Carrageenan: A Food-Grade and Biocompatible Support for Immobilisation Techniques

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Dedicated Prof. Dr. Roger A. Sheldon on the occasion of his 60th birthday.

Abstract: Immobilisation of both enzymes and whole-cell systems is of major importance in the improvement of the stability, activity and reusability of these biocatalysts. This review describes the use of the naturally occurring polysaccharide carrageenan as a support for the immobilisation of biocatalysts. Carrageenan is a food-grade and biocompatible support material extracted from red seaweeds. Before focusing on the use of carrageenan as an immobilisation support, an overview is given of the present uses of biocatalysts in industrial processes. The basic concepts of enzyme and whole-cell immobilisation are discussed, as well as the background of carrageenan as a biopolymer. Several examples of enzymes and whole-cell systems immobilised in carrageenan are discussed. A list of the most relevant patents in this field is presented as well as a list of enzymes and cell systems immobilised in carrageenan as described in the literature.

Keywords: biocatalysis; biotransformations; carbohydrates; carrageenan; enzyme catalysis; supported catalysts; whole-cell catalysis

1 Introduction

Biocatalysts are traditionally used in aqueous media.[1–4] History has shown us that the use of enzymes in biotechnological processes has been largely focused on their hydrolytic activity towards biopolymers.[1–7] Replacing the aqueous reaction medium with an organic solvent in order to perform the reverse synthesis reaction widened the potential of biocatalysis enormously.[1] Application of biocatalysts in organic solvents

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Fred van de Velde studied chemical engineering at the Delft University of Technology. In 1995 he received his degree in organic chemistry and catalysis for a study on ruthenium-catalysed epoxidations. He received his PhD from the same university in 2000, studying the use of peroxidases as catalysts for the synthesis of fine chemicals in the group of Prof. R. A. Sheldon. He continued as a post-doc for the Wageningen Centre for Food Sciences (WCFS) employed by TNO Nutrition and Food Research Institute in Zeist. The main research topic is the relation between the chemical/molecular structure of food biopolymers and their macroscopic properties, e. g., rheological behaviour. Fred is especially interested in structure/function relation of carrageenans, and is the authors of several publications and book sections about carrageenans. In January 2002 he moved to NIZO food research (Ede, the Netherlands) and continued his work for the Wageningen Centre for Food Sciences as scientist.

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Martin Bakker studied organic chemistry at the University of Amsterdam. He worked for one year at the Bioorganic Department of Prof. U. K. Pandit, on the synthesis of transition state analogues for catalytic antibodies. Afterwards, he worked at Solvay Pharmaceuticals in Weesp at the development of new methods for the synthesis of new biological active compounds for serotonine and dopamine receptors. In 1995 he got his Master of Science degree in the organic chemistry and went for his PhD to Delft University of Technology. Under supervision of Prof. R. A. Sheldon he worked at the immobilisation of metallo-enzymes and their application in non-natural conversions. In 2000 Martin joined Avantium Technologies, a company working in high throughput experimentation and simulation. As a project leader in fine chemicals and pharmaceuticals he was involved with the business development of biocatalysis within the company. Recently Martin joined Pharma Bio-Research Group in Zuidlaren as a Study Director. He is involved in bioanalytical studies in compliance with the OECD Principles of GLP.

stimulated the development of immobilisation techniques to improve their operational stability. The original work of Klibanov, who presented a large number of applications of biocatalysts in non-aqueous media, greatly stimulated further research in the field of biotransformations.[8,9] Nowadays, the ability of enzymes to act as regiospecific and enantioselective catalysts in aqueous as well as in organic media is widely recognised in the production of foods,[10] fine chemicals,[11] pharmaceuticals,[12,13] agrochemicals, and detergents,[14,15] and they are used for fuel and waste gas biodesulphurisation,[16–19] biopulping,[20] and wastewater treatment.[21,22]

Moreover, biocatalysts – either native or immobilised – are progressively being more applied by chemical industries, integrated in traditional, multi-step chemical processes, particularly for high added value and low volume speciality products.[12] Hence, a slightly higher number of enzyme-catalysed reactions in organic solvents has been reported in the last decade as compared to biotransformations in aqueous media.[1,23–27] During the last decades several industrial processes were developed using enzymes in native or immobilised form or whole cells as biocatalysts, as summarised in Table 1.

The production of high-fructose corn syrup (HFCS, top of Table 1) is probably the most well-known example of an enzyme application in industrial processes.[10,28] Not surprisingly, starch and sugar processing is the largest application of food enzymes with a processing segment worth about $58 million (annually), the produced HFCS being primarily used by the soft-drink market.[29] Moving downwards in Table 1, from the manufacturing of food ingredients to that of fine-chemicals and pharmaceuticals, the scale in tons per year decreases with the increase in the price per kg of product. For instance, fructose has a bulk price of $1 per kg whereas enantiopure compounds, e.g., amines or L-DOPA, are marketed at over $100 per kg. In the food industries the profit margins of even the most profitable processes are much smaller than in the pharmaceutical or fine-chemical industries. This trend is mainly due to the prices of the biocatalyst and the final product and the scale of the industrial process. Competition and large-scale production push down the prices of the major enzymes.

Many industrially relevant hydrolases, such as proteases, lipases, and aminoacylases (see Table 1), are derived from fungal or bacterial sources, which are eminently suitable for high-productivity large-scale culture. The enzymes for, e.g., food, laundry and agrochemical purposes are produced in bulk quantities and generally used without further purification.[23,30,31] Enzymes for the production of pharmaceuticals are generally marketed as a preparation with a high degree of purity and, therefore, in relatively small quantities.[4,12] In most cases, this results in higher enzyme production costs, which also significantly influences the cost-price of the final product for which the enzyme is used. Immobilisation of these expensive enzymes improves the reusability and, thereby, reduces the cost-price for the catalyst. Ultimately, the commercial viability of a bioconversion depends on the enzyme-derived costs per kg of product, which are determined by the cost of obtaining the enzyme and by its productivity.

The productivity of an enzyme-catalysed reaction is, in turn, determined by the enzyme productivity

Table 1. Industrial applications of biocatalysts.[11,13,166]

Substrate	Product	Biocatalyst	Scale (tons/year)	Company
Glucose	Fructose	Glucose isomerase; immobilised enzyme	$>10^6$	UOP
Lactose	Glucose and galactose	β-Galactosidase; immobilised enzyme	$>10^6$	DSM (Gist), Sumitomo
Triglycerides	Cocoa butter	Lipase	$>10^6$	Unilever
Acrylonitrile	Acrylamide	Nitrile hydratase; whole cell	$>10^3$	Nitto Chemical
Adiponitrile	5-Cyanovaleramide	Nitrile hydratase; whole cell	$>10^2$	Dupont
Racemic alcohols and amines	Enantiomeric pure alcohols and amines	Lipases	$>10^3$; $>10^2$	BASF
D,L-Methionine	L-Methionine	Aminoacylases; membrane reactor or immobilised enzyme	$>10^3$	Degussa/Tanabe[a]
Aliphatic acids	L-Carnitin	Hydroxylase; whole cell	$>10^2$	Lonza
3-Cyanopyridine	Nicotinamide	Nitrile hydratase Immobilized enzyme	$>10^3$	Lonza
Penicillins	6-Aminopencillanic acid (6-APA)	Penicillin amidohydrolase	$>10^3$	DSM
Pyrocatechol	L-3-(3,4-Dihydroxyphenyl)alanine (L-DOPA)	Tyrosin phenollyase; whole cell	$>10^2$	Ajinomoto

[a] Degussa is producing with a membrane reactor and Tanabe's process is running with a fixed-bed reactor.

(g product per g biocatalyst per unit time) which is related to the turnover number (TON), or rather the turnover frequency (TOF), and to the volumetric productivity or space-time yield (STY). The turnover number (TON: moles of product produced per mole of catalyst) and turnover frequency (TOF: moles of product per mole of catalyst per unit time) are important parameters in the determination of catalyst costs for a given process. Immobilisation techniques (see next section) have resulted in biocatalysts with increased operational stability as compared to their native forms. This significantly reduces the incidence of enzyme costs since a higher total turnover number is achieved, although the price of the carrier should also be taken into account.[32] The second important parameter, STY, is the amount of product produced in a certain reactor volume per unit time (which has a bearing on the required installation costs). An industrial biocatalytic process will stand or fall depending on the above-mentioned parameter values and still further effort is necessary to screen for new processes and/or increase the viability of the existing ones. Immobilisation techniques play an important role in the development of new or improved biocatalytic processes.

2 Immobilisation Techniques for Biocatalysts

2.1 Overview

Biocatalysts have the ability to catalyse reactions under mild conditions of pH, temperature and pressure. Moreover, they can be used under such environmental conditions with high efficiency, specificity and selectivity. Enzymes and whole-cell systems containing specific enzymes or enzyme systems are therefore used in many industrial processes (see previous section). However, the industrial application of enzymes can be limited by several factors, such as their availability in small amounts, their generally high costs and their frequent instability under the desired reaction conditions. Moreover, enzymes are mostly soluble in aqueous solvents, which makes it difficult and expensive to recover the biocatalyst once the catalytic process is complete. The same general limitations apply to the use of whole cells as a source of enzymes and enzyme systems.

To facilitate the recycling of biocatalysts Chibata and coworkers[33] pioneered in 1972 the use of immobilised enzymes in industrial processes. Since then numerous methods of immobilisation on a variety of different materials have been developed in order to improve the stability, activity and reusability of enzymes and whole cells.[28,34–36] However, the immobilisation of enzymes and whole cells has also some disadvantages which are summarised in Table 2, together with its advantages.

The decision to use either an immobilised or a free enzyme or whole-cell system as biocatalyst in a given procedure depends on economical, technical and practical considerations.[4] The more expensive the enzyme preparation, the greater the incentive to use it in immobilised form to minimise its associated costs per unit weight of product. However, some disadvantages, such as the loss of biocatalyst selectivity and activity, could disfavour immobilisation.[37] Furthermore, certain enzymes can be produced cheaply in bulk quantities and immobilisation is not economically justified in this case.[2]

As in any industry, the driving force for biocatalysis is economics. Therefore, several objectives for enzyme immobilisation could be defined in order to ensure a highly profitable process development:

(1) Retain a large portion of the enzyme's native activity;
(2) Maintain structural stability under reaction conditions;
(3) Exhibit high storage stability;
(4) Attain a high protein concentration to reduce the fraction of reactor volume occupied by the catalyst;
(5) Allow low costs for carrier preparation and immobilisation method.

All these objectives will serve as a template to obtain higher efficiencies and yields for industrial processes with low additional costs. The biocatalysts to be immobilised can be either whole cells or the isolated enzymes. The techniques for immobilisation can be divided into five different categories, i.e., adsorption, ionic binding, covalent attachment, cross-linking, and entrapment (see Figure 1).

Table 2. Advantages and disadvantages of enzyme and whole cell immobilisation.

Advantages	Disadvantages
Easy recycling of catalyst	Extra costs
More simple down-stream processing	Loss of activity
Stabilisation of enzymes	Diffusion limitations
Good reaction control	Increased catalyst volume
Flexibility of reactor choice (continuous or fixed bed)	A time-consuming effort to obtain a suitable carrier

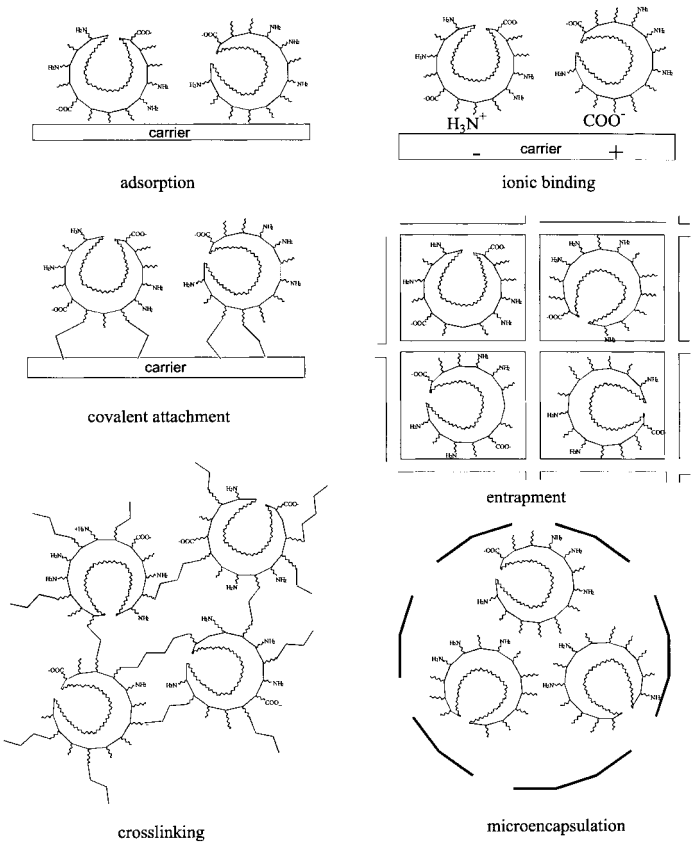


Figure 1. Schematic representation of enzyme immobilisation methods.

2.2 Adsorption

Adsorption of enzymes is based on mostly non-specific, physical interactions between the biocatalyst and the support surface. The bond is generally weak and affected by pH, temperature, and contact with salts and solvents. Carriers that have been used range from inorganic oxides to organic polymers, such as Accurel EP100 (polypropylene),[38] silica,[39] mesoporous molecular sieves,[40] Eudragit S-100 (an enteric methacrylate polymer),[41] and Teflon.[42] Most are macroporous and adsorb the enzyme at the internal pore surface. However, immobilisates prepared *via* adsorption can be subject to leaching of the enzyme to the aqueous medium. Adsorption followed by cross-linking prevents leaching and can increase enzyme stability, although activity can be adversely affected.[43]

2.3 Ionic Binding

In ionic binding procedures, enzymes are regarded as polyelectrolytes that will bind to supports of opposite charge. Ionic binding of enzymes gives (operationally) much more stable preparations than physical adsorption, although the stability of the preparation may be sensitive to changes in pH and ionic strength. Hence, the carrier is easy to regenerate. Whether the carrier has an anionic or a cationic character, the resulting enzyme load and activity will depend on the enzyme charge. Reported examples of positively charged (anion exchange) enzyme supports are DEAE-cellulose,[44] chitosan beads,[45] Amberlite (IRA),[46] and DEAE-Sephadex.[47] Typical negatively charged (cation exchange) supports are cellulose phosphate,[48] Amberlite (ICR),[49] and dextran sulphate.[50]

2.4 Covalent Attachment

The immobilisation of enzymes via covalent attachment is probably the most widely applied method.[4] The bond is created through the reaction of reactive groups at the protein surface, e.g., the N-terminus, lysine amino groups or hydroxy, sulphydryl, or phenolic functional groups, with corresponding substrate groups at the surface of water-insoluble matrices. Examples of carriers which are used for the covalent immobilisation of enzymes are activated cellulose[51], dextran,[52] and starch[53] (pre-activated *via* the formation of reactive imidocarbonates from native hydroxy groups and cyanogen bromide), isocyanate prepolymers (Hypol 3000),[33,54,55] and the very popular Eupergit C (copolymer of methylacrylamide containing oxirane groups).[56,57] For the latter, the protein amino groups react with the oxirane groups *via* nucleophilic substitution. Sulphydryl and hydroxy groups of enzymes also react with epoxide groups but their reactivity is very low when compared with that of the amino groups.

One of the advantages of covalent attachment of enzymes as an immobilisation method is an increased stability, caused by the strong interaction with the carrier. However, the strong bond decreases the enzyme's molecular flexibility, which may have an effect on activity. Moreover, the multipoint attachment may induce structural changes resulting in loss of activity. Finally, the often expensive carrier material generally cannot be reused.

2.5 Cross-Linking

Cross-linking involves the attachment of molecules of enzyme to each other *via* covalent bonds. Bi- and multifunctional compounds are used as reagents for the intermolecular cross-linking of enzymes, such as glutaraldehyde,[58] diazobenzidine,[59] tannic acid,[60] and dimethyl adipimidate.[61] They usually react with amino groups at the outer surface of the protein. The method is very attractive due to its simplicity and an almost pure immobilised enzyme is obtained. The cross-linking of dissolved enzymes is hard to control, however.[23] Cross-linking of protein precipitates affords a higher degree of

control over the properties of the final product. Robust catalysts have been obtained *via* the cross-linking of enzyme crystals (CLEC's)[62–65] and, recently, good results were obtained with so-called cross-linked enzyme aggregates (CLEA's).[66–68] Although CLEC's are chemically and mechanically quite robust catalysts, the development of suitable protocols for crystallisation and cross-linking is lengthy and labour-intensive, which is a distinct disadvantage of these preparations, when compared with CLEA's.

2.6 Entrapment

Entrapment is a mild immobilisation method that does not involve any modification or binding of the enzyme. The method is based on the encapsulation of enzymes within matrices, membranes, or lattice structures, effectively preventing enzyme leakage from the porous structure. Entrapment in a polymeric matrix can be accomplished by polymerising or cross-linking, e.g., thermally or chemically, a monomer or polymer solution containing the enzyme. Many variations are possible with regard to the constraining structure, e.g., gel or fibre entrapment[69–72], or microencapsulation (see next section) and to the polymers used, for example polyacrylamides[73,74] and carrageenan.[75,76] As opposed to the other immobilisation methods, this procedure can be applied to every kind of biocatalyst, which makes the method more or less universal. However, a disadvantage of this method is that a large pore size could cause enzyme leakage whereas a small pore size could prevent the diffusion of large substrate molecules into the matrix to reach the biocatalyst. Furthermore, the conditions during polymerisation can destroy enzymes.

2.7 Microencapsulation

Microencapsulations are performed with semipermeable polymer membranes, prepared from nylon,[77] κ-carrageenan,[78] cellulose nitrate,[79] or 1,6-diaminohexane and adipoyl chloride[80], in such a way that enzymes or whole cells are enclosed in microcapsules. The usual membrane pore size ranges from 1 to 100 nm, which is sufficient to prevent enzyme or cell leakage and to allow substrates to dialyse freely across the membrane.[81] Microcapsules can be likened to artificial cells. The use of permeated, whole dead cells is a closely related technique. A small pore size can be a disadvantage for high molecular weight substrates. Moreover, in case of whole-cell encapsulated enzymes the cell loading of capsules is limited by the size and the material properties of the polymer bead.[78] Furthermore, the application of microencapsulation is, similarly to fibre entrapment, limited to aqueous media.

2.8 Choosing Carrageenan as Immobilisation Support

The choice of immobilisation method and of support depends on the nature of the biocatalyst (e.g., whole cells or purified enzymes, from fungal or bacterial origin, either native or genetically modified), the envisaged process conditions, the type of reactor to be used, and the specific application of the biocatalyst. In general, the important characteristics are operational stability, particle size, solubility (or insolubility), biodegradability, diffusivity of substrates and/or reactants, and in the case of cell immobilisation, the growth parameters inside the support. Most processes in the pharmaceutical industry involve small-scale operations with high research and development costs. These costs are usually recouped by charging relatively high prices for the produced drugs. In contrast, the food industry is mainly composed of large-scale operations with a low profit margin.[75] Consumers expect a safe product, readily available for a reasonable price. Moreover, in contrast with the production of pharmaceuticals, it is difficult to significantly improve a food production process so as to make it economically highly profitable.

For food and pharmaceutical applications the required protocols are different than those used for (fine) chemical applications. Regulatory entities, such as the FDA, require more extensive investigations when new polymers, cross-linking agents, and other chemical additives are introduced during enzyme immobilisation. Hence, in most cases a time-consuming effort, such as the determination of the leaching levels of materials and their toxicity, has to be instigated before the process will be FDA-approved. To save in time and costs it could be helpful to use food-grade chemicals, as well as cheap (bio)polymers generally recognised as safe (GRAS), as suitable carriers. Carrageenan has a long history of safe food applications and is, therefore, a suitable support for applications in food and pharmaceutical industries.

The solubility of carrageenan in aqueous media is strongly dependent on the presence of counter ions and temperature (see Section 3). The presence of gel-inducing counter ions increases the stability of the gel beads, whereas the beads dissolve in reaction media with low ionic strength.

Thermoreversible gels, such as carrageenan, melt at elevated temperature. Lowering the temperature results in the gelation of the biocatalyst/biopolymer mixture. The temperature-induced gelation allows for the easy formation of gels of different shapes. For carrageenan the melting temperature increases with increasing salt concentration in the reaction medium. The application of carrageenan is thus limited by the composition of the reaction medium. On the other hand, the possibility of melting the beads or of redissolving them could be a positive aspect for the final disposal of the spent support material through biodegradation. The biodegradability of biopolymers is a major issue in cases

where large amounts of biocatalyst have to be discarded. On the other hand, if immobilised biocatalysts are to be used for industrial wastewater treatment, a support is needed that will resist degradation by microorganisms. Follow-up treatments can improve the mechanical stability of the carrageenan beads.

3 Carrageenan

3.1 Introduction

Carrageenan is a generic name for a family of gel-forming and viscosifying polysaccharides, which are obtained by extraction from certain species of the red seaweeds (Rhodophyta). They are produced on a commercial scale in Argentina, Chile, the Philippines, Indonesia, Morocco, France, Canada, and the North Atlantic region. Carrageenans are used in a wide variety of applications, especially in food products, such as frozen desserts, chocolate-milk, cottage cheese, whipped cream, yoghurt, jellies, and sauces. In addition to this, carrageenans are used in pharmaceutical and cosmetic formulations and in other industrial applications, such as in oil-well drilling fluids.[82]

The use of carrageenan for food applications started almost 600 years ago. Due to this long and safe use, carrageenan is generally recognised as safe (GRAS) by experts of the US Food and Drug Administration (21 CFR 182.7255) and is approved as a food additive (21 CFR 172.620). In the EU, carrageenan is included in the list of permitted emulsifiers, stabilisers, thickening, and gelling agents. The World Health Organisation (WHO) Joint Expert Committee of Food Additives has concluded that it is not necessary to specify an acceptable daily intake limit for carrageenan. These registrations made carrageenans applicable in food products, a subject which has been reviewed in several textbooks.[82–84] Since the 19th century, carrageenan has been used for industrial applications. In the 1970's, the use of carrageenan for the immobilisation of enzymes and microorganisms was introduced by the pioneering work of Chibata and coworkers.[85–87]

3.2 Production

Carrageenan-containing seaweeds are harvested both from naturally occurring populations and cultivated species.[88] The latter option is called seaweed farming and is of importance to remove the harvest pressure on target species in sensitive eco-systems.[82] The harvesting of carrageenan-containing seaweeds is labour-intensive, as the largest part is manually collected.[83,88] Harvested seaweed is first washed to remove sand and stones. In the production plant the seaweed is extracted under alkaline conditions to produce carrageenans of good quality. The extraction lasts several hours at a temperature near the boiling point of the aqueous alkaline solution ($\geqslant 110\,^{\circ}C$). The extract is subsequently filtered and concentrated to a final carrageenan concentration of 3%. The carrageenan is precipitated by the addition of 2-propanol, dried, and finally ground to an appropriate particle size.[82] Commercial carrageenan preparations are often blended with additives such as sucrose, glucose, or galactomannans of some higher plants to adjust their viscosity or improve their gelling properties.

3.3 Chemical Structure

Carrageenan is not a single biopolymer but a mixture of water-soluble, linear, sulphated galactans. Several types of carrageenans differ in the number and position of sulphate groups. The most common types of carrageenan are traditionally identified by a Greek prefix. The three commercially most important carrageenans are called ι-, κ-, and λ-carrageenan. ι- and κ-carrageenans are gel-forming, whereas λ-carrageenan is a thickener/viscosity builder and, therefore, not applicable for immobilisation techniques. The gel-forming ι- and κ-carrageenans are composed of alternating, 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-D-galactopyranose units, forming the disaccharide repeating unit or diad of the carrageenans (see Figure 2).

κ-Carrageenan has one sulphate group per repeating diad (attached to O-3 of the galactopyranose ring), whereas ι-carrageenan has an additional sulphate group (attached to the O-2 of the anhydrogalactopyranose ring). Besides galactose and sulphate, other carbohydrate residues (e.g., xylose, glucose, and uronic acids) and substituents (e.g., methyl ethers and pyruvate groups) are present in carrageenans. The molecular composition of commercial carrageenan batches can routinely be analysed by NMR spectroscopy,[89] either alone or in combination with monosaccharide composition analysis and sulphate content determination.[90]

3.4 Gel Formation

Gel formation is the most important feature of carrageenans and essential for their application in immobilisation techniques. As said above, only κ- and

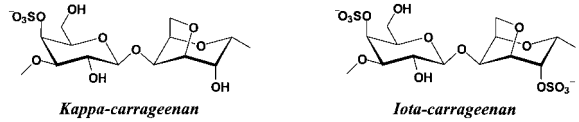


Figure 2. Idealised structure of the repeating disaccharide unit of carrageenan.

ι-carrageenans are able to form gels, as the other carrageenans lack the essential $^1C_4$-conformation that results from the 3,6-anhydro bridge.[91] The transition from a disordered (random coil) to the ordered (helical) state is the first step in the gelation of carrageenan.[92] The change to the helical conformation is promoted by the addition of salts or by lowering the temperature.[93,94] Both mechanisms are used for the preparation of gel beads for immobilisation purposes. ι-Carrageenan forms a gel in the presence of calcium, whereas the gelation of κ-carrageenan is specifically promoted by some monovalent cations (K^+, Rb^+, Cs^+, and NH_4^+). The strength of both types of gels is controlled by the concentration of gel-inducing cations.

In general terms, ι-carrageenan forms soft and weak gels that are freeze/thaw stable, whereas κ-carrageenan gels are hard, strong and brittle gels that are freeze/thaw instable. Both types of gels are thermoreversible, which means that the gels will melt when heated and form a gel again upon cooling. Galactomannan gums, especially locust bean gum, modulate the morphology of the carrageenan gels. The synergistic interaction between κ-carrageenan and locust bean gum is widely used in the food industries.[84,95] The interaction between locust bean gum and carrageenans is also studied for the application in immobilisation techniques.[96]

4 Methods for Biocatalyst Immobilisation in Carrageenan

4.1 General

The different techniques for the immobilisation of biocatalysts, either purified enzymes or whole cells, into carrageenan beads and matrices of other shapes are facilitated by the fact that under non-gelling conditions carrageenan solutions exhibit moderate viscosity and are relatively easy to handle. Gelling is promoted by lowering the temperature or by the addition of gel-inducing agents, such as cations,[82] water-soluble amines, or water-miscible organic solvents.[87] The different methods described in the literature can be roughly divided into four groups: gel method, droplet method, emulsion method, and dehydration method. These four methods are described below. In addition to these different methods, several follow-up treatments and modifications are applied to improve the stability of the beads. A few of these follow-up treatments are summarised in a separate section.

4.2 Gel Method

The simplest immobilisation technique is the gel method. An aqueous solution of carrageenan is heated above the gel temperature of the carrageenan. After dissolution of the carrageenan the solution is mixed with a heated solution or suspension of the biocatalyst and the mixture is allowed to cool and gel. The gel is then cut into the desired shape, such as cubes,[86] or crushed into small particles. The latter is described for gelatine beads,[97] and is also applicable for brittle carrageenan gels, such as κ-carrageenan/potassium gels.[98] Membranes of immobilised enzymes or whole cells can be obtained through the preparation of a thin film of carrageenan gel.[86]

The limitation of this method is the thermosensitivity of the biocatalyst. The biocatalysts need to be stable above the gel temperature of the carrageenan solution. As discussed in Section 3, both the gel temperature and the gel strength are controlled by the concentration of gel-inducing ions. Thus, by lowering the salt concentration, the gel temperature can be decreased to below the temperature to which the biocatalyst is substantially thermosensitive. However, lowering the salt concentration also decreases the gel strength of the final gel. To produce strong gels this method is only applicable to thermostable biocatalysts. The advantages of this method is the fact that the gels can be produced in any shape, either by cutting, shredding, moulding, or other shaping techniques.[98,99]

4.3 Droplet Method

The droplet method is widely used for the preparation of spherical gel beads (see Figure 3). In contrast to the gel and emulsion method, for this approach the carrageenan/biocatalyst mixture requires no heating and can be prepared at room temperature. The carrageenan is dissolved in distilled water and mixed with a solution or suspension of the bioactive material, resulting in a solution/suspension of low ionic strength and moderate viscosity. This solution/suspension is extruded dropwise into a stirred potassium chloride solution using techniques such as, a pump with a needle,[100] a syringe,[101] or a nozzle,[102] depending on the scale of the process and the desired bead size. A typical KCl concentration used is 0.3 M at or below room temperature. Under these conditions the carrageenan solution immediately sol-

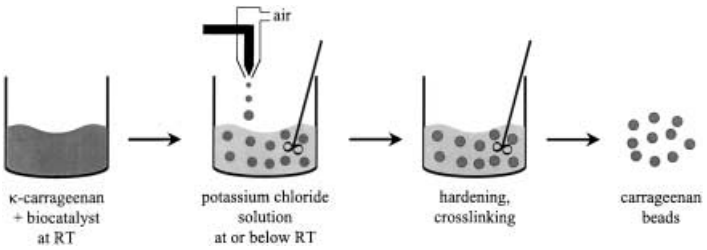


Figure 3. Schematic representation of the preparation of carrageenan beads by the droplet method.

idifies. Instead of a KCl solution, other gel-inducing conditions can also be used, such as alcohols or a combination of alcohol and KCl.[87,100] Gel beads obtained via this droplet method normally have an average diameter in the order of a few millimetres.[100,102,103]

The gel beads are stored in the KCl solution to increase their stability. A limitation of this method is that the beads will dissolve when placed in reaction media with low ionic strength. The stability of the beads can be improved by hardening and/or cross-linking (see Section 4.6).

4.4 Emulsion Method

As for the gel method, the emulsion method (see Figure 4) is based on the temperature-induced gelation of the carrageenan solution. This method has, for example, been described for the preparation of immobilised yeast cells for brewing applications[104] and for the immobilisation of *Acidovorax focilis* 72W cells for the chemoenzymatic production of 1,5-dimethyl-2-piperidone.[98] In this method an aqueous solution/suspension of carrageenan and biocatalyst is heated above the gel temperature. This aqueous phase is then mixed with a heated, non-reactive food-grade oil phase (canola oil or soybean oil has been used in the above-mentioned examples). The two phases are vigorously mixed to obtain biocatalysts/carrageenan droplets of the desired size. Under continuous stirring the droplets are gelled by lowering the temperature of the oil. The oil is than decanted from the resulting beads and the beads are washed with appropriate buffer and/or salt solutions. By controlling the stirring rate and optionally through the addition of emulsifiers, this method is eminently suited to prepare beads of a particular desired size. However, similar to the gel method, this method is limited to rather thermostable biocatalysts as the biocatalyst/carrageenan mixtures are heated above the gel temperature.

The above-described technique is used in an advanced apparatus, developed by Lockheed Martin Energy Sys[105] for the continuous production of gel beads containing a biocatalyst. In this approach the heated carrageenan/biocatalyst solution is sprayed at the top of a column into a heated non-interactive liquid, typically an organic solvent. In this solution spherical droplets of the carrageenan solution are formed. The droplets are allowed to fall through the liquid, due to the force of gravity. In the bottom part of the column the non-interactive liquid is kept at a low temperature, and the droplets are allowed to gel. The gelled beads are continuously removed from the bottom of the column. This approach allows the continuous and large-scale production of carrageenan gel beads.

4.5 Dehydration Method

The dehydration method is often used for the immobilisation of enzymes for applications in non-aqueous solutions, both in industrial biocatalysis[106] and in enzyme electrodes.[107,108] For this method carrageenan beads or films are prepared without bioactive material. Gel beads can be prepared by the above-mentioned droplet method or by spray drying, the latter resulting in much smaller beads (around 50 μm).[109] Prepared carrageenan beads or films are dehydrated, either by drying or lyophilisation. Immersing or suspending the dehydrated gels in an aqueous solution/suspension of enzymes or cells results in swelling of the gel beads or films and embedding of the bioactive material. These biocatalysts can be used in non-aqueous solutions either directly or after a second dehydration step.

4.6 Modifications and Follow-Up Treatments

4.6.1 Addition of Other Hydrocolloids

κ-Carrageenans show synergism with galactomannan gums, especially with locust bean gum and less with konjac gum and guar gum. Galactomannans have a (1 → 4)-β-D-mannopyranosyl backbone with single (1 → 6)-α-D-galactose side units and are obtained from higher plants. The different galactomannans differ in their galactose to mannose ratio. A synergistic effect means that the minimum gelling polymer (κ-carrageenan) concentration can be reduced even though locust bean gum alone is non-gelling. The addition of galactomannans to κ-carrageenan gels changes their morphology from brittle, rigid gels to strong elastic gels with low syneresis. The synergistic effect between carrageenan and galactomannans is often used in the preparation of food products.[82,84]

The addition of galactomannans is also used in immobilisation protocols. In contrast to food indus-

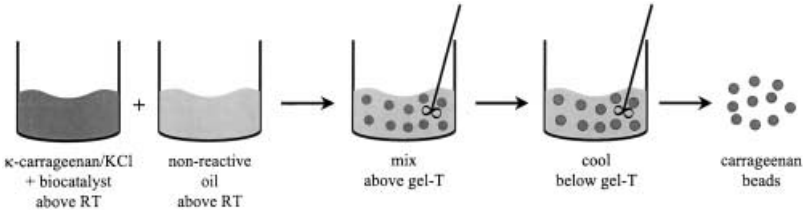


Figure 4. Schematic representation of the preparation of carrageenan beads by the emulsion method.

tries, this is not applied to reduce the amount of hydrocolloid needed, but to improve the properties of the carrageenan gel beads. Czaczyk et al.[110] studied the effect of locust bean gum (lbg) addition to κ-carrageenan on the mechanical properties and acid resistance of cylindrical gel samples. It turned out that the addition of lbg to κ-carrageenan in the ratio 1:2 made the gels softer but increased the resistance to acids. The latter is of importance for applications under acidic conditions, as carrageenan alone is sensitive to acid hydrolysis.[82]

Besides galactomannans, also glucomannan is claimed to increase the gel strength of carrageenan gel beads.[111] The carrier properties of κ-carrageenan beads have also been modified by the addition of gelatin.[102] The mechanical strength and thermal stability of carrageenan beads is improved by the addition of chitosan to the KCl hardening solution.[112]

4.6.2 Gel Hardening

The mechanical stability of carrageenan gel beads and matrices can be improved by so-called gel hardening methods. This is done by soaking the particles in a concentrated solution of gel-inducing ions. Potassium chloride is often used[86], but also $CaCl_2$ is described for this purpose.[100] The use of Al^{3+} ions and Fe^{2+} ions results in an increased stability of the beads.[86,100,113,114] The addition of Fe_2O_3 has been applied to increase the density of the gel beads.[115]

4.6.3 Cross-Linking

Cross-linking is a frequently applied technique to reduce the leakage of enzymes and cells from the immobilisation support. Carrageenan beads containing enzymes and/or cells can also be cross-linked to improve their stability and reusability.[76] The carrageenan matrix of these beads can prevent the loss of enzyme activity during cross-linking, in comparison to the cross-linking of free enzymes and cells (see Section 2.5). In fact, the stability of the beads can be increased with a minimal loss of enzyme activity. The particle size of the biocatalyst is also larger than in simple cross-linking (see Section 2.4) which gives a higher flexibility for reactor set-up. Cross-linking agents, such as glutaraldehyde, hexamethylenediamine,[86,116] chlorohydrin, diepoxides, polyamines,[100] tannins, diisocyanates, or carbodiimides[87] are useful for this purpose. The degree of cross-linking is an optimum between biocatalyst deactivation, due to chemical modification by the cross-linking agent, and the increase in stability and/or reusability of the final product. The ideal level is strongly dependent on the specific process conditions and has to be carefully optimised.

4.6.4 Optimisation

Several examples of immobilised biocatalyst optimisation methodologies have been described in the literature. For example, the response surface methodology (RSM) has been used to optimise the immobilisation of β-galactosidase from *Kluyveromyces fragilis* in a hydrocolloid matrix. The concentration of hydrocolloids, both κ-carrageenan and sodium alginate, and the enzyme concentration has been varied to obtain an immobilised biocatalyst with high activity and stability.[103] Another example of the RSM approach is given by Asanza Teruel et al.[117] for the immobilisation of mycelia of *Streptomyces aureofaciens* used for the production of antibiotics belonging to the tetracycline group. They varied the carrageenan and potassium chloride concentration to optimise the immobilisation method. The tetracycline production was enhanced more than eight-fold (from 1.5 to 12.3 mg tetracycline per g immobilised cells) upon optimised immobilisation.

5 Applications in Enzyme Immobilisation

5.1 Overview

In comparison to the immobilisation of whole cells into carrageenan matrices (see Section 6), the immobilisation of enzymes into carrageenan is applied in fewer examples. Several examples of enzyme immobilisation into carrageenan matrices are discussed in the sections below, whereas the reminder are summarised in Table 3.

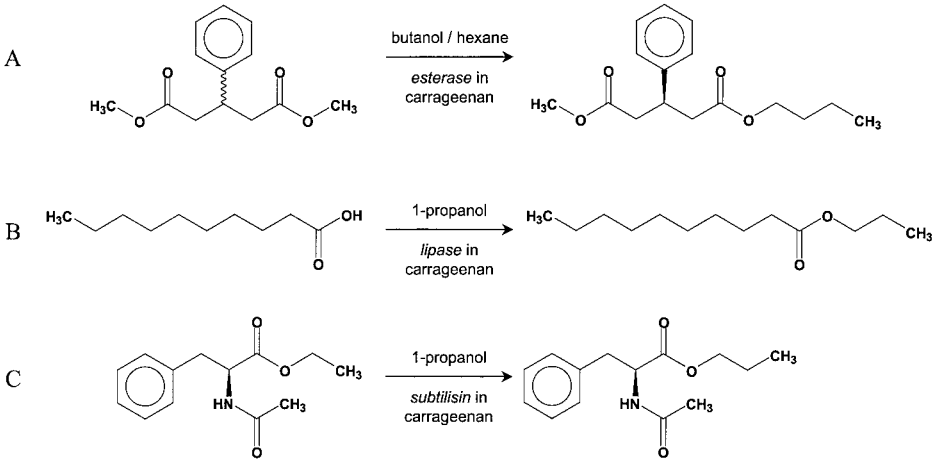
5.2 Asymmetric Synthesis in Non-Aqueous Solvents

History shows that for the immobilisation of enzymes carrageenan is not a very popular carrier, as compared with the entrapment of whole cells. Kitchell and coworkers were among the first inventors who adopted carrageenan as a gel for the entrapment of enzymes.[118] Esterases were immobilised into beads by dropping an enzyme/carrageenan solution into a cold alcohol, saturated with inorganic salts. The latter were essential to increase the stability, hardening and curing of the beads in organic media. The immobilised esterase catalysed the transesterification of 3-phenylglutaric acid diethyl ester into the butyl methyl ester with 60% yield after three days (Scheme 1A).

A lipase from *Pseudomonas* species was immobilised into carrageenan matrices via the gel preparation method.[99] High conversions (80%) were obtained for the synthesis of propyl laurate catalysed by a microemulsion-based gel of lipase and carrageenan (Scheme 1B). However, after one bioconversion cycle the operational stability was found to be very low, since

Table 3. Overview of applications of enzymes immobilised in carrageenan matrices.

Enzyme	Application	Method	Ref.
Aminoacylase	Immobilisation studies	droplet, gel	[86]
Aspartase	Immobilisation studies	droplet, gel	[86]
Catalase	Hydrogen peroxide determination	dehydration	[108,119]
Choline oxidase + butyrylcholinesterase	Analysis of organophosphorus pesticides	dehydration	[120,121]
Choline oxidase + phospholipase D	Lecithin analysis in food and drugs	dehydration	[108,123]
Esterase	Transesterification of 3-phenylglutamic acid esters	droplet	[118]
Fumarase	Immobilisation studies	droplet, gel	[86]
β-Galactosidase	Hydrolysis of lactose	droplet	[103,167]
Glucoamylase	Ethanol production from corn starch	droplet	[115]
Lipase	Esterification of laurate to propyl laurate	gel, emulsion	[99]
Naringinase	Debittering of citrus juice	droplet	[164]
Subtilisin	Transesterification of *N*-acetyl-L-phenylalanine esters	dehydration	[106]
Proteases	Production of casein hydrolysates	–	[168]
Tannase	Hydrolysis of tea tannins	droplet	[163]
Tyrosinase	Monitoring the rancification process of olive oils	dehydration	[107]



Scheme 1. Reactions catalysed by carrageenan-immobilised enzymes.

only 40% of the biocatalytic activity was left. As mentioned previously, the gel-preparation method gives rather unstable beads or microemulsions and additional salt-hardening or cross-linking steps are recommended to increase the operational stability.

For entrapment of enzymes into carrageenan Prud Homme and coworkers[106] used the dehydration method. The beads were prepared by dehydrating the corresponding hydrocolloid gels. Imbedding the enzyme into the pre-formed hydrocolloid beads resulted in biocatalyst particles with an average particle size of 5 to 150 microns. Subtilisin Carlsberg was used as an example of this technique and an enzyme loading of 0.05 to 0.5 g per g of dehydrated gel bead was obtained. The beads were used in organic solvent for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester into a propyl ester (Scheme 1C). After one hour 60% of the starting material was converted with the subtilisin Carlsberg imbedded carrageenan bead, which was a higher yield than that given by the non-imbedded enzyme, with only 16% conversion. However, a disadvantage of this method was that the embedded enzyme beads absorbed 25% of the substrate.

5.3 Biosensors

Biosensors are of growing importance, due to their high specificity. This high specificity is obtained by selecting a specific enzyme or whole-cell system that specifically catalyses the conversion of the desired compound. A new class of biosensors are the organic phase enzyme electrodes (OPEE's) that are active in non-aqueous solvents. This type of sensors is based on recent research on enzyme-catalysed synthesis in organic solvents. The combination of enzymes working in organic solvent with the techniques applied in conventional biosensors resulted in the development of electrodes which have several challenging features as described by Campanella et al.[108]:

- determination of compounds that are soluble in organic solvent, but insoluble in aqueous systems;
- enhanced operational stability due to immobilised enzymes working in organic solvents;
- decrease of microbial contamination of the electrode;
- a simple immobilisation technique due to the fact that enzyme loss is hindered by the organic solvent.

OPEE's, as assembled by Campanella et al.,[108] have a commercial-type gas diffusion amperometric electrode for oxygen as their base. The selectivity of these electrodes results from the immobilised enzymes that are placed on top of this oxygen electrode. Enzymes are immobilised by adsorption of an enzyme solution into a dry κ-carrageenan film, using the dehydration method (see Section 4.5). By selecting the suitable enzyme or combination of enzymes, electrodes for specific functions can be designed:

- tyrosinase yields an electrode for the determination of the polyphenol content in olive oil (see detailed description below);[107,108]
- catalase yields an electrode for the determination of hydrogen peroxide;[108,119]
- butyrylcholinesterase combined with choline oxidase yields an electrode that is selective for organophosphorus pesticides and carbamates;[108,120,121]
- phospholipase D combined with choline oxidase yields an electrode for the analysis of lecithin in food and pharmaceutical products.[108,122,123]

Polyphenols are the main natural antioxidants present in olive oil. The rancidification process in olive oils can be related to the decrease of their concentration. A biosensor to measure the polyphenol content of olive oil samples dissolved in *n*-hexane was developed based on mushroom tyrosinase and an oxygen electrode.[107] The enzyme was immobilised by entrapment in κ-carrageenan using the dehydration method. This electrode was used to monitor the rancidification process occurring in any sample of olive oil by a rapid determination of the polyphenol content. This demonstrated the reliability of this type of biosensors regarding both the precision and the accuracy of the measurement.

The biological component of a biosensor is commonly an enzyme but some developments have been made in the use of whole-cells as cheaper biological components. Two strains of mutant *E. coli* with defects in the carbohydrate transport system were entrapped in κ-carrageenan at the surface of platinised gold electrodes.[124,125] When a carbohydrate which can be carried by the specific transport system of *E. coli* is present it is metabolised and depletion of oxygen in the vicinity of the electrodes occurs. An example of such a highly selective analytical system is the biosensor for simultaneous determination of glucose and lactose.[125]

6 Applications in Whole-Cell Immobilisation

6.1 Overview

Due to the enormous amount of literature dealing with whole-cell immobilisation using carrageenan, only some important examples of applications in this area will be described. An overview of carrageenan-entrapped cells from different sources and immobilised for different purposes is given in Table 4.

6.2 Wastewater Treatment

6.2.1 Nitrogen Removal

The removal of nitrogen from wastewater is normally achieved by two biological steps, nitrification and denitrification. Considering that the nitrifying bacteria are strict aerobes and that the denitrifying bacteria are facultative anaerobes, it is difficult to combine these two steps in a single wastewater treatment unit. An efficient integrated nitrogen removal system was achieved by the co-immobilisation of *Nitrosomonas europaea* and *Pseudomonas* sp. in κ-carrageenan, taking advantage of the oxygen gradient inside the entrapment beads.[126]

6.2.2 Morpholine Degradation

Morpholine (1,4-tetrahydrooxazine) and its derivatives can be found in a significant number of industrial effluents due to their wide range of applications as optical brighteners, antioxidants, rubber additives, solvents, inhibitors of corrosion, and in the production of some drugs and herbicides. In the presence of nitrites morpholine can undergo an *N*-nitrosation reaction, leading to the formation of the strong mutagen *N*-nitrosomorpholine, which makes morpholine removal from wastewaters an important requirement.[127] Most of the isolated morpholine-degrading bacteria have been identified as *Mycobacterium* sp. but these bacteria are known to grow in aggregates, resulting in inefficient morpholine degradation. The immobilisation of *M. aurum* in κ-carrageenan and its use in an air-bubble fermentor resulted in an improvement of its morpholine-degrading capacity.[127,128]

6.2.3 Degradation of Chlorophenol Pollutants

A chlorophenol-degrading microorganism isolated from activated sludge was entrapped in κ-carrageenan by a new method of immobilisation based on hardening with KCl and chitosan.[112] This new method resulted in

Table 4. Overview of applications of whole-cell systems immobilised in carrageenan matrices.

Source	Application	Method	Ref.
Acetobacter aceti	Production of vinegar	droplet	[142]
Acidovorax facilis	Nitrilase activity for the chemoenzymatic synthesis of 1,5-dimethyl-2-piperidone	emulsion, gel	[98]
Aspergillus niger strain As9.203	Production of pectinase (polygalacuronase)	–	[169]
Bacillus amyloliquefaciens CCRC40268	Production of α-amylase in annular packed bed reactor	–	[170]
Bacillus cereus	Oxidation of naphthalene to naphthol	–	[171]
Bacillus subtilis	Production of α-amylase	droplet	[172]
Brevibacterium ammoniagenes	Production of L-malic acid	emulsion, gel	[132]
Brevibacterium ammoniagenes	Fumarase activity/immobilisation studies	droplet, gel	[86]
Brevibacterium flavum	Production of L-malic acid	–	[132]
Dioscorea deltoidea	Synthesis of carveol and carvone from (−)-limonene	gel (cubes)	[173]
Escherichia coli	Production of L-aspartic acid	gel	[130,131]
Escherichia coli	Aspartase activity/immobilisation studies	droplet, gel	[86]
E. coli KFCC-84-3	Penicillin G acylase activity for the production of 6-amino penicillanic acid (6-APA)	droplet	[154]
E. coli (recombinant)	Production of D-*p*-hydroxyphenylglycine	droplet	[171]
E. coli (recombinant)	Biosensor for the simultaneous determination of glucose and lactose	–	[124,125]
Lactococcus lactis	Continuous lactic starter production	–	[174]
Megasphaera elsdenii ATCC25940	Hexanoic acid production	–	[155]
Pseudomonas dacunhae	Synthesis of L-alanine using a column reactor	droplet	[175]
Pseudomonas dacunhae	Decarboxylase activity for the synthesis of L-alanine from L-aspartic acid	–	[116]
Pseudomonas putida	Biodegradation of cyanides, cyanates and thiocyanates	–	[176]
Pseudomonas sp BA2	Aminoacylase activity for the production of L-alanine	droplet	[135]
Rhodotorula rubra	Conversion of *trans*-cinnamic acid into L-phenylalanine	–	[177]
Saccharomyces cerevisiae LCC3021	Beer production	–	[178]
Saccharomyces cerevisiae W 96	Beer volatile by-product formation	droplet	[179]
Solunum aviculare	Synthesis of carveol and carvone from (−)-limonene	gel (cubes)	[173]
Streptomyces aureofaciens	Production of chlortetracycline and tetracycline	droplet	[117]
Streptomyces phaeochromogenes	Glucose isomerase activity/immobilisation studies	droplet, gel	[86]
Unidentified	Degradation of 2,4,6-trichlorophenol	droplet	[102]
Zymomonas mobilis ATCC 31821	Sorbitol production using 2-stage continuous packed bed reactor	droplet	[180]
Zymomonas mobilis CP4(pZB5)	Ethanol production from glucose and xylose	droplet	[114]
Zymomonas mobilis NRRL B14023	Ethanol production from corn starch	droplet	[115,181]

improved mechanical strength and thermal stability of the carrageenan gel and the immobilised microbial cells were used for 4-chlorophenol degradation with higher activity than the free cells.[112]

Aerobic and anaerobic microbial communities were co-immobilised into κ-carrageenan/gelatin gel beads.[102] Under air-limited conditions these immobilisates catalyse the degradation of 2,4,6-trichlorophenol.

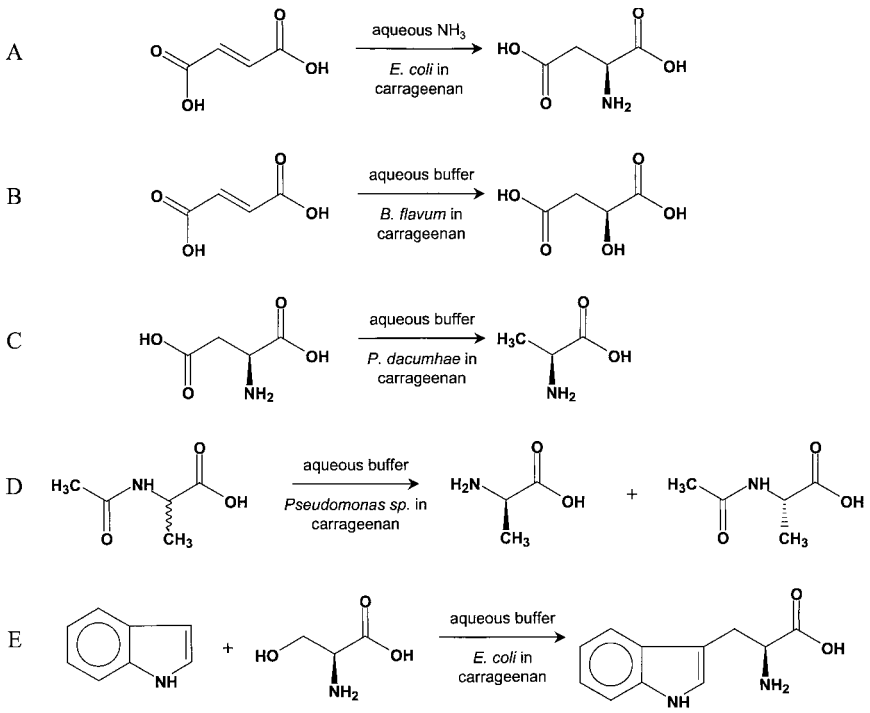
Pentachlorophenol pesticide degradation in soil decontamination was attempted by the use of *Pseudomonas* sp. UG30 cells immobilised in κ-carrageenan. The cell encapsulation provided a protective effect, allowing dechlorination and mineralisation of high levels of pentachlorophenol, i.e., 600 mg/L in 21 days, when only 200 mg/L were converted by free cells.[129]

6.3 Asymmetric Synthesis

Amino acids are the building blocks of proteins and consequently play an important role in human and animal nutrition. However, only the L-form can be metabolised, explaining the high interest in the selective production of this form for use in food and medical applications.[130]

6.3.1 L-*Aspartic Acid Production*

L-Aspartic acid can be obtained either by microbial fermentation or from an enzymatic reaction between fumaric acid and ammonia catalysed by L-aspartase (Scheme 2A). The method, still in use for the industrial preparation of L-aspartic acid, was developed by Chibata (Tanabe Seiyaku, Japan) in 1973[131] and is often referred to as the first industrial application of



Scheme 2. Amino acids produced by carrageenan immobilised whole-cells.

immobilised whole cells. The initial method consisted in immobilising *Escherichia coli* cells with intracellular L-aspartase activity using polyacrylamide gel as the support. Since 1978 this support was replaced by κ-carrageenan, not only due to a resulting increase in the initial activity and half-life of the biocatalyst, but also because the immobilisation method was simpler, cheaper and used milder conditions. The cell suspension is mixed with carrageenan at 45–50 °C, cooled to 10 °C and the resulting gel is washed in a 0.3 M KCl solution. Ammonium fumarate (1 M solution) is used as the substrate in column reactors of 1 m^3 volume. The stability of the biocatalyst can be improved by the addition of magnesium (0.1 mmol Mg^{2+} per litre of substrate) or by a cross-linking treatment with glutaraldehyde and hexamethylenediamine[130].

6.3.2 L-*Malic Acid Production*

L-Malic acid is commonly used as an acidulant in juices, carbonated drinks, and jams but is also used in the pharmaceutical industry in amino acid infusions. Fumaric acid is the starting material for malic acid production in a reaction catalysed by fumarase (Scheme 2B). Tanabe Seiyaku (Japan) introduced this reaction at industrial scale in 1974 using cells of *Brevibacterium ammoniagenes* immobilised in polyacrylamide gel and later in κ-carrageenan[131,132] due to the advantages already observed for aspartic acid production. Succinic acid is produced by a side reaction, but this reaction can be eliminated through the treatment of the immobilised cells with bile extracts. In 1993 Takata and Tosa[132] screened for microbial cells with higher fumarase activity than *B. ammoniagenes* and reported a nine-fold increase in productivity using cells of *Brevibacterium flavum* immobilised in κ-carrageenan when compared to that of *B. ammoniagenes* immobilised in polyacrylamide. The stability of the fumarase of *B. flavum* cells immobilised in carrageenan can be further improved by the addition of polyethyleneimine, Chinese gallotannin or by carrageenan modification with amines[132].

6.3.3 L-*Alanine Production*

L-Alanine is an amino acid that can be used for medical purposes and as a food additive for flavour improvement. It started to be industrially produced in 1965 by an enzymatic batch reaction using the L-aspartate β-decarboxylase activity of intact *Pseudomonas dacunhae* cells and using L-aspartic acid as substrate (Scheme 2C). In 1980 Yamamoto et al.[133] developed a more efficient system for the production of L-alanine using a conventional column reactor with *P. dacunhae* cells immobilised in κ-carrageenan. Calik et al. further investigated the potential scale-up of this immobilised system for the industrial production of L-alanine.[116] Considering that the L-aspartic acid produced by *E. coli* aspartase can be converted to L-alanine by L-aspartate β-decarboxylase, L-alanine should be more

efficiently produced by the co-immobilisation of *E. coli* and *P. dacunhae* with ammonium fumarate as substrate. However, the co-immobilisation strategy resulted in a lower L-alanine productivity than that of the association of separately immobilised cells of *E. coli* and *P. dacunhae*.[134] Since 1982 the industrial production of L-alanine is performed in a continuous system using a sequence of two column reactors with immobilised *E. coli* cells in the first reactor and immobilised *P. dacunhae* cells in the second.[134] In 1998 Santoyo et al. reported a different method for L-alanine production using a new L-aminocyclase-producing *Pseudomonas* sp. immobilised in κ-carrageenan. These cells with L-aminocyclase activity were used for the stereospecific production of L-alanine from *N*-acetyl-D/L-alanine (Scheme 2D) in a batch reactor with conversion levels up to 100%.[135]

6.3.4 L-Tryptophan Production

L-Tryptophan can be produced by a condensation reaction between indole and L-serine catalysed by tryptophanase (Scheme 2E). *E. coli* cells with tryptophanase activity were immobilised in κ-carrageenan gel beads and used in a liquid-impelled reactor containing two liquid phases, an organic solvent (*n*-dodecane) containing indole and an aqueous phase containing L-serine and pyridoxal phosphate as the co-factor.[136,137]

6.3.5 Synthesis of 1,5-Dimethyl-2-piperidone

Nitrilases catalyse the conversion of a nitrile into the corresponding acid with the release of ammonia. In comparison to the chemical conversion of nitriles, which requires a strongly acid or basic media and results in unwanted side products, the enzymatic alternative is attractive because it runs under ambient temperature and neutral pH with the formation of very pure products.[138] The biocatalyst is mainly applied in whole-cell preparations due to the instability of the enzyme in its purified form.[139–141]

Besides the production of acylamides as mentioned in Table 1, another attractive industrial biocatalytic process is known with nitrilases.[98] A chemoenzymatic process was developed by Dupont for the preparation of 1,5-dimethyl-2-piperidone from 2-methylglutaronitrile (Scheme 3). The hydrolysis of 2-methylglutaronitrile was catalysed with an aliphatic nitrilase from *Acidovorax facilis* species. The concentrated ammonium salt solution was hydrogenated in the presence of methylamine resulting in an efficient and easily scaleable process for the production of 1,5-dimethyl-2-piperidone. When free cells were used to produce the ammonium salt intermediate, the biocatalyst lost a significant part of its activity with each reuse. Therefore, the enzyme in whole cells was immobilised into κ-carregeenan beads using the gel preparation method as well as the emulsion method. For the latter, the beads were stabilised by cross-linking and for the gel preparation method ammonium salts were added. The immobilised cells catalysed the hydrolysis reaction resulting in a 100% conversion with a regioselectivity of 98% at concentrations of 170 – 210 g product per litre. The catalyst was recycled several times resulting in a productivity of 1 ton of product per kg of cell dry weight. The product has properties similar to those of the industrial solvent *N*-methyl-2-piperidone and could also be used for electronics and coatings.

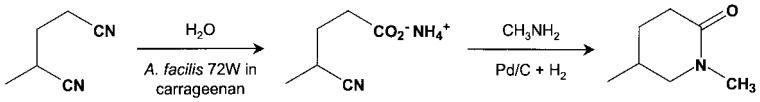
6.4 Food Applications

6.4.1 Vinegar Production

Vinegar has been used for a long time in food seasoning and its industrial production is a biochemical process using bacteria. In 1984 Osuga et al.[142] described the use of a bubble-mixed reactor and κ-carrageenan gel beads as carriers for the continuous production of acetic acid during 120 days, but longer runs of 460 days could be achieved.[143] An improvement was attempted through the use of an air-lift reactor[144] using the culture of *Acetobacter* species K1024 isolated from a commercial vinegar broth. More recently, a successful continuous production of vinegar was reported using a bubble-mixed tabletop bioreactor with κ-carrageenan immobilised *Acetobacter suboxydans* cells.[145]

6.4.2 Milk Prefermentation for Fresh Cheese Production

Fermented milk products like yoghurt and fresh cheese can be obtained by simultaneous acidification and inoculation of skim milk by immobilised mixed cultures of lactic acid bacteria. This inoculation can be ten times higher than that of the traditional batch method, resulting in a 50% reduction in the total fermentation time for fresh cheese production.[146,147] Three different strains of *Lactococcus lactis* and one strain of *Leuco-*



Scheme 3. Chemoenzymatic synthesis of 1,5-dimethyl-2-piperidone.

nostoc mesenteroides were separately immobilised in κ-carrageenan/locust bean gum gel (2.75% and 0.25% w/w, respectively) and used in a 2-L stirred reactor. Prefermented milk correctly acidified (pH 6.0) and highly inoculated could be obtained with this immobilised system using a starting procedure with a varying milk flow rate.[146,147]

6.4.3 Beer Production

The brewing industry is very well established and very traditional in what concerns the production processes. However, the use of immobilised yeast cells has been widely reported in the literature. In the typical fermentation process, the conversion of the fermentable sugars into ethanol, carbon dioxide and flavour-active compounds by free yeast cells can take five to seven days. The flavour maturation of the resulting liquid requires one to three weeks longer. The main advantage of immobilised yeast technology for the brewing industry is a faster fermentation step by supplying a high cell concentration in the bioreactor. Several applications of this technology have been reported but research in primary fermentation of beer is still in progress.[148,149] Labbat Breweries together with the University of West Ontario is developing a new immobilisation system of yeast cells (*Saccharomyces* sp.) for beer production using κ-carrageenan beads continuously produced by a static mixer process. In these studies the continuous primary beer fermentation was performed in a 50-L gas lift draft tube bioreactor with 20 hours minimum residence time.[148]

6.4.4 Ethanol Production

Ethanol production from glucose using cells of *Zymomonas mobilis* immobilised in κ-carrageenan was investigated in a fluidised bed fermentor. This research was extended to study the production of ethanol from starch[150] using the bacteria co-immobilised with an industrial glucoamylase in carrageenan gel beads and used in a glass column fermentor.

The carrageenan gel matrix was reported to provide protection of immobilised *Saccharomyces cerevisiae* cells[151] and continuous ethanol production from pineapple cannery waste was attempted using these yeast cells immobilised in κ-carrageenan. The reactor used was a packed bed tapered glass column reactor.[113] The volumetric ethanol productivity was around 11.5 times higher than that of free cells and the maximum productivity achieved was 42.8 g ethanol L^{-1} h^{-1} at a dilution rate of 1.5 h^{-1}.

6.5 Pharmaceutical Applications

6.5.1 Tetracycline and Chlorotetracycline Production

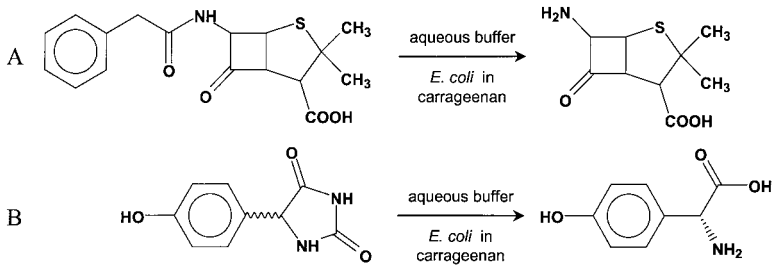
Tetracyclines represent one of the most important groups of antibiotics in the medical field and the method normally used for its industrial production is conventional fermentation. Asanza-Teruel et al.[117] used *Streptomyces aureofaciens* immobilised in κ-carrageenan in order to improve the production of tetracycline and chlorotetracycline.

6.5.2 D-Aspartic Acid Production

A number of D-amino acids have been shown to be important intermediates in drug production. D-Aspartic acid can be used as a component of synthetic penicillins.[134]. When D/L-aspartic acid is used as a substrate for the L-aspartate β-decarboxylase of *P. dacunhae* cells, L-aspartic acid is converted to L-alanine but D-aspartic acid remains unchanged due to the high stereospecificity of the biocatalyst. In this way D-aspartic acid and L-alanine can be produced simultaneously using *P. dacunhae* cells immobilised in carrageenan.[134] D/L-Aspartic acid is chemically produced from fumaric acid and ammonia. D-Aspartic acid is crystallised by acidification of the reactor effluent and recovered by centrifugation. L-Alanine is also recovered by centrifugation after crystallisation by the addition of ammonia to the resulting liquor followed by concentration and cooling. This system for the continuous production of D-aspartic acid and L-alanine using *P. dacunhae* cells immobilised in κ-carrageenan has been industrialised since 1988.

6.5.3 Semisynthetic Antibiotic Production

The increasing demand for antibacterial drugs pushes the production of semisynthetic antibiotics, such as amoxicillin (>15000 ton/year), cephalexin (3000 ton/year), cephradine (800 ton/year), and cefadroxil (600 ton/year).[152] These semisynthetic antibiotics are prepared via the coupling of a β-lactam core (6-aminopenicillanic acid or 7-aminodesacetoxycephalosporanic acid) with the so-called side chain, such as phenylacetic acid, D-phenylglycine, or D-*p*-hydroxyphenylglycine. 6-Aminopenicillanic acid (6-APA) is obtained by the enzymatic hydrolysis of penicillin G produced by fermentation (Scheme 4A). The suitability of κ-carrageenan as a support for 6-APA production was tested with *E. coli* cells with penicillin-amidase activity.[153,154] The cells were immobilised with an efficiency of 90% and could be used for 20 repeated cycles retaining 60% of the initial penicillin-amidase activity. The carrageenan gel beads were hardened with gluteraldehyde.



Scheme 4. Preparation of semisynthetic antibiotics. **A**: synthesis of 6-aminopenicillanic acid; **B**: synthesis of D-*p*-hydroxyphenylglycine.

Among the side chains, D-*p*-hydroxyphenylglycine is one of the most important precursors, as it is used for the synthesis of amoxicillin and cefadroxil. Recombinant *E. coli* cells expressing both dihydropyrimidinase and carbamoylase were immobilised in κ-carrageenan and were able to convert D/L-hydroxyphenylhydantoin in to D-*p*-hydroxyphenylglycine (Scheme 4B). In a single-step reaction a conversion of 93% was obtained, while a 20% value was observed with the strain of *Agrobacterium radiobacter*, which contained the original dihydropyrimidinase gene cloned in *E. coli*.[155]

7 Relevant Patents

The pioneering work of Chibata and coworkers on the immobilisation of enzymes and microorganisms has resulted in a patent covering the entrapment into sulphated polysaccharides.[87] Ammonium and metal ions, water-soluble amines, and water miscible organic solvents are described as gel-inducing agents. Gel hardening or cross-linking is described with dialdehydes, tannins, epichlorohydrin, diisocyanates, carbodiimides, and diamines.

The standard droplet method results in an average bead size in the order of millimetres. The preparation of much smaller beads (around 50 μm) is claimed by FMC Corp. using a spray-drying technique.[109] These dehydrated microbeads can be loaded with enzymes or microorganisms by suspending the beads in an aqueous solution containing the bioactive material.[106]

An essential aspect for the industrial application of immobilised biocatalysts is the possibility to produce the catalyst on a large scale. Lockheed Martin Engery Sys. developed an apparatus to produce immobilised biocatalysts in a continuous process.[105] This apparatus is based on the emulsion method.

8 Outlook and Perspectives

Carrageenan is a suitable support material for the immobilisation of whole cells, as proven by several applications in different industrial processes. The approval of carrageenan as food-grade additive and the easiness of the immobilisation protocol stimulate applications in the food industries. The mild immobilisation and reaction conditions applied for carrageenan-immobilisation of whole cells allows their application in highly (enantio)selective production processes for pharmaceutical compounds and fine chemicals.

However, the low number of published applications of enzymes entrapped into carrageenan in comparison to the number of whole-cell applications is rather surprising. In our opinion carrageenan is a high-potential immobilisation support for the immobilisation of most industrial or newly discovered enzymes. For example, several enzymes are rather difficult to immobilise by other methods. Certain lipases (e.g., from *Mucor miehei*) and peroxidases (e.g., chloroperoxidase[156]) contain functional groups (e.g., lysine residues, haeme groups), which are essential for catalysis, that are affected by reagents used for covalent attachment. Therefore, these enzymes are liable to deactivation as a result of reaction with these functional groups during covalent attachment or cross-linking. A mild entrapment immobilisation into carrageenan beads will overcome this problem.

Cofactor regeneration is an important issue in industrial processing, as this accounts for high costs.[157] Dehydrogenases and monooxygenases are promising redox enzymes for synthetic applications,[157–159] but require stoichiometric amounts of an expensive cosubstrate (NADH) as cofactor. Dehydrogenases are highly selective in catalysing oxidations and reductions of carbonyl groups and alcohols under mild reaction conditions (e.g., environmentally friendly solvents, ambient pH and temperature). Monooxygenases catalyse a wide variety of synthetically useful enantioselective oxidations. Entrapment of the cofactor together with the enzyme in the carrageenan beads will reduce the diffusion limitations between cosubstrate and biocatalyst. Moreover, co-immobilisation of the biocatalyst and a cofactor regeneration system, either chemical or biochemical, in carrageenan matrices will significantly improve the applicability of these enzymes for industrial processing.

When the adequate enzymatic system is available, immobilised biocatalysts can be used not only for the degradation of any compound but also for the accumu-

lation of certain compounds such as heavy metals or phosphate in the biocatalyst. For the particular case of bacteria immobilised in gel beads, the compounds accumulated in the biomass are confined to the gel and are consequently easier to separate from the pollutant-containing medium, allowing the recovery of the waste as raw material.

Multi-enzyme systems or cascade catalysis combining chemo- as well as biocatalysts in one reactor has a very promising future for industrial processes. An example of multi-enzyme systems is the highly efficient method for the preparation of carbohydrates from glycerol using 5 different enzymes in a one-pot process.[160] Co-immobilisation of such enzymes by entrapment into carrageenan could improve the operational stability and cycle time. Separated immobilisation of the different enzymes into carrageenan matrices allows for the creation of enzyme-specific reaction conditions inside the beads.

Resolution of racemates is an often applied technique in biocatalytic processes for the production of optically pure products, such as secondary amines and alcohols. The maximum yield of such a process is inherently limited to 50%. To overcome this maximum yield limitation, the biocatalytic resolution is increasingly being combined with a metal-catalysed racemisation process.[161,162] Co-immobilisation of these chemo-enzymatic processes into carrageenan matrices will decrease the diffusion limitations and increase the efficiency and productivity of the catalyst. Hence, the carrageenan entrapment method is, in addition to the entrapment of a single biocatalyst, suitable for immobilisation of more than one homogeneous catalyst as well.

The application of carrageenan as carrier material is hampered by the low stability of native carrageenan beads in environments of low ionic strength. Dissolution and bead instability has been reported for several carrageenan-immobilised enzyme preparations, such as tannase used for the hydrolysis of tea tannins[163] and naringinase employed for the debittering of citrus juices.[164] For industrial processes several follow-up treatments were developed to increase the stability. But, on the other hand, this sensitivity to environmental factors makes carrageenan a promising material for the controlled release of pharmaceuticals, (pro)biotica, and bioactive materials. For example, most therapeutic proteins currently on the market are delivered by injection,[165] as the oral intake is difficult to achieve. Entrapment of these proteins and other bioactive materials into carrageenan beads could solve this problem by protecting these materials against degradation processes. The change of pH in the digestive system could cause degradation of the beads. By improving bead stability, the release of the entrapped materials could be fine-tuned. The possibility to fine-tune the physical properties of carrageenan gels makes it a promising material for controlled release applications.

References

[1] H. Uhlig, *Industrial enzymes and their applications*, John Wiley & Sons, New York, **1998**.
[2] W. Crueger, A. Crueger in *Biotechnology: A textbook of industrial microbiology*, (Eds.: W. Crueger, A. Crueger), Sinauer Associated, Sunderland, **1989**, pp. 150 – 174.
[3] G. A. Tucker, L. F. J. Woods, *Enzymes in food processing*, Blackie Academic & Professional, Glasgow, **1995**.
[4] G. Walsh, D. Headon, *Protein biotechnology*, John Wiley & Sons Ltd, Chichester, **1994**.
[5] Y. Izumi, I. Chibata, T. Itoh, *Angew. Chem. Int. Ed.* **1978**, *17*, 176 – 183.
[6] W. Gerhartz, *Enzymes in industry; production and application*, VCH Verlagsgesellschaft GmbH, Weinheim, **1990**.
[7] J. Kamphuis, W. H. J. Boesten, B. Kaptein, H. F. M. Hermes, T. Sonke, Q. B. Broxterman, W. J. J. Van den Tweel, H. E. Schoemaker, in *Chirality in Industry*, (Eds.: A. N. Collins, G. N. Sheldrake, J. Crosby), John Wiley and Sons, Chichester, **1992**, pp. 187 – 208.
[8] A. M. Klibanov, *Chemtech* **1996**, *16*, 354 – 359.
[9] A. M. Klibanov, *Nature* **2001**, *409*, 241 – 246.
[10] V. J. Jensen, S. Rugh, *Methods Enzymol.* **1987**, *136*, 356 – 370.
[11] A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258 – 268.
[12] A. Zaks, D. R. Dodds, *Drug Disc. Today* **1997**, *2*, 513 – 531.
[13] A. Zaks, *Curr. Opinion Chem. Biol.* **2001**, *5*, 130 – 136.
[14] M. McCoy, *Chemistry & Engineering News* **2001**, *79 (Jan 15th)*, 19 – 24.
[15] J. M. Neugebaurer, *Methods Enzymol.* **1990**, *182*, 239 – 253.
[16] J. M. Valentine (personally), *US Patent* 5,874,294, **1997**.
[17] G. J. Olson (Patent Clean Diesel Technologies Inc.), *US Patent* 6,124,130, WO 00/09761, **1998**.
[18] B. L. McFarland, *Curr. Opin. Microbiol.* **1999**, *2*, 1369 – 5274.
[19] T. Zerlia, *Riv. Combust.* **2000**, *54*, 267 – 276.
[20] A. Gutierrez, J. C. d. Rio, M. J. Martinez, A. T. Martinez, *Trends Biotechnol.* **2001**, *19*, 340 – 348.
[21] C. Benthack, B. Srinivasan, D. Bonvin, *Biotechnol. Bioeng.* **2001**, *72*, 34 – 40.
[22] K. Watanabe, *Curr. Opinion Biotechnol.* **2001**, *12*, 237 – 241.
[23] K. Faber, *Biotransformations in Organic Chemistry*, Springer-Verlag, Heidelberg, **1997**.
[24] I. Gill, R. L. López-Fandino, X. Jorba, E. N. Vulfson, *Enzyme Microbiol. Technol.* **1996**, *18*, 162 – 183.
[25] C. H. Wong, G. M. Whitesides, *Enzymes in synthetic organic chemistry*, Elsevier science Ltd, Oxford, **1994**.
[26] A. M. P. Koskinen, A. M. Klibanov, *Enzymatic reactions in organic media*, Blackie Academic & Professional, Glasgow, **1996**.
[27] U. T. Bornscheuer, R. J. Kazlauskas, *Hydrolases in organic synthesis; regio- and stereoselective biotransformations*, Wiley VCH, Weinheim, **1999**.
[28] S. F. D'Souza, *Curr. Sci.* **1999**, *77*, 69 – 79.

[29] L. Jarvis, *Schnell Publishing Company, Inc, June* **2001**, *11*, 11.
[30] E. N. Vulfson, I. Gill, D. B. Sarney, in *Enzymatic reactions in organic media*, (Eds.: A. M. P. Koskinen, A. M. Klibanov), Blackie Academic & Professional, Glasgow, **1996**, pp. 244 – 263.
[31] R. A. Sheldon, in *Enzymatic reactions in organic media* (Eds.: A. M. P. Koskinen, A. M. Klibanov), Blackie Academic & Professional, Glasgow, **1996**, pp. 266 – 302.
[32] B. Marrs, S. Delagrave, D. Murphy, *Curr. Opin. Microbiol.* **1999**, *2*, 241 – 245.
[33] I. Chibata, T. Tosa, T. Sato, T. Mori, Y. Matsuo, in *Fermentation technology today: Proceedings of the IVth international fermentation symposium*, (Ed.: G. Terui), Society of Fermentation Technology, Osaka, **1972**, pp. 383 – 389.
[34] E. Katchalski-Katzir, *Trends Biotechnol.* **1993**, *11*, 471 – 478.
[35] U. Kragl, in *Industrial enzymology*, (Eds.: T. Godfrey, S. West), Basingstoke, Macmillan, **1996**, pp. 274 – 283.
[36] M. Bakker, F. Van de Velde, F. Van Rantwijk, R. A. Sheldon, *Biotechnol. Bioeng.* **2000**, *70*, 342 – 348.
[37] M. D. Klein, R. Langer, *Trends Biotechnol.* **1986**, *4*, 179 – 186.
[38] F. Van Rantwijk, A. C. Kock-Van Dalen, R. A. Sheldon, in *Stability and stabilization of biocatalysts*, (Eds.: A. Ballesteros, F. J. Plou, J. L. Iborra, P. J. Halling), Elsevier, New York, **1998**, pp. 447 – 452.
[39] E. Marczak, I. Makulec, B. Baranowska, A. W. Lipkowski, in *Peptides 1994: Proceedings of the 23th European Peptide Symposium*, (Eds.: H. L. S. Maia), ESCOM, Leiden, **1995**, pp. 224 – 225.
[40] J. F. Díaz, K. J. Balkus, *J. Mol. Catal. B: Enzymatic* **1996**, *2*, 115 – 126.
[41] M. Sardar, R. Agarwal, A. Kumar, M. N. Gupta, *Enzyme Microbiol. Technol.* **1997**, *20*, 361 – 367.
[42] T. Zoungrana, G. H. Findenegg, W. Norde, *J. Colloid Interface Sci.* **1997**, *190*, 437 – 448.
[43] M. Woudenberg-Van Oosterom, H. J. A. Van Belle, F. Van Rantwijk, R. A. Sheldon, *J. Mol. Catal. A: Chem.* **1998**, *134*, 267 – 274.
[44] T. Barth, H. Maskova, *Collect. Czech. Chem. Commun.* **1971**, *36*, 2398 – 2400.
[45] E. De Vroom (Gist-brocades B. V.), PCT Int. Appl. *WO* 97 04086, **1997**.
[46] H. E. Klei, D. W. Sundstrom, R. Gargano, *Biotechnol. Bioeng.* **1978**, *20*, 611 – 617.
[47] I. Chibata, T. Tosa, T. Sato, T. Mori, *Methods Enzymol.* **1976**, *44*, 746 – 759.
[48] H. Yamada, *J. Biochem.* **1978**, *83*, 1577 – 1581.
[49] P. S. J. Cheetham, *J. Appl. Biochem.* **1979**, *1*, 51 – 59.
[50] S. V. Klinov, N. P. Sugrobova, B. T. Kurganov, *Mol. Biol.* **1979**, *13*, 421 – 428.
[51] M. D. Lilly, *Methods Enzymol.* **1976**, *44*, 46 – 53.
[52] M. Ibrahim, P. Humbert, E. Dellacherie, J. Magdalou, J. Muller, G. Siest, *Enzyme Microbiol. Technol.* **1985**, *7*, 66 – 72.
[53] P. Monsan, D. Combes, *Biotechnol. Bioeng.* **1984**, *26*, 347 – 361.
[54] K. E. LeJeune, A. J. Russell, *Biotechnol. Bioeng.* **1996**, *51*, 450 – 457.
[55] F. J. Hartdegen, P. A. Hahn, L. L. Wood (Grace W R & Co.), *US Patents* 4,312,946, 4,342,834, **1982**.
[56] R. Binder, J. Brown, G. Romancik, *Appl. Environ. Microbiol.* **1994**, *60*, 1805 – 1809.
[57] M. Bakker, F. Van Rantwijk, R. A. Sheldon, *Can. J. Chem.* **2002**, in press.
[58] D. R. Walt, V. I. Agayn, *Trends Anal. Chem.* **1994**, *13*, 425 – 430.
[59] F. A. Quiocho, F. M. Richards, *Biochemistry* **1966**, *5*, 4062 – 4076.
[60] R. Ohba, H. Chaen, S. Hayashi, S. Ueda, *Biotechnol. Bioeng.* **1978**, *20*, 665 – 676.
[61] J. E. Seip, S. K. Fager, J. E. Gavagan, D. L. Anton, R. D. Cosimo, *Bioorg. Med. Chem.* **1994**, *2*, 371 – 378.
[62] A. Margolin, *Trends Biotechnol.* **1996**, *14*, 223 – 230.
[63] M. A. Navia, N. L. StClair (Vertex Pharma), PCT Int. Appl. *WO* 92 02617, **1992**.
[64] N. K. Khalaf (Altus Biologics Inc.), PCT Int. Appl. *WO* 97 44445, **1997**.
[65] N. K. Khalaf (Altus Biologics Inc.), *US Patent* 5,932,212, **1999**.
[66] L. Cao, F. Van Rantwijk, R. A. Sheldon, *Org. Lett.* **2000**, *2*, 1361 – 1364.
[67] L. Cao, L. M. Van Langen, M. H. A. Janssen, R. A. Sheldon (DSM N. V.), *EP* 1088887, **2001**.
[68] L. Cao, L. M. Van Langen, F. Van Rantwijk, R. A. Sheldon, *J. Mol. Catal. B: Enzymatic* **2001**, *11*, 665 – 670.
[69] R. J. P. Corriu, D. Leclercq, *Angew. Chem. Int. Ed.* **1996**, *35*, 1420 – 1436.
[70] M. T. Reetz, A. Zonta, J. Simpelkamp, *Angew. Chem. Int. Ed.* **1995**, *34*, 301 – 303.
[71] M. T. Reetz, A. Zonta, J. Simpelkamp, *Biotechnol. Bioeng.* **1996**, *49*, 527 – 534.
[72] B. Benita, E. Klein, J. L. Lopez, *Biotechnol. Bioeng.* **1996**, *51*, 327 – 341.
[73] A. A. Prabhune, B. Rao, B. S. Pundle, A. V. Sivaraman, *Enzyme Microb. Technol.* **1992**, *14*, 161 – 163.
[74] A. Deshpande, S. F. D'Souza, G. B. Nadkarni, *J. Biosci.* **1987**, *11*, 137 – 144.
[75] I. Takata, K. Yamamoto, T. Tosa, I. Chibata, *Enzyme Microb. Technol.* **1980**, *2*, 30 – 35.
[76] T. Sato, Y. Nishida, T. Tosa, I. Chibata, *Biochim. Biophys. Acta* **1979**, *570*, 179 – 186.
[77] J. C. W. Ostergaard, S. C. Martiny, *Biotechnol. Bioeng.* **1973**, *15*, 561 – 563.
[78] J. K. Park, H. N. Chang, *Biotechnol. Adv.* **2000**, *18*, 303 – 319.
[79] D. T. Waliack, R. G. Carbonell, *Biotechnol. Bioeng.* **1975**, *17*, 1157 – 1181.
[80] T. M. S. Chang, *Methods Enzymol.* **1976**, *44*, 201 – 218.
[81] R. Angold, G. Beech, J. Taggart, *Food Biotechnology*, Cambridge University Press, Cambridge, **1989**.
[82] F. Van de Velde, G. A. De Ruiter, in *Biopolymers Vol. 6: Polysaccharides II: Polysaccharides from Eukaryotes*, (Eds.: A. Steinbüchel, S. DeBaets, E. J. VanDamme), Wiley-VCH, Weinheim, **2002**, pp. 245 – 274.

[83] G. H. Therkelsen, in *Industrial gums: Polysaccharides and their derivatives*, (Eds.: R. L. Whistler, J. N. BeMiller), Academic Press Inc., San Diego, **1993**, pp. 145–180.
[84] A. P. Imeson, in *Handbook of hydrocolloids*, (Eds.: G. O. Phillips, P. A. Williams), Woodhead Publishing Ltd., Cambridge, **2000**, pp. 87–102.
[85] I. Takata, T. Tosa, I. Chibata, *J. Solid-Phase Biochem.* **1977**, *2*, 225.
[86] T. Tosa, T. Sato, T. Mori, K. Yamamoto, I. Takata, Y. Nishida, I. Chibata, *Biotechnol. Bioeng.* **1979**, *21*, 1697–1709.
[87] I. Chibata, T. Tosa, I. Takata (Tnabe Seiyaku Co.), *US Patent* 4,138,292, **1979**.
[88] B. Rudolph, in *Marine and freshwater products handbook*, (Eds.: R. E. Martin, E. P. Carter, L. M. Davis, G. J. Flich), Technomic Publishing Company, Inc., Lancaster, USA, **2000**, pp. 515–529.
[89] F. Van de Velde, S. H. Knutsen, A. I. Usov, H. S. Rollema, A. S. Cerezo, *Trends Food Sci. Technol.* **2002**, in press.
[90] C. N. Jol, T. G. Neiss, B. Penninkhof, B. Rudolph, G. A. De Ruiter, *Anal. Biochem.* **1999**, *268*, 213–222.
[91] F. Van de Velde, H. S. Rollema, N. V. Grinberg, T. V. Burova, V. Ya. Grinberg, R. H. Tromp, *Biopolymers* **2002**, in press.
[92] C. Viebke, L. Piculell, S. Nilsson, *Macromolecules* **1994**, *27*, 4160–4166.
[93] L. Piculell, in *Food polysaccharides and their applications* (Eds.: A. M. Stephan), Marcel Dekker, New York, **1995**, pp. 205–244.
[94] M. Lahaye, *J. Appl. Phycology* **2001**, *13*, 173–184.
[95] S. E. Harding, S. E. Hill, J. R. Mitchell, *Biopolymer mixtures*, Nottingham University Press, Nottingham, **1995**.
[96] K. Czaczyk, A. Olejnik, K. Trojanowska, *Acta Biotechnol.* **1999**, *19*, 147–156.
[97] S. Backlund, G. Hedstroem, F. Eriksson, S. Karlsson (personally), *US Patent* 6,280,983, **2001**.
[98] F. B. Cooling, S. K. Fager, R. D. Fallon, P. W. Folsom, F. G. Gallagher, J. E. Gavagan, E. C. Hann, F. E. Herkes, R. L. Phillips, A. Sigmund, L. W. Wagner, W. Wu, R. DiCosimo, *J. Mol. Catal. B: Enzymatic* **2001**, *11*, 295–306.
[99] H. Stamatis, A. Xenakis, *J. Mol. Catal. B: Enzymatic* **1999**, *6*, 399–406.
[100] J. L. Iborra, A. Manjón, M. Cánovas, in *Methods in Biotechnology* (Eds.: G. F. Bickerstaff), Humana Press Inc., Totowa (NJ), **1997**, pp. 53–60.
[101] T. P. West, *J. Basic Microbiol.* **2000**, *40*, 397–401.
[102] H. Gardin, A. Pauss, *Appl. Microb. Biotechnol.* **2001**, *56*, 517–523.
[103] E. J. Mammarella, A. C. Rubiolo, *Biotechnol. Prog.* **1996**, *12*, 612–617.
[104] R. J. Neufeld, D. J. C. M. Poncelet, S. D. J. M. Norton (Labatt Brewing Company Ltd.), *US Patent* 6,217,916, **2001**.
[105] B. H. Davison, C. D. Scott, T. C. Scott (Lockheed Martin Energy Sys. Inc.), *US Patent* 5,725,888, **1998**.
[106] R. K. Prud-Homme, H. A. Pfeffer, C.-A. Cukras (personally), *US Patent* 6,268,191, **2001**.
[107] L. Campanella, G. Favero, M. Pastorino, M. Tomassetti, *Biosensors Bioelec.* **1999**, *14*, 179–186.
[108] L. Campanella, G. Favero, M. P. Sammartino, M. Tomassetti, *J. Mol. Catal. B Enzym.* **1999**, *7*, 101–113.
[109] S. Tomko, H. A. Pfeffer, B. A. Guiliano, C. J. Sewall, W. R. Thomas (FMC Corp.), *US Patent* 5,662,840, **1997**.
[110] K. Czaczyk, A. Albrecht, A. Mroczkowski, K. Trojankowska, *J. Biotechnol.* **1997**, *53*, 13–20.
[111] W. Chen, S. Zhang (Dazhong Lingwei Biochemical Co.), *CN* 1275619, **2000**.
[112] W. Jianlong, L. Ping, S. Hanchang, Q. Yi, *J. Environ. Sci.* **1997**, *9*, 283–287.
[113] J. N. Nigam, *J. Biotechnol.* **2000**, *80*, 189–193.
[114] M. S. Krishnan, M. Blanco, C. K. Shattuck, N. P. Nghiem, B. H. Davison, *Appl. Biochem. Biotechnol.* **2000**, *84-86*, 525–541.
[115] M. S. Krishnan, N. P. Nghiem, B. H. Davison, *Appl. Biochem. Biotechnol.* **1999**, *77-79*, 359–372.
[116] G. Çalik, H. Savasçi, P. Çalik, T. H. Özdamar, *Enzyme Microb. Technol.* **1999**, *24*, 67–74.
[117] M. L. Asanza-Teruel, E. Gontier, C. Bienaime, J. E. Nava-Saucedo, J. N. Barbotin, *Enzyme Microb. Technol.* **1997**, *21*, 314–320.
[118] B. S. Kitchell, R. W. Henkens, P. Brown, S. W. Baldwin, C. H. Lochmueller, J. P. O'Daly (Andcare Inc.), *US Patent* 5,262,313, **1993**.
[119] L. Campanella, R. Roversi, M. P. Sammartino, M. Tomassetti, *J. Pharm. Biomed. Anal.* **1998**, *18*, 105–116.
[120] L. Campanella, L.-S. De, M. P. Sammartino, M. Tomassetti, *Anal. Chim. Acta* **1999**, *385*, 59–71.
[121] L. Campanella, L. Persi, M.-P. Sammartino, M. Tomassetti, S. Zannella, *Annali di Chimica* **2000**, *90*, 35–49.
[122] L. Campanella, F. Pacifici, M. P. Sammartino, M. Tomassetti, *J. Pharm. Biomed. Anal.* **1998**, *18*, 597–604.
[123] L. Campanella, F. Pacifici, M. P. Sammartino, M. Tomassetti, *Bioelec. Bioenergetics* **1998**, *47*, 25–38.
[124] M. Fritzen, W. Shuhmann, J. W. Lengeler, H.-L. Schmidt, in *Immobilized Cells: Basics and Applications*, (Eds.: R. H. Wijffels, R. M. Buitelaar, C. Bucke, J. Tramper), Elsevier Science, Amsterdam, **1996**, pp. 821–827.
[125] M. Fritzen, W. Schumann, J. W. Lengeler, H. L. Schmidt, *Prog. Biotechnol.* **1996**, *11*, 821–827.
[126] V. A. P. M. DosSantos, J. Tramper, R. H. Wijffels, in *Immobilized Cells: Basics and Applications*, (Eds.: R. H. Wijffels, R. M. Buitelaar, C. Bucke, J. Tramper), Elsevier Science, Amsterdam, **1996**, pp. 486–493.
[127] A. Swain, K. V. Waterhouse, W. A. Venables, *Appl. Microbiol. Biotechnol.* **1991**, *35*, 110–114.
[128] P. Poupin, N. Mazure, N. Truffaut, *Prog. Biotechnol.* **1996**, *11*, 770–776.
[129] M. B. Cassidy, K. W. Shaw, H. Lee, J. T. Trevors, *Appl. Microbiol. Biotechnol.* **1997**, *47*, 108–113.
[130] W. H. Pitcher, Jr., in *Immobilized Enzymes for Food Processing*, (Eds.: W. H. Pitcher), CRC Press, Inc., Boca Raton, Florida, **1980**, pp. 186–196.

[131] W. Hartmeier, in *Immobilized Biocatalysts. An introduction*, (Eds.: W. Wieser, A. Hartmeier), Springer-Verlag, Berlin, **1986**, pp. 95 – 100.
[132] I. Takata, T. Tosa, in *Industrial Application of Immobilized Biocatalysts*, (Eds.: A. Tanaka, T. Tosa, T. Kobayashi), Marcel Dekker Inc., New York, **1993**, pp. 53 – 65.
[133] K. Yamamoto, T. Tosa, I. Chibata, *Biotechnol. Bioeng.* **1980**, *22*, 2045 – 2052.
[134] S. Takamatsu, T. Tosa, in *Industrial Application of Immobilized Biocatalysts*, (Eds.: A. Tanaka, T. Tosa, T. Kobayashi), Marcel Dekker Inc., New York, **1993**, pp. 25 – 35.
[135] A. B. Santoyo, J. B. Rodriguez, J. L. G. Carrasco, E. G. Gomez, I. A. Rojo, M. L. A. Teruel, *J. Chem. Technol. Biotechnol.* **1998**, *73*, 197 – 202.
[136] D. M. R. Mateus, S. S. Alves, M. M. R. daFonseca, *Bioprocess Eng.* **1996**, *14*, 151 – 158.
[137] M. M. R. daFonseca, D. M. R. Mateus, S. S. Alves, *Prog. Biotechnol.* **1996**, *11*, 511 – 517.
[138] T. Nagasawa, H. Yamada, *Pure Appl. Chem.* **1995**, *67*, 1241 – 1256.
[139] M. Kobayashi, S. Shimizu, *FEMS Microbiol. Lett.* **1994**, *120*, 217 – 224.
[140] H. Ohta, *Chimia* **1996**, *50*, 434 – 436.
[141] T. Sugai, T. Yamazaki, M. Yokoyama, H. Ohta, *Biosci. Biotech. Biochem.* **1997**, *61*, 1419 – 1427.
[142] J. Osuga, A. Mori, J. Kato, *J. Ferment. Technol.* **1984**, *62*, 139 – 149.
[143] A. Mori, N. Matsumoto, C. Imai, *Biotechnol. Lett.* **1989**, *11*, 183 – 188.
[144] A. Mori, in *Industrial Application of Immobilized Biocatalysts*, (Eds.: A. Tanaka, T. Tosa, T. Kobayashi), Marcel Dekker Inc., New York, **1993**, pp. 291 – 313.
[145] T. Tosa, T. Shibatani, *Ann. New York Acad. Sci.* **1995**, *750*, 364 – 375.
[146] I. Sodini, G. Corrieu, C. Lacroix, in *Immobilized Cells: Basics and Applications*, (Eds.: R. H. Wijffels, C. Bucke, J. Tramper), Elsevier Science, Amsterdam, **1996**, pp. 687 – 694.
[147] I. Sodini, C.-Y. Boquien, G. Corrieu, C. Lacroix, *Enzyme Microbial Technol.* **1997**, *20*, 381 – 388.
[148] N. A. Mensour, A. Margaritis, C. L. Briens, H. Pilkington, I. Russel, in *Immobilized Cells: Basics and Applications*, (Eds.: R. H. Wijffels, R. M. Buitelaar, C. Bucke, J. Tramper), Elsevier Science, Amsterdam, **1996**, pp. 661 – 671.
[149] N. A. Mensour, A. Margaritis, C. L. Briens, H. Pilkington, I. Russel, *Prog. Biotechnol.* **1996**, *11*, 661 – 671.
[150] N. P. Nghiem, M. Y. Sun, B. H. Davison, P. R. Bienkowski, *Abstr. Pap. Am. Chem. Soc. Meet., Pt.1, BIOT245* **1997**, 213.
[151] S. Norton, K. Watson, T. D'Amore, *Appl. Microbiol. Biotechnol.* **1995**, *43*, 18 – 24.
[152] J. Verweij, E. De Vroom, *Recl. Trav. Chim. Pays-Bas* **1993**, *112*, 66 – 81.
[153] V. Nagalakshmi, J. S. Pai, *Indian J. Microbiol.* **1997**, *37*, 17 – 20.
[154] I.-S. Chang, G.-M. Chong, S. So (Pacific Chemistry Industry Co.), *KR* 8600698, **1986**.
[155] Y. P. Chao, H. Y. Fu, T. E. Lo, P. T. Chen, J. J. Wang, *Biotechnol. Prog.* **1999**, *15*, 1039 – 1045.
[156] F. Van de Velde, F. Van Rantwijk, R. A. Sheldon, *Trends Biotechnol.* **2001**, *19*, 73 – 80.
[157] A. S. Bommarius, K. Drauz, U. Groeger, C. Wandrey, in *Chirality in industry*, (Eds.: A. N. Collins, G. N. Sheldrake, J. Crosby), Wiley, Chichester, **1992**, pp. 371 – 397.
[158] N. S. Clair, F. W. Y, A. L. Margolin, *Angew. Chem. Int. Ed.* **2000**, *39*, 380 – 383.
[159] K. Drauz, H. Waldmann, *Enzyme catalysis in organic synthesis*, VCH, Weinheim, **1995**.
[160] R. Schoevaart, F. Van Rantwijk, R. A. Sheldon, *Chem. Commun.* **1999**, *24*, 2465 – 2466.
[161] A. E. L. Larsson, B. Anders-Persson, J. E. Backvall, *Angew. Chem. Int. Ed.* **1997**, *36*, 1211 – 1212.
[162] R. Azerad, D. Buisson, *Curr. Opinion in Biotechnol.* **2000**, *11*, 565 – 571.
[163] D. K. Boadi, R. J. Neufeld, *Enzyme Microb. Technol.* **2001**, *28*, 590 – 595.
[164] M. Puri, S. S. Marwaha, R. M. Kothari, *Enzyme Mircrob. Technol.* **1996**, *18*, 281 – 285.
[165] C. Henry, *Chemistry & Engineering News* **2000**, *78 (38)*, 49 – 65.
[166] *The catalyst group* **2001**, *March 21st*.
[167] A. Bodalo, E. Gomez, J. L. Gomez, J. Bastida, M. F. Maimo, F. Diaz, *Process Biochem.* **1991**, *26*, 349 – 353.
[168] J. Idziak, P. Moszczynski, *Polish J. Food Nutr. Sci.* **2000**, *9*, 43 – 46.
[169] Y. H. Lin, X. M. Liu, Y. F. Shen, Y. L. Chen, *Food Sci. China* **1997**, *18*, 16 – 20.
[170] C. S. Lin, C. S. Hong, C. Y. Chen, T. L. Chen, *J. Chem. Technol. Biotechnol.* **1997**, *69*, 433 – 437.
[171] F. A. Roddick, M. L. Britz, *J. Chem. Technol. Biotechnol.* **1997**, *69*, 383 – 391.
[172] P. E. Duran, K. O. Garcia, J. F. Hervagault, D. Thomas, J. N. Barbotin, *Appl. Biochem. Biotechnol.* **2000**, *84-86*, 479 – 486.
[173] T. Vanek, I. Valterova, R. Vankova, T. Vaisar, *Biotechnol. Lett.* **1999**, *21*, 625 – 628.
[174] L. Lamboley, C. Lacroix, C. P. Champagne, J. C. Vuillemard, *Biotechnol. Bioeng.* **1997**, *56*, 502 – 516.
[175] K. Yamamoto, T. Tosa, I. Chibata, *Biotechnol. Bioeng.* **1980**, *22*, 2045 – 2054.
[176] K. D. Chapatwala, G. R. V. Babu, O. K. Vijaya, K. P. Kumar, J. H. Wolfram, *J. Ind. Microbiol. Biotechnol.* **1998**, *20*, 28 – 33.
[177] R. N. Chu, Y. Qian, S. Y. Li, P. Y. Cheng, Z. Y. Yuan, *Chinese J. Biotechnol.* **1997**, *13*, 70 – 75.
[178] H. Pilkington, A. Margaritis, N. Mensour, J. Sobczak, I. Hancock, I. Russel, *J. Institute Brewing* **1999**, *105*, 398 – 404.
[179] D. Smogrovicova, Z. Domeny, *Process Biochem.* **1999**, *34*, 785 – 794.
[180] H. J. Ki, J. J. Sung, S. C. Hyun, H. C. Uck, *Process Biochem.* **1996**, *31*, 485 – 492.
[181] M. Y. Sun, N. P. Nghiem, B. H. Davison, O. F. Webb, P. R. Bienkowski, *Appl. Biochem. Biotechnol.* **1998**, *70-72*, 429 – 439.