stirred tank fermentation system with uncoated and chitosan/alginate/chitosan coated (CAC-coated) alginate beads containing *E. faecium* CTC 492. In order to produce CAC-coated beads, uncoated alginate beads containing *E. faecium* CTC 492 (initial cell concentration in gel: 1.0×10^{10} cfu/ml) were sequentially coated with chitosan/alginate/chitosan. Uncoated and CAC-coated beads were introduced to mMRS-broth and fermented batch-wise at pH-values between 6.0 and 7.0. After the first fermentation, the beads were removed from

the fermentation broth, rinsed with calcium chloride solution and introduced to fresh mMRS-broth. The enterocin activity, glucose concentration, and biomass concentration in the fermentation broth were measured at regular intervals. Figure 2 shows measured enterocin activities and glucose concentrations during the first of the sequential batch fermentations. Table 1 presents data from the first and second sequential fermentation operated at pH 6.0 and pH 7.0, with both types of beads as well as free cell data. The maximal enterocin activity in each of the fermentations, the corresponding concentrations of free cells and glucose, the yields of enterocins A + B on glucose ($Y_{E/G}$) and the time necessary to reach maximum bacteriocin activity are shown.

As expected, a different pattern of fermentation was observed in the immobilized fermentations, compared to the traditional free-cell fermentations. In batch-operated systems, the use of gel-immobilized cells causes a dilution of the medium. Therefore, yields of enterocins on glucose as well as maximum enterocin concentrations are used for comparison. In the first sequential fermentation with uncoated beads, the maximum enterocin activities were somewhat lower than in fermentations with freely suspended cells. However, maximum enterocin activities and yields of enterocins on glucose were less affected by fermentation pH than in free cell fermentations. With immobilized cells, almost equal yields of enterocins on glucose was seen in the first sequential fermentation at pH 6.0 and pH 7.0. The use of CAC-coatings resulted in lower maximal enterocin activities and lower yields of enterocins on glucose. At pH 6.0, the yield of enterocins on glucose with these beads was 25% of the yield in fermentations with freely suspended cells. At pH 7.0, the corresponding value was 73%. Furthermore, the use of CAC-coatings decreased rates of enterocin production and caused almost a doubling of the time of the first sequential fermentation, as compared to uncoated beads. The time of fermentation was, however, still shorter with both types of immobilization than with freely suspended cells.

The amount of free cells in the fermentation broth at the maximum bacteriocin activity was, as expected, significantly lower in fermentations with immobilized cells, compared to traditional free cell fermentations. However, the use of CAC-coatings on the beads caused a significant further reduction in the amount of free cells in the fermentation broth in the first sequential fermentation. At the maximal bacteriocin concentration, the amount of free cells was 3.3 log units lower than in the comparable fermentations with freely suspended cells operated at the same pH (pH 6.0). This reduction was dependent on fermentation pH and was somewhat less in fermentations operated at higher pH-values. Even though the absolute levels of bacteriocin production was somewhat lower in the first sequential fermentation with

immobilized cells, the reduction of the amount of free cells was significantly larger than the concomitant reduction in bacteriocin activity. As a consequence, the ratio of product to free cells was greatly increased in immobilized fermentations compared to traditional free cell fermentations.

In the second of the sequential fermentations with uncoated beads, yields of enterocins on glucose and maximum enterocin activities were lower at both pH values as compared to the first sequential fermentation. With CAC-coated beads, only pH 7.0 showed a further reduction in maximal activity and yield of enterocins. The time needed to reach maximum bacteriocin activity with each type of bead was shorter in the second fermentation, compared to the first. However, the time of fermentation needed in the second fermentation with CAC-coated beads at pH 6.0 was still nearly twice as long as that with uncoated beads. As in the first of the sequential batch fermentations, the use of CAC-coatings also caused an initial reduction in the amount of free cells in the fermentation broth in the second sequential fermentation (data not shown). However, in the second sequential fermentation the use of CAC-coatings did not result in a lower number of free cells than with uncoated beads at the time of maximum bacteriocin concentration.

In addition, a difference in the pattern of utilization of glucose between uncoated and CAC-coated beads was observed in these experiments. In both the first and the second batch fermentations with uncoated beads operated at pH 7.0, a maximum in enterocin activity was seen before glucose was exhausted from the fermentation broth. This was not the case for fermentations with uncoated beads operated at pH 6.0, or for all fermentations with CAC-coated beads. In these fermentations, the maximum enterocin activity seemed to coincide with depletion of glucose. In light of the similar pH related effects observed in free cell fermentations, these effects might indicate a lower pH in the active parts of CAC-coated beads, compared to uncoated beads.

Assessment of bacteriocin production and cell release using continuous fermentations

In batch fermentations, cells released to the fermentation broth can grow and may contribute significantly to biomass density and bacteriocin activity in the fermentation broth. Due to the nearly doubled time of fermentation, these effects might be more pronounced in fermentations with CAC-coated beads. An experimental system based on continuous fermentations was used in order to get a more accurate understanding of the kinetics of enterocin production and cell release. Continuous fermentation at a high dilution rate facilitates direct measure-

ments of rates of bacteriocin production and rates of cell release during fermentation, and can provide data on the behavior of an immobilized system on a longer-term basis.

Uncoated beads and CAC-coated beads were transferred to reactors and fermented continuously at a dilution rate of 3 h^{-1} for 46 hours in mMRS-broth. This represents a dilution rate well above the maximum growth rate of *E. faecium* CTC 492 in this medium. Uncoated beads were fermented at a pH of 6.3 while CAC-coated beads were fermented at pH 6.3, 7.0 and 7.5. Separate fermentations operated at a reduced dilution rate (1.3 h^{-1}) were used to investigate effects of dilution rate. Samples were taken at regular intervals throughout the fermentations in order to monitor bacteriocin production, cell release, glucose consumption and lactate production. Calculated rates of bacteriocin production during fermentation are shown in Figure 3, while the biomass densities in the effluents are shown in Figure 4.

The initial rates of bacteriocin production in continuous fermentations with both types of beads were similar to the initial rates of bacteriocin production observed in batch fermentations (Data not shown). As the data from batch fermentations indicated, the use of CAC-coatings influenced the courses of bacteriocin production. With uncoated beads, a sharp increase in the rate of bacteriocin production was observed during the first 10 hours of fermentation. This increase was followed by a slow reduction. With CAC-coated beads fermented at the same pH, only a slow increase in the rate of bacteriocin production was observed during the first 30 hours of fermentation. In comparison to rates of bacteriocin production from uncoated beads fermented at the same pH, the rates of bacteriocin production of CAC-coated beads were lower, initially by a factor of nearly 2. This lower rate lasted throughout the 46 hours of fermentation examined. However, the rates of bacteriocin production from CAC-coated beads were higher in fermentations at higher pH-values. After 19 hours of fermentation, the rate of bacteriocin production of CAC-coated beads fermented at pH 7.5 was nearly twice the comparable rate at pH 6.3 (factor = 1.91). A reduction in dilution rate from $D = 3 \text{ h}^{-1}$ to 1.3 h^{-1} did not significantly influence the rate of bacteriocin production of CAC-coated beads fermented at pH 7, but did result in an increase in the bacteriocin activity of the effluent of the fermentor.

The amount of free cells in the fermentation broth stabilized after 10.5 hours in the fermentation with uncoated beads operated at pH 6.3. Only minor changes in the biomass concentration of the effluent were observed during the rest of the fermentation period. The use of CAC-coatings affected the amount of free cells in the effluent, as the initial biomass concentration in the effluent was reduced by a factor of 10^4 , compared to fermentation with uncoated beads at the same pH (The initial data points are not shown in Figure 4). This difference was quickly

reduced, but remained nonetheless significant during the first 27 hours of fermentation (factor 5.9 at 27 hours). An increased fermentation pH from 6.3 to 7.5 led to an increase in the effluent biomass density in fermentations with CAC-coated beads. Similarly, a decreased dilution rate led to an increased effluent biomass density in fermentations with CAC-coated beads operated at pH 7.0.

The rate of bacteriocin production and the rate of cell release are both affected simultaneously by the choice of immobilization matrix, and both rates seem to vary extensively throughout the fermentation. As these fermentations are continuous, the ratio of these rates can provide information on the extent of an altered relationship between bacteriocin production and cell release at any given time. These ratios are illustrated in figure 5.

In the fermentation with uncoated beads at pH 6.3, an initial decline in the rates of bacteriocin production to cell release was observed the first 10 hours of fermentation. Beyond this, there were no significant changes in this ratio throughout the fermentation. In fermentations with CAC-coated beads operated at all pH-values, this ratio was initially nearly 3 log units lower than that obtained with uncoated beads at pH 6.3 (The initial data points are not shown in Figure 5). In these fermentations, a sharp increase in the ratio of bacteriocin production to cell release was observed in the first period of fermentation. However, this ratio remained higher with CAC-coated beads, as compared to uncoated beads, during the first 20 hours of fermentation at pH 6.3. Beyond 35 hours of fermentation, there was no difference in this ratio for uncoated beads and CAC-coated beads fermented at the same pH. In the case of CAC-coated beads fermented at higher pH values, a sharper initial increase was observed. In these fermentations, this ratio stabilized after a shorter time of fermentation than seen at lower operating pH-values. In the case of CAC-coated beads fermented at different dilution rates, no significant effects were seen on the ratio of rates of bacteriocin production to cell release.

pH-gradients within alginate beads immobilizing E. faecium CTC 492

In fermentations with freely suspended *E. faecium* CTC 492 growing in mMRS, a sharp optimum at pH 6.0 was observed for production of enterocin A and B. At pH values above this pH, less total activity was measured, and the rate of bacteriocin production was reduced. In the immobilized fermentations, a different pattern seems to appear. In continuous fermentations with an increased pH from 6.3 to 7.5, an increasing rate of bacteriocin production was observed. Previously, several authors (Masson *et al.* 1994, Cachon *et al.* 1997) have demonstrated large pH-gradients within alginate beads immobilizing lactic acid bacteria. Due to this, the pH-profiles within uncoated and CAC-coated beads were measured at different times of

fermentation. Measurements of local pH within uncoated and CAC-coated alginate beads are shown as a function of position within the beads in Figure 6.

In both types of beads, only minor gradients in local pH within the beads were observed after 2.5 hours of fermentation. However, a significant pH-gradient evolved in both types of beads during the next 16.5 hours of fermentation. At this time, the pH-gradient was slightly steeper in CAC-coated beads, compared to uncoated beads. The local pH at the center of CAC-coated beads was at this time 4.5, in comparison to 4.9 in uncoated beads. The measurements in the outer regions of these beads indicate the presence of a pH-gradient across the initially cell free CAC-coating at this time.

In the following 25 hours, a slight increase in the steepness of the pH-gradient was found within uncoated beads. This increase was not observed in CAC-coated beads. However, the pH-gradient was still slightly steeper within CAC-coated beads compared to that within uncoated beads fermented at the same pH.

An increased fermentation pH seemed to enhance the magnitude of the pH gradient within the beads. A significantly steeper pH-gradient was found within CAC-coated beads fermented at pH 7.5, compared to similar beads fermented at 6.3. When compared after 19 hours of fermentation, the local pH in these beads was equal at a distance of 0.5 mm from the surface. Thus, the pH-gradient across the initially cell free layer within CAC-coated beads is larger in beads fermented at the higher pH.

Comparison of various modes of enterocin production.

In this study, both traditional and immobilized production systems for production of the bacteriocins enterocin A and B has been evaluated. A comparison of the maximum enterocin activity obtained, the volumetric productivity of each mode of fermentation and the ratio of bacteriocin concentration to free biomass concentration in the product is shown in Figure 7.

Both the highest bacteriocin concentration and the highest biomass concentration in the fermentation broth were obtained in fermentations with freely suspended cells. In batch systems with gel-immobilized cells, the presence of the gel-matrix causes a relative dilution of the growth medium. However, a maximum enterocin activity of 30% - 70% of the maximal activity in fermentations with freely suspended cells was obtained in the immobilized batch fermentations, with concomitant reduction of the biomass concentration in the fermentation broth by up to 3,3 log units. In addition, these fermentations are less sensitive to changes in fermentation pH. However, in comparison to batch fermentations continuous fermentations with immobilized cells have a greatly improved volumetric productivity.

These data suggest that the use of immobilized cells might be a valuable tool in enterocin production. The possibility to alter the product composition with regard to in enterocin activity and biomass concentration, combined with an improved productivity, might even be exploited in order to produce enterocins more efficiently in novel applications.

DISCUSSION

During recent years the use of immobilized lactic acid bacteria has been proposed for a variety of fermentation processes. The use of immobilized lactic acid bacteria in bacteriocin production has, however, only recently attracted interest (Bhugaloo-Vial *et al.* 1997). In this study, the production of enterocin A and B by alginate immobilized *Enterococcus faecium* CTC 492 has been examined in batch and continuous fermentations.

In order to avoid instability-problems with alginate beads during fermentation (Wan *et al.* 1995), a modified MRS-broth was constructed. Growth kinetics and bacteriocin production of *E. faecium* CTC 492 in this medium were investigated using batch fermentations with freely suspended cells at controlled pH-values. There were no adverse effects noted for *E. faecium* CTC 492 growth and bacteriocin production in mMRS, as compared to MRS-broth. Previously, Nilsen *et al.* (1998) reported a pH optimum for enterocin production at pH 6.2 in MRS-broth supplied with extra glucose. In our study, the highest enterocin activity was reached at pH 6.0 in mMRS. However, in all fermentations operated at pH 6.0 and above, a maximum of enterocin activity was reached before glucose was exhausted from the fermentation broth. The reason for this arrest of enterocin production seen at the higher pH-values is, however, not known.

As expected, the amount of free cells in the fermentation broth at the end of batch fermentations with alginate immobilized *E. faecium* CTC 492 was significantly lower than in fermentations with freely suspended cells. However, a significant amount of cells was still released from the immobilization matrix. This normally occurs in fermentations with immobilized lactic acid bacteria (Champagne *et al.* 1994). Also, maximum bacteriocin activities and yields of enterocins on glucose in these fermentations were lower, compared to freely suspended cells, but this reduction was not nearly so large as the reduction of the amount of free cells in the fermentation broth. The use of CAC-coatings on alginate beads immobilizing *E. faecium* CTC 492 caused an even further reduction of the amount of free cells in the fermentation broth in the first of the sequential fermentations. Several authors report the use of polycationic coatings on alginate beads in order to reduce cell release. Champagne *et al.* (1992) used multiple coatings of poly L-lysine and alginate, which reduced the final concentration of free cells by a factor of 10 in 2-hour batch fermentations of milk. Zhou *et al.* (1998) have previously demonstrated the use of chitosan coatings in batch fermentations, and Klinkenberg *et al.* (2001a) have previously demonstrated the use of chitosan coatings and sequential chitosan and alginate coatings in batch and continuous fermentations. In contrast to those studies, the

use of CAC-coating in the present study did not significantly lower the amount of free cells in the fermentation broth at the end of the second sequential batch fermentations. However, in the previous studies the beads were exposed to the fermentation broth a significantly shorter time in each batch, giving less time for bead colonization. In those studies, the beads were exposed to the fermentation medium only a sufficient time to produce a given amount of lactic acid in the product. A similar method of application might be considered for enterocin production – allowing the immobilized cells to produce only a sufficient amount of bacteriocin for product conservation in each batch.

In this and in previous studies (Klinkenberg *et al.* 2001a) the use of CAC-coatings seemed to reduce the metabolic activity of the beads, resulting in a prolonged time of fermentation. This might both be due to a reduction in the amount of biomass immobilized, and to diffusional limitations posed by the coatings. In our study, a total bead volume of 100 ml was used in all fermentations. However, beads with CAC-coating initially contain less biomass per unit volume due to the presence of a cell free layer of alginate. As the biomass in CAC-coated beads is situated beneath a layer of alginate and chitosan, the diffusional resistance experienced by these cells might also be higher than in uncoated beads. In these batch fermentations, freely suspended cells will give an unknown contribution to the amount of free cells and bacteriocin activity in the final suspension. Due to the observed differences in time of fermentation, these contributions can be significantly different for CAC-coated beads and uncoated beads.

In order to be better able to distinguish between effects of the immobilized and freely suspended biomass, continuous fermentation at high dilution rates were used. The use of a high dilution rate reduces the contribution to the bacteriocin activity and biomass concentration in the effluent by growth and bacteriocin production of freely suspended cells. The assessment of bead performance was done in continuous reactors operated at a dilution rate of 3 h^{-1} , which is 3.75 times the maximum growth rate of the organism. At this dilution rate, less than 20 % of the glucose in the mMRS-broth was converted to lactate. In these fermentations, the amount of free cells and product produced by freely suspended cells can be quantified through the aid of mass balances (Klinkenberg *et al.* 2001a). Thus, this experimental system allows direct measurements of the kinetics of bacteriocin production, metabolic activity and cell release during the course of fermentation.

In continuous fermentations with uncoated beads, the biomass concentration in the product stabilized after 10 hours of fermentation – indicating a stable rate of cell release. Previously, several authors have reported that the biomass concentration in the outermost layers increases

significantly during the first hours of fermentation in alginate beads containing growing lactic acid bacteria (Audet *et al.* 1991, Cachon *et al.* 1993, Cachon *et al.* 1995b, Klinkenberg *et al.* 2001b). Eventually, the biomass concentration in the outermost parts of the beads stabilizes, as excess biomass growth is released to the fermentation broth. As a result of this process, most of the active biomass is concentrated close to the surface of the beads (Cachon *et al.* 1995a, 1995b, Klinkenberg *et al.* 2001b). Cachon *et al.* (1995a, 1997) used mathematical simulations in combination with practical experiments to study this phenomenon. These simulations indicated that this phenomenon was caused by diffusional limitations. However, these limitations did not affect the cells by reducing the accessibility to the growth limiting substrate, but by limiting lactate efflux, which results in acidification of the microenvironment (Cachon *et al.* 1995a).

The amount of free cells in the fermentation broth with CAC-coated beads was significantly reduced during the first 27 hours of fermentation compared to fermentations with uncoated beads. Data on the ratio of rates of cell release to lactate production (not shown) indicate that this reduction could not simply be explained by a reduction in the metabolic activity of the immobilized biomass. Analyses of the ratios of rates of bacteriocin production to cell release indicates that the use of CAC-coatings significantly alters the ratio of these rates during the first 20 hours, allowing bacteriocin production to continue while at the same time giving a reduced rate of cell release. The use of CAC-coating seems to slow down the formation of large concentrations of loosely bound biomass close to the surface. As these beads contain an outer layer without initial biomass, this layer must be penetrated before release is possible. Previous experiments have shown that the use of chitosan coatings significantly enhances the effect of an alginate layer in reducing rates of cell release (Klinkenberg *et al.* 2001a). The actual mechanism behind this effect is not known. Several studies have illustrated that chitosan diffuses into alginate beads and interacts with the alginate gel-network, forming an alginate-chitosan complex coacervate membrane (Gåserød *et al.* 1998, Hugunet *et al.* 1996). This membrane might possibly mechanically increase the resistance to cell release. Another possible explanation is that the process of chitosan coating may kill parts of the biomass in the outermost parts of the bead. This may cause a reduced biomass density in the outermost layers of the beads, which in turn reduces the rate of cell release.

The initial rates of bacteriocin production of both types of beads were similar when examined in batch and continuous fermentation. However, larger differences between the experimental systems appeared during fermentation. The highest rates of bacteriocin production in batch fermentations were observed early in the second of the sequential batch fermentation

(Table 2). These rates were, however, nearly 5 times less than rates of bacteriocin production observed at a similar time of continuous fermentation. Even though the medium is high in glucose and low in lactate, it is important to realize that the cell population in the batch fermentations has experienced a cycle of medium depletion – that is low glucose and high lactate concentrations during the first batch. This medium depletion might influence the growth pattern within these beads. In the continuous fermentations, however, high concentrations of glucose and low concentrations of lactate are preserved throughout the fermentation period. These differences may have a significant effect on the production kinetics observed in the two experimental systems.

In batch fermentations with freely suspended cells, the pH optimum for enterocin production in mMRS was pH 6.0. In fermentations at pH-values above this pH, both the rates of bacteriocin production and the maximum bacteriocin activity obtained in the fermentations were reduced. In immobilized fermentations, a different effect of fermentation pH was observed. In continuous fermentations with CAC-coated beads operated at pH-values from pH 6.3 to 7.5, rates of bacteriocin production increased with fermentation pH. As seen from measurements of the pH within the beads, this effect might be due to pH-gradients within the beads. In both uncoated and CAC-coated beads severe pH-gradients evolve during continuous fermentation. After 16 hours of fermentation, the pH in the interior of the beads was below 5. At such low pH-values, minimal growth and bacteriocin production was recorded in fermentations with freely suspended *E. faecium* CTC 492. These measurements suggest that only the biomass situated in the outer parts of the beads are metabolically active. Previously, several authors have reported similar observations (Masson *et al.* 1994. Cachon *et al.* 1997, Klinkenberg *et al.* 2001b). However, in CAC-coated beads, the presence of an initially cell-free layer in the outermost parts of the beads may limit the amount of biomass close to the surface. Measurements of the local pH within these beads indicate that the use of an increased fermentation pH may have increased the potential for metabolic activity in the layers situated beneath this cell-free layer. In these layers, the pH might be in compliance with high bacteriocin production even though the pH at the surface is too high. The observed production rates and patterns of production seem to be consistent with the hypothesis of such an advantageous pH-shift.

Compared to the biomass in uncoated beads, most of the biomass in CAC-coated beads might experience a somewhat lower pH due to the presence of the cell free coatings. If so, the different utilization of glucose for bacteriocin production observed with uncoated and CAC-coated beads in batch fermentations might be a reflection of this difference in local pH. Data from fermentations with freely suspended cells indicate that the pattern of glucose utilization

and bacteriocin production of *E. faecium* CTC 492 in mMRS-broth is influenced by pH. With freely suspended cells the maximum enterocin activity occurred before glucose was exhausted in fermentations operated at pH 6.0 and below. In the immobilized fermentations, a similar effect was seen with uncoated beads fermented at pH 7.0. This effect was, however, not seen in fermentations with CAC-coated beads (data not shown).

The development of the pH-gradient within uncoated beads might also explain the reduction in rate of bacteriocin production observed in continuous fermentation with uncoated beads. This reduction was present without a concomitant reduction in rate of lactate production, or a reduction in the immobilized biomass. In fact, both the rate of lactate production and the amount of immobilized biomass increased during this time period (data not shown). During the same time period, a significant increase in the steepness of the pH-gradient was observed. This increase may have introduced changes in the local environment experienced by most of the active biomass in these beads, which may have caused the observed reduction in the rate of bacteriocin production.

In this work the use of alginate immobilized *E. faecium* CTC 492 for the production of enterocin A and B has been evaluated. Both continuous and batch fermentations with immobilized *E. faecium* CTC 492 have been examined. In fermentations with immobilized lactic acid bacteria both immobilized and freely suspended cells contribute to product production. In our study a high dilution rate was used for the assessment of the kinetics of bead performance. A reduction of the dilution rate from 3 h⁻¹ to 1.3 h⁻¹ did not influence the rate of bacteriocin production in continuous fermentations with CAC-coated beads operated at pH 7.0. This suggests that bacteriocin production by freely suspended cells was not significant at a dilution rate of 3 h⁻¹. Such a high dilution rate does, however, reduce the bacteriocin concentrations in the product. If the main goal is a high bacteriocin concentration in the product and a good utilization of the growth media, lower dilution rates must be used.

In continuous fermentations with immobilized *E. faecium* CTC 492 the volumetric productivity is significantly increased, compared to traditional batch fermentations. In this study, beads with a diameter of ca. 3.0 mm were used. Previously, several authors have reported that the bead size in fermentations with alginate immobilized lactic acid bacteria has a significant impact on the activity of the beads (Yabannavar and Wang 1991, Champagne *et al.* 1994, Cachon *et al.* 1995b). In a previous study we reported that a reduction in bead diameter from 3.5 mm to 0.8 caused an increase in the volumetric rate of lactate production by a factor of approximately 3 in fermentations with *L. lactis* ssp. *lactis* NCIMB 6681 (Klinkenberg *et al.* 2001b). This effect is due to an accumulation of high biomass concentrations in the outermost

parts of the beads. In a similar volume of beads there is a larger volume situated close to the surface in smaller beads, compared to larger beads (Yabannavar and Wang 1991, Cachon *et al.* 1995b). There may, however, be practical difficulties related to using small beads in continuous reactors due to the problems with bead-liquid separation and bead instability (Audet *et al.* 1991).

The use of immobilized *E. faecium* CTC 492 in enterocin production significantly alters the relation between the amount of bacteriocin activity and the amount of free cells produced in the product, although with some influence on the amount of bacteriocin produced. Within a limited amount of time, the use of CAC-coating may be used to further reduce the amount of free cells produced in the product. In addition, the use of immobilized cells in batch fermentations allows reactors to be operated in a fill and drain modus, thus greatly improving process handling and reactor operation. In light of these findings the use of immobilized cell technology might be a valuable tool in increasing the performance of enterocin production - and might even open new applications of these bacteriocins.

ACKNOWLEDGEMENT

This work was financed by The Research Council of Norway (Project nr. 110682/420), and is part of SINTEF/NTNU's strategic institute program: Technology for competitive food processing. The authors would like to thank Dr. Trine Nilsen, Matforsk – The Norwegian Food Research Institute, and Dr. Erik Selmer-Olsen, Tine – The Norwegian Dairies Association, for supplying the organisms used in this study.

LITERATURE:

Audet, P., Lacroix, C., and Paquin, C. (1991) Continuous fermentation of a supplemented whey permeate medium with immobilized *Streptococcus salivarius* subsp. *thermophilus*. *International Dairy Journal*, 1, 1 - 15

Aymerich, T., Holo, H., Håvardstein, L.S., Hugas, M., Garriga, M., and Nes, I.F. (1996) Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bactericins. *Applied and Environmental Microbiology* 62, 1676-1682.

Bhugaloo-Vial, P., Wlodzimierz, G., Dousset, X. and Boyaval, P. (1997) Continuous bacteriocin production with high cell density bioreactors. *Enzyme and Microbial Technology* 21, 450-457.

Cachon, R., and Divies, C. (1993) Localization of *Lactococcus lactis* ssp. *lactis* by *diacetylactis* in alginate gel beads affects biomass density and synthesis of several enzymes involved in lactose and citrate metabolism. *Biotechnology Techniques* 7 (6), 453-456.

Cachon, R., Molin, P. and Divies C. (1995a) Modeling of Continuous ph-stat stirred tank reactor with *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* immobilized in calcium alginate gel beads. *Biotechnology and Bioengineering*, 47, 567 - 574

Cachon, R., Catté, M., Nommé, R., Prévost, H. and Divies, C. (1995b) Kinetic behavior of *Lactococcus lactis* ssp. *lactis* bv. *diacetylactis* immobilized in calcium alginate gel beads. *Process Biochemistry* 30, 503-510.

Cachon, R., Lacroix, C. and Divies, C. (1997) Mass transfer analysis for immobilized cells of *Lactococcus lactis* sp. using both simulations and *in-situ* pH measurements. *Biotechnology Techniques* 11 (4), 251-255.

Casaus, P., Nilsen, T., Cintas, L.M., Nes, I.F., Hernández, P.E., and Holo, H. (1997) Enterocin B., a new bacteriocin from *Enterococcus faecium* T136, which can act synergistically with enterocin A. *Microbiology*, 143, 2287-2294.

Champagne, C.P., Gaudy, C., Poncelet, D. and Neufeld R.J. (1992) *Lactococcus lactis* release from calcium alginate beads. *Applied and Environmental Microbiology* 58, 1429-1434.

Champagne, C.P., Lacroix, C., and Sodini-Gallot, I. (1994) Immobilized cell technologies for the dairy industry. *Critical Reviews in Biotechnology* 14, 109-134.

De Vuyst, L. and Vandamme, E.J. (1991) Microbial manipulation of nisin biosynthesis and fermentation. In *nisin and novel Antibiotics* ed. Jung, G. and Sahl, H.G. pp.397-400. Leiden: ESCOM.

De Vuyst, L., Vandamme E.J. (1994) Lactic acid bacteria and bacteriocins: Their practical importance. In *Bacteriocin of lactic acid bacteria* ed. De Voust, L., Vandamme, E.J. pp 1-11. Glasgow: Blackie academic & professional

Delves-Broughton, J. (1990) Nisin and its application as a food preservative. *Journal of the Society of Dairy Technology* 43, 73-76

Groboillot, A., Boadi, D.K., Poncelet, D. and Neufel, R.J. (1994) Immobilization of cells for application in the food industry. *Critical Reviews in Biotechnology* 14, 75-108.

Holo, H., Nilssen, Ø., and Nes, I.F. (1991) Lactococcin A, a new bacteriocin from *Lactococcus Lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. *Journal of Bacteriology* 173, 3879-3887.

Hüsken, L.E., Tramper, J., and Wijffels, R.H. (1996) Growth and eruption of gel-entrapped microcolonies. In *Immobilized Cells: Basics and Applications* ed. Wijffels R.H., Buitelaar, R.M., Bucke, C. and Tramper J. pp. 336-340 Amsterdam: Elsevier Science.

Jack, R.W., Tagg, J.R. and Kay, B. (1995) Bactericins of gram-positve bacteria. *Microbiological Reviews* 59, 171-200.

Klinkenberg, G., Lystad, K.Q., Levine, D.W and Dyrset, N. (2001) (a) Cell release of alginate immobilized *L. lactis* ssp. *lactis* immobilized in chitosan and alginate coated beads. *Journal of Dairy Science*, 84, 1118-1127.

Klinkenberg, G., Lystad, K.Q., Levine, D.W. and Dyrset, N. (2001) (b) pH controlled cell release and biomass distribution of alginate immobilized *Lactococcus lactis* ssp. *lactis*. *Journal of Applied Microbiology* 91, 705-714.

Lille, A., Pirhonen, T., Skjerve, E., Rosengren, Å., Wallin, H. and Skår Nordli, H. (1999) *NMKL-Procedure no. 8: Measurement of uncertainty in microbiological examination of foods*. pp. 2-23 Oslo: Nordic committee on food analysis.

Masson, F., Lacroix, C., Paquin, C. (1994) Direct measurements of pH profiles in gel beads immobilizing *Lactobacillus helveticus* using a pH sensitive microelectrode. *Biotechnology techniques* 8,8, 551-556.

Nilsen, T., Nes, I.F. and Holo H. (1998) An exported inducer peptide regulates Bacteriocin production in *Enterococcus faecium* CTC 492. *Journal of bacteriology* 180, 7, 1848-1854.

Pasini, G., Crapisi, A., Lante, A., Curioni, A, Zamorani, A., and Spettoli, P. (1995) Evaluation of Bacteriocin activity produced in milk by *lactococcus lactis*, subspecies *lactis*, immobilized in barium alginate beads. *Annals New York academy of sciences* 750, 465-468.

Scannell, A.G.M, Hill, C., Ross, R.P, Marx, S., Hartmeier, W. and Arendt, E.K. (2000) Continuous production of lactacin 3147 and nisin using cells immobilized in calcium alginate. *Journal of Applied Microbiology* 89, 573-579.

Wan, J., Hickey, W. and Coventry, M.J. (1996) Continuous production of bacteriocins, brevicin, nisin, and pediocin, using calcium alginate-immobilized bacteria. *Journal of Applied Bacteriology* 79, 671-676.

Willaert, R. and Baron G. (1996) Gel entrapment and micro-encapsulation: Methods, applications and engineering principles. *Reviews in Chemical Engineering*. Vol. 12, Nos. 1-2.

Willaert, R. and Baron, G. (1993) Growth kinetics of gel-immobilized yeast cells studied by on-line microscopy. *Applied Microbiology and Biotechnology* 39, 347-352.

Yabannavar, V. and Wang, D.I.C. (1991) Analysis of mass transfer for immobilized cells in an extractive lactic acid fermentation. *Biotechnology and Bioengineering* 38, 544-550

Zeza, N., Pasini, G., Lombardi, A., Mercenier, A., Spettoli, P., Zamorani, A. and Nuti, M.P. (1993) Production of a bacteriocin active on lactate fermenting clostridia by *Lactococcus lactis* subsp. *lactis* immobilized in coated alginate beads. *Journal of Dairy Research* 60, 581-591.

Zhou, Y., Martins, E., Groboillot, A., Champagne, C.P. and Neufeld, R.J. (1998) Spectrophotometric quantification and control of cell release with chitosan coating. *Journal of Applied Microbiology* 84, 342-348.

LABELS:

Figure 1: Growth and bacteriocin production in batch fermentations with freely suspended *E. faecium* CTC 492 operated at various pH values. The maximum bacteriocin concentration (◆) and specific growth rate (■) recorded in each fermentation are shown as function of fermentation pH.

Figure 2: Batch fermentations with *E. faecium* CTC 492 immobilized in uncoated (figure 2A) and CAC coated (figure 2B) alginate beads operated at various pH values. Bacteriocin concentration (closed symbols) and glucose concentration (open symbols) are shown as function of time of fermentation. ◆ pH = 6.0, ● pH = 6.3, ▲ pH = 6.7, ■ pH = 7.0.

Table 1: Sequential Batch fermentations with free *E. faecium* CTC 492 and immobilized *E. faecium* CTC 492 in uncoated and CAC coated beads – data on bacteriocin activity, free biomass concentration, glucose concentration and time of fermentation at the maximum bacteriocin concentration. The highest bacteriocin concentrations achieved, together with the corresponding biomass and glucose concentrations in the fermentation broth, the corresponding yield of enterocins on glucose ($Y_{E/G}$) and the corresponding time of fermentation are shown for 2 sequential batch fermentations operated at pH 6.0 and pH 7.0. The corresponding numbers obtained in fermentations with freely suspended cells at pH 6.0 and 7.0 are included for comparison.

Figure 3: Continuous fermentations with *E. faecium* CTC 492 immobilized in uncoated and CAC coated alginate beads operated at various pH-values and dilution rates. Recorded rates of bacteriocin production, expressed as AU/h·ml beads, are shown as function of time. ◆ Uncoated beads (pH = 6.3, $D \approx 3$), ■ CAC coated beads (pH = 6.3, $D \approx 3$), ▲ CAC coated beads (pH = 7.0, $D \approx 3$), △ CAC coated beads (pH = 7.0, $D \approx 1$), × CAC coated beads (pH = 7.5, $D \approx 3$).

Figure 4: Continuous fermentations with *E. faecium* CTC 492 immobilized in uncoated and CAC coated alginate beads operated at various pH values and dilution rates. The concentrations of free cells in the effluent of the reactors, expressed as cfu/ml, are shown as function of

time. ◆ Uncoated beads (pH = 6.3, $D \approx 3$), ■ CAC coated beads (pH = 6.3, $D \approx 3$), ▲ CAC coated beads (pH = 7.0, $D \approx 3$), △ CAC coated beads (pH = 7.0, $D \approx 1$), × CAC coated beads (pH = 7.5, $D \approx 3$).

Figure 5: Continuous fermentations with *E. faecium* CTC 492 immobilized in uncoated and CAC coated alginate beads operated at various pH values. The ratio of the rates of bacteriocin production and the rate of cell release, expressed as AU/cfu, are shown as function of time. All fermentations were run at a dilution rate of 3 h^{-1} . ◆ Uncoated beads (pH = 6.3), ■ CAC coated beads (pH = 6.3), ▲ CAC coated beads (pH = 7.0), × CAC coated beads (pH = 7.5).

Figure 6: Measured local pH within beads as function of distance from the bead surface in beads containing immobilized *E. faecium* CTC 492 during continuous fermentation. Uncoated beads (figure 6A) fermented at a pH of 6.3 and CAC coated beads (figure 6B) fermented at pH 6.3 (closed symbols) and pH 7.5 (open symbols) were harvested at different times of fermentation. The values are average values of two independent measurements. ◆ Beads harvested at 2.5 hours, ■ □ beads harvested at 19.5 hours, ▲ beads harvested at 44 hours. The arrow indicates the initial thickness of the CAC-coating.

Figure 7: Comparison of the performance of batch fermentations, immobilized batch fermentations and immobilized continuous fermentations with *E. faecium* CTC 492. Immobilized fermentations were run with *E. faecium* CTC 492 immobilized in uncoated and CAC coated alginate beads. All batch fermentations were run at a pH of 6.3, while continuous fermentations were run at a dilution rate of 1.3 and pH 7.0. The productivities at maximum bacteriocin concentration (AU/h·ml), and the corresponding bacteriocin concentrations (AU/ml) and ratios of bacteriocin to biomass (AU/cfu), are shown for the batch fermentation. Data at 10 hours are shown for the continuous fermentations. U = Uncoated beads, CAC = Chitosan/alginate/chitosan coated beads. F. cell. = Freely suspended cells.

Figures, publication enterocin

Figure 1:

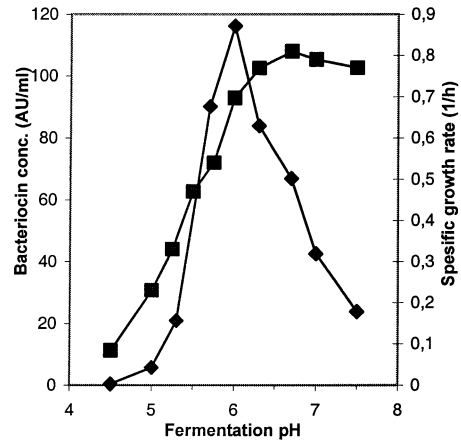


Figure 2:

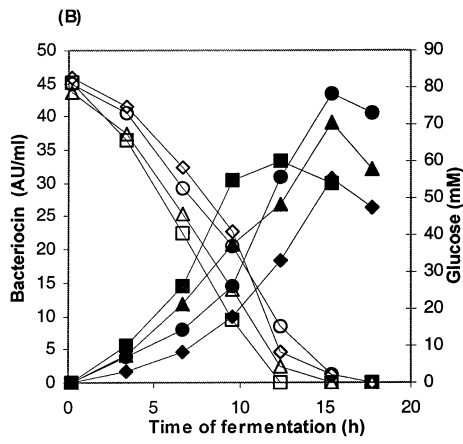
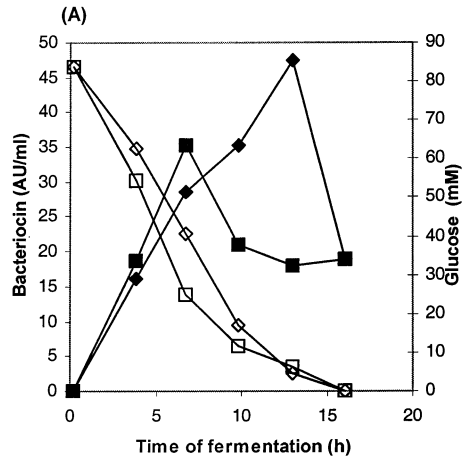


Table 1:

Fermentation	Enterocin (AU/ml)	Free cells (cfu/ml)	Glucose (mM)	Y _{E/G} * (AU/mmol glu.)	Time of fermentation (h)
Free cells, pH = 6.0	116	1.1×10 ¹⁰	36.5	1443	17.0
Uncoated, Batch 1, pH = 6.0	48	5.2 × 10 ⁷	4.6	592	9.8
Uncoated, Batch 2, pH = 6.0	22	6.7 × 10 ⁷	8.3	286	6.7
CAC, Batch 1, pH = 6.0	31	5.0 × 10 ⁶	2.3	372	15.4
CAC, Batch 2, pH = 6.0	35	7.7 × 10 ⁷	13.9	474	7.2
Free cells, pH = 7.0	43	1.3×10 ¹⁰ †	30.9	542	11.2
Uncoated, Batch 1, pH = 7.0	35	5.9 × 10 ⁷	24.9	587	6.8
Uncoated, Batch 2, pH = 7.0	19	2.5 × 10 ⁷	34.9	398	3.5
CAC, Batch 1, pH = 7.0	33	1.0 × 10 ⁷	0	397	12.4
CAC, Batch 2, pH = 7.0	20	4.0 × 10 ⁸	2.3	254	7.2

* Y_{E/G}: Amount of enterocin activity produced divided on the amount of glucose consumed. Data at the time of maximum enterocin activity were used.

† Value estimated from OD measurements and a correlation between cfu/ml and OD.

Figure 3:

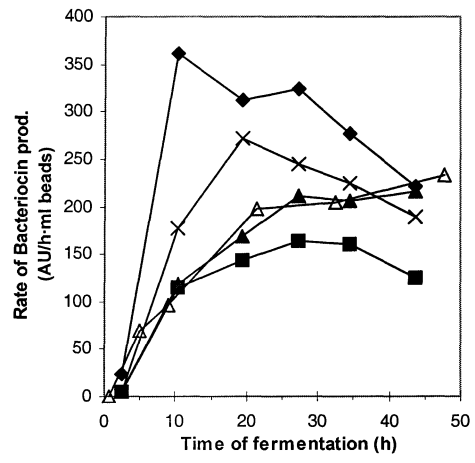


Figure 4

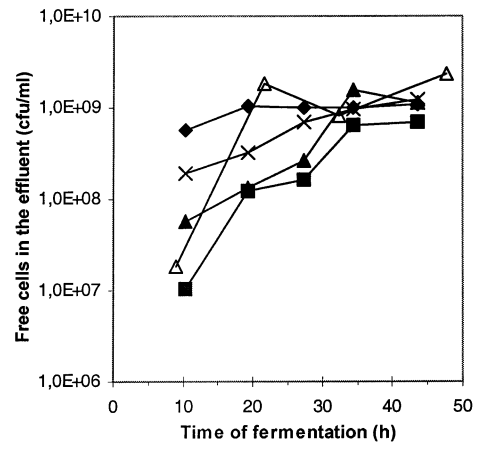


Figure 5

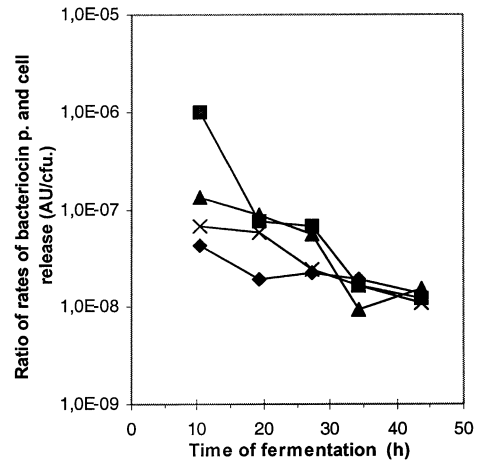


Figure 6:

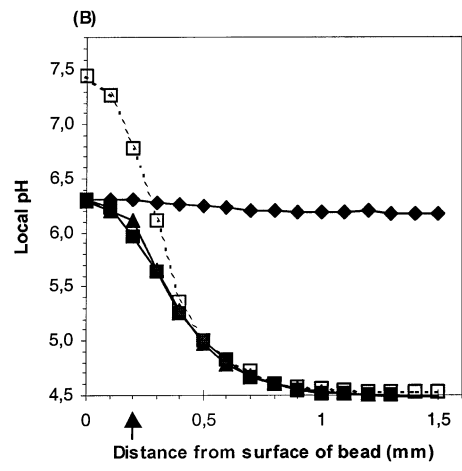
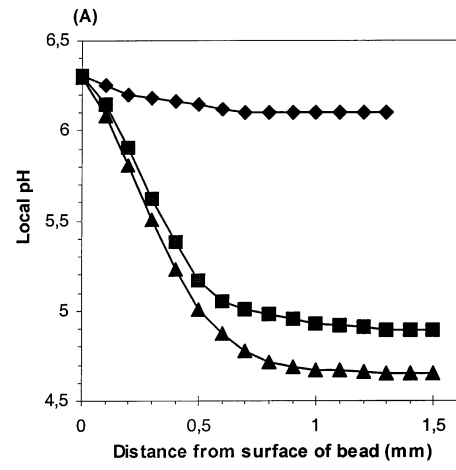


Figure 7

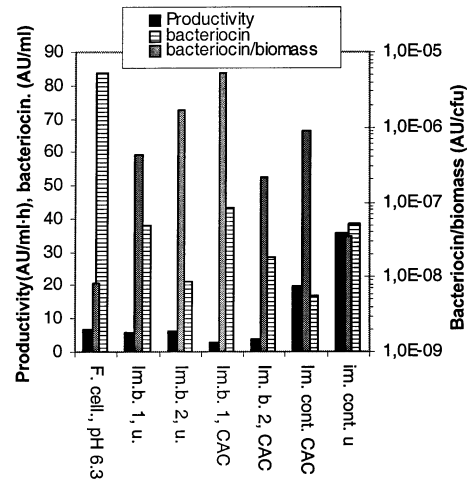


Table 2:

Fermentation pH	6.3	7.0
CAC batch (AU/ml bead·h)*	27.1	26.0
CAC cont. (AU/ml bead·h)†	143	169

* Maximum rates of bacteriocin production in batch fermentations.

† Rates of bacteriocin production in continuous fermentations a time of fermentation corresponding to the maximum rate in batch fermentations.