Perspectives and Applications of Immobilised β-Galactosidase in Food Industry – a Review

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Abstract

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β-Galactosidase is an important industrial enzyme in the hydrolysis of milk and whey lactose. The enzymatic hydrolysis of lactose allows to avoid health and environmental problems posed by this disaccharide. In addition, this enzyme catalyses the formation of galacto-oligosaccharides, which are prebiotic additives for the so-called "healthy foods". β-Galactosidase is one of the relatively few enzymes that have been used in large-scale processes in both free and immobilised forms. This article presents a review of recent trends in immobilisation of β-galactosidase and their application in food industry.

Keywords: immobilisation; β-galactosidase; lactose hydrolysis; galacto-oligosaccharides; bioreactors

One of the major applications of enzymes in industry is the preparation of lactose-hydrolysed milk and whey, using β-galactosidase (EC 3.2.1.23). This enzyme occurs widely in nature and has been isolated from animals, plants as well as microorganisms. Compared to animal and plant sources, the microbial is produced at higher yields and is more technologically important. The major enzymes of commercial interest are isolated mainly from the yeast *Kluyveromyces lactis*, *K. fragilis*, *K. marxianus*, *Candida kefyr* and the fungi *Aspergillus niger* or *A. oryzae* (Holsinger & Kligerman 1991).

The enzyme parameters and price are the main attributes which determine the technology and relative costs of the lactose hydrolysis process. The price of β-galactosidases is rather high. Compared to the low value of the waste product whey, the direct addition of β-galactosidase to the substrate is economically unacceptable (Mahoney 1997). This problem should be overcome by enzyme immobilisation employing a variety of techniques and matrices. Immobilised biocatalyst can be reused several times, which decrease the costs of the process (Genari *et al*. 2003). In addition, the application of immobilised enzyme compared to free enzyme has several benefits; e.g. easy separation from reaction solution, no contamination of product by the enzyme (especially useful in the food technologies), operational and long-term stability, continuous processing, multienzyme reaction systems (Dervakos & Webb 1991).

During the last three decades, the application of β-galactosidase to the hydrolysis of lactose in dairy process has been in increased attention. Although

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most industries still hydrolyses lactose with free enzyme, the immobilisation of β-galactosidase is an area of great interest, mainly in whey processing (German 1997). Nowadays, immobilised β-galactosidase is intensively used with other enzymes also in large-scale processes (Table 1). Perspectives of β-galactosidase immobilisation, as well as the benefits of this technology in lactose hydrolysis process and production of galacto-oligosaccharides are reviewed in this paper.

Enzymatic hydrolysis of lactose

Lactose hydrolysis can be performed either by acid treatment at higher temperature (150°C), or by enzymatic catalysis carried out with β-galactosidase enzyme that permits milder operating conditions of both temperature and pH (Gekas & Lopez-Leiva 1985). The enzymatic hydrolysis of lactose offers some benefits mainly in three areas: health, food technology and environment.

Health. It is well known that consumption of milk and other dairy products is limited for the majority of the world's adult population (75%). These people are unable to digest lactose present in these products because of the lack of β-galactosidase in the mucosa of the small intestine. Consuming milk products causes abdominal pain, diarrhea, cramps, or flatulence. This problem is circumvented if lactose in the products is hydrolysed by β-galactosidase to the readily utilisable sugars, glucose and galactose (Sieber *et al*. 1997).

Another advantage of the enzymatic lactose hydrolysis is the simultaneous formation of galacto-oligosaccharides (GOS), used as prebiotic food ingredients. These compounds are indigestible, acting as dietary fibre. They promote the growth of intestinal bifidobacteria, with the subsequent healthy effect in the intestine and the liver. Nowadays, the demand for GOS production, as well as the development of an effective and inexpensive GOS manufacture has increased significantly (Tuohy *et al*. 2003).

Food technology. The high lactose content in milk products such as ice-cream, frozen milks, whey spreads and condensed milk, can lead to excessive lactose crystallisation resulting in products with a mealy, sandy or gritty texture. Using β-galactosidase to process such products could reduce lactose concentrations to acceptable values, and

Table 1. Industrial application of immobilised enzymes (Marconi & Morisi 1978; Tosa & Shibatani 1995; Schmid *et al*. 2001; Velde *et al*. 2002)

Substrate	Product	Immobilised enzyme	Scale (t/year)	Company (organisation)
Glucose	fructose (high fructose syrups)	glucose isomerase	> 10^6	UOP, SnamProgetti, Sanmatsu, Clinton Corn Processing
Lactose	glucose, galactose	β-galactosidase	> 10^6	Gist Brocades, Sumitomo, Valio Laboratory, Nutrisearch Co, Snam-Progetti, Corning Glass Works, Rohm GmbH, Union Laitierre Normande
Starch hydrolysates	glucose	glucoamylase	> 10^6	Corning Glass Works, Kyowa Hakko, SnamProgetti, Minmedbioprom
Saccharose	glucose, fructose (invert sugar)	invertase	–	SnamProgetti
D,L-Methionine	L-methionine	aminoacylase	> 10^3	Degussa, Tanabe Seiyaku
3-Cyanopyridine	nicotinamide	nitrile hydratase	> 10^3	Lonza
Penicillins	6-aminopencillanic acid (6-APA)	penicillin amidohydrolase	> 10^3	DSM, Brychem, Rohm GmbH
Fumaric acid	L-malic acid	fumarase	–	SnamProgetti, Kyowa Hakko, Tanabe Seiyaku
Progesterone	17-α-hydroxy-progesterone	17-hydroxylase	> 10^6	Brychem

so improve some technological and sensorial quality of dairy foods; e.g. increasing the digestibility, softness, creaminess, etc. (Zadow 1993).

Environment. In the cheese industry lactose is a waste, which causes several economical and environmental problems. Approximately 47% of whey produced annually worldwide is disposed off. The problems arise because lactose is associated with the high biochemical and chemical oxygen demand and because of lactose uncertain solubility (Guimaraes *et al*. 1992). Hydrolysis of lactose present in whey converts whey into very useful sweet syrup, which can be used in the dairy, confectionery, baking and soft drinks industries (Pivarnik *et al*. 1995). In addition, several studies demonstrate the opportunity to degrade whey lactose using β-galactosidase for manufacturing galacto-oligosaccharides (Foda & Lopez-Leiva 2000; Novalin *et al*. 2005).

Immobilisation of β-galactosidase

The immobilised enzyme is defined as "the enzyme physically confined or localised in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously" (Chibata 1978). β-Galactosidase is one of the most studied enzymes in term of its immobilisation. Although many studies described the effective immobilisation of β-galactosidase isolated from recombinant *Escherichia coli*, its application in food industry is complicated, because this microorganism is not generally recognised as safe (GRAS) (Ladero *et al*. 2001; Di Serio *et al*. 2003). Currently, GRAS status is valid for *A. niger*, *A. oryzae*, *K. lactis*, *K. fragilis,* which are the main producers of β-galactosidase used in food industry. The choice of suitable enzymatic preparation depends on its properties and the purpose of its application. Yeast β-galactosidases are habitually used for the hydrolysis of lactose in milk and sweet whey, whereas fungal β-galactosidases are more suited for acidic whey hydrolysis. Compared to yeast enzymes, fungal β-galactosidases are more thermostable, but they are more sensitive to product inhibition, mainly by galactose (Boon *et al*. 2000).

Immobilisation of β-galactosidases can dramatically affect enzyme's properties; e.g. pH and temperature stability, kinetic parameters, etc. (Rossi *et al*. 1999; Sun *et al*. 1999; Ladero *et al*. 2000). If an adequate technique is applied, immobilisation can improve properties of β-galactosidases such as stability of the enzyme at high or low pH and temperatures. Therefore, in term of prevention of microbial growth in the reactor, these forms of enzymes are more useful for lactose hydrolysis processes (Zhou & Chen 2001b; Tanriseven & Dogan 2002). Moreover, the immobilisation technology shows a promising role in reducing the product inhibition, what permits to reach higher conversion of lactose hydrolysis (Jurado *et al*. 2002; Pessela *et al*. 2003).

Techniques and matrices for immobilisation of β-galactosidase

β-Galactosidases were immobilised by several methods to a variety of matrices, including entrapment, cross-linking, adsorption, covalent binding or the combination of these methods (Table 2). Since each method has its own advantages and drawbacks, the selection of suitable immobilisation method depends on the enzyme (different properties of various β-galactosidases, such as molecule weight, protein chain length, and position of the active site), matrix, reaction conditions, reactor, etc. (Tanaka & Kawamoto 1999).

Covalent binding. This method is mostly used for β-galactosidases immobilisations (Table 2). Enzymes are covalently linked to the support through the functional groups in the enzymes that are not essential for the catalytic activity. Compare to other techniques this method has the following advantages: enzymes does not leak or detach from the carrier, the biocatalyst can easily interact with the substrate, since being on the surface of the carrier. On the other hand, the major disadvantages are high costs and low activity yield owing to exposure of the biocatalyst to toxic reagents or severe reaction conditions (Tanaka & Kawamoto 1999). There were several matrices used for β-galactosidase immobilisation. Oxides materials such as alumina, silica, silicated alumina were used for covalent binding of β-galactosidase from *K. marxianus* and applied in lactose hydrolysis processes. In spite of the immobilisates showed good stability, the immobilisation yields were less than 5% (Di Serio *et al*. 2003). β-Galactosidase from *K. fragilis* was covalently linked to silanised porous glass beads via amino groups, using glutaraldehyde. The coupling efficiency was very high, since more than 90% of the enzyme was active and 87.5% of the protein was bound to the support.

Table 2. The immobilisation of β-galactosidase by different methods

Source of enzyme	Immobilisation method	Recovery of activity (%)	References
K. fragilis	covalent binding on corn grits	8	Siso *et al.* (1994)
	covalent binding on cellulose beads	82	Roy & Gupta (2003)
	covalent binding on porous silanised glass modified by glutaraldehyde	90	Szczodrak (2000)
	entrapment in alginate-carrageenan gels		Mammarella & Rubiolo (2005)
	adsorption on phenol-formaldehyde resin	23	Woudenberg-van Oosterom *et al.* (1998)
	adsorption onto bone powder	83	Carpio *et al.* (2000)
K. lactis	covalent binding onto glutaraldehyde-agarose covalent binding onto thiolsulfinate-agarose	36–40 60	Giacomini *et al.* (2001)
	covalent binding on graphite surface	0.01	Zhou & Chen (2001a)
K. marxianus	covalent binding on oxides supports: alumina, silica, silicated alumina	< 5	Di Serio *et al.* (2003)
E. coli	covalent binding onto glutaraldehyde-agarose covalent binding onto thiolsulfinate-agarose	39 75–85	Giacomini *et al.* (2001)
	entrapment in liposomes	28	Rodriguez-Nogales & Delgadillo-Lopez (2006)
	covalent binding onto gelatin cross-linking with chromium (III) acetate covalent binding onto gelatin cross-linking with glutaraldehyde	25 22	Sungur & Akbulut (1994)
	adsorption on chromosorb-W	–	Bodalo *et al.* (1991)
B. circulans	adsorption onto a ribbed membrane made from polyvinylchloride and silica	–	Bakken *et al.* (1992)
A. oryzae	fibers composed of alginate and gelatine cross-linking with glutaraldehyde	56	Tanriseven & Dogan (2002)
	carbodiimide coupling to alginate beads	76	Dominguez *et al.* (1988)
	entrapment in a spongy polyvinyl alcohol cryogel	–	Rossi *et al.* (1999)
	entrapment in cobalt alginate beads cross-linked with glutaraldehyde	83	Ates & Mehmetoglu (1997)
	microencapsulation in alginate beads	64	Dashevsky (1998)
	encapsulation into gelatin and cross-linking with transglutaminase	8–46	Fuchsbauer *et al.* (1996)
	adsorption on phenol-formaldehyde resin	54	Woudenberg-van Oosterom *et al.* (1998)
	adsorption on polyvinylchloride (PVC) adsorption on silica gel membrane	–	Bakken *et al.* (1990)
	adsorption on celite covalent binding to chitosan cross-linked aggregation by glutaraldehyde	2 18.4 13.5	Gaur *et al.* (2006)
	covalent binding in polyurethane foams	–	Hu *et al.* (1993)
	covalent binding to the tysolated cotton cloth	55	Albayrak & Yang (2002c)
A. niger	adsorption on a porous ceramic monolith	80	Papayannakos & Markas (1993)
Chicken bean	immobilised on cross-linked polyacrylamide gel	72	Sun *et al.* (1999)

(Szczodrak 2000). As well graphite was used for immobilisation of β-galactosidase (from *K. lactis*). The immobilisation of the enzyme on graphite slabs was achieved by means of the Schiff's base reaction between the active groups of graphite and the enzyme molecules to form covalent linkage using glutaraldehyde as a cross-linking reagent. The immobilisation increased the enzyme thermal stability and shifts the optimal pH to a more alkaline value (7.7) compared to the free enzyme (6.6) (Zhou & Chen 2001a). Yeast β-galactosidase was covalently linked by glutardaldehyde to chemically modified corn grits. Although the immobilisation material showed great characteristics; e.g. inexpensive, highly stable, good mechanical properties, the immobilisation yield was very low (8%) (Siso *et al*. 1994). Among different fibrous matrices tested (non-woven polyester fabric, cotton wool, terry cloth, rayon non-woven cloth, etc.), β-galactosidase (from *A. oryzae*) covalently bounded to cotton cloth activated with tosyl chloride showed the highest immobilised enzyme activity with coupling efficiency of 85% and enzyme activity yield of 55%. Thermal stability of the enzyme was increased by 25-fold upon immobilisation and the immobilised enzyme had a half-life of 50 days at 50°C and more than one year at 40°C (Albayrak & Yang 2002c). Giacomini *et al*. (1998) compared the properties of immobilised *K. lactis* β-galactosidase using two different coupling carriers: glutaraldehyde-agarose gel and thiosulfinate-agarose gel. Glutaraldehyde-agarose exhibited lower yield after immobilisation (36–40%) than thiosulfinate-agarose (60–85%), but better thermal properties.

Entrapment. The entrapment method is based on the localisation of an enzyme within the lattice of a polymer matrix or membrane. Entrapment has been evolved and extensively used mostly for the immobilisation of cells, but not for enzymes. The major limitation of this technique for the enzymes immobilisation is the possible leakage during repeated use due to the small molecular size compared to the cells. Next disadvantages of the method are diffusion limitations. Entrapment method is classified into five major types: lattice, microcapsule, liposome, membrane, and reverse micelle (Tanaka & Kawamoto 1999).

For β-galactosidase immobilisation, the lattice method is the most widely used. The enzyme is entrapped in the matrix of the various synthetic or natural polymers. Alginate, a naturally occurring polysaccharide that forms gels by ionotropic gelation, is the most popular one. Mammarella and Rubiolo (2005) entrapped *K. fragilis* β-galactosidase in alginate-carrageenan gels to form beads. The presence of κ-carrageenan had favourable influence on the enzymatic reaction, because this gel is formed with K^+ ions, which increased the enzyme activity. Alginate as an immobilisation matrix was also used in combination with gelatin to immobilised *A. oryzae* β-galactosidase in fibers. The immobilised enzyme showed good operational stability (35 days without decrease of activity). Furthermore, at 70°C immobilised enzyme retained 27% of its initial activity (free enzyme lost all activity) and at pH 9 immobilised enzyme had 25% of initial activity (free enzyme retained just 11.5%) (Tanriseven & Dogan 2002). From synthetic polymers used for β-galactosidase entrapment, polyvinylalcohol gel was shown as very attractive because of its mild conditions of preparation, stability, biocompatibility, structural strength and diffusive properties (Rossi *et al*. 1999). Fungal β-galactosidase entrapped in polyvinyl alcohol cryogel beads was more thermostable than free enzyme, retaining 70% of activity after 24 h at 50°C and 5% activity at 60°C (Batsalova *et al*. 1987).

The microcapsule type involves an entrapment to a semipermeable polymer. The preparation of enzyme micro capsules requires extremely well-controlled conditions. Taqieddin and Amiji (2004) developed a new encapsulation method in which the alginate-chitosan core-shell microcapsules were formed to immobilise β-galactosidase. The enzyme was localised and protected in the inner biocompatible alginate core while the outer chitosan shell dictated the transport properties. While using Ca^{2+} ions for crosslinking alginate, microcapsules with liquid core were produced with 60% loading efficiency. While using Ba^{2+} ions, microcapsules with solid core were produced and 100% loading efficiency was obtained.

The liposome type employs entrapment within an amphiphatic liquid-surfactant membrane prepared from lipid (usually phospholipids) (Kirby & Gregoriadis 1984). For the hydrolysis of lactose milk, β-galactosidase – containing lipid vesicle (liposome) is added to milk and is disrupted into the stomach by the presence of bile salts, allowing *in situ* degradation of the lactose (Kim *et al*. 1999). The entrapment in liposome is increasingly recognised as a method of protecting biocatalysts from inactivation by proteolytic enzymes. β-Galactosidase immobilised in liposomes prepared

by the dehydration-rehydration method showed high resistance to proteolysis, retaining about 93% and 75% of its initial activity after 6 h and 24 h of exposure to protease. Free enzyme retained only 7% of its activity after 24 h of exposure to protease. Moreover, liposomal enzyme offers a noticeable increase in thermal protection. At 55°C, β-galactosidase-containing lipid vesicle retained 86% of its activity, compared to only 65% of the free enzyme activity at the same temperature (Rodriguez-Nogales & Delgadillo 2006).

In the reversed micelle type, β-galactosidase is entrapped within the reversed micelles, which are formed by mixing a surfactant with an organic solvent, for example aerosol OT/isooctane reverse micelles (Chen & Ou-Yang 2004). And in the membrane type, the enzyme is separated from the reaction solution by an ultrafiltration membrane, a microfiltration membrane, or a hollow fiber (Chockchaisawasdee *et al*. 2005).

Physical adsorption. Physical adsorption is the simplest and the oldest method of immobilising enzymes onto carriers. Immobilisation by adsorption is based on the physical interactions between the biocatalyst and the carrier, such as hydrogen bonding, hydrophobic interactions, van der Waals force, and their combinations. Despite its simplicity, this immobilisation method is significantly limited by the tendency of enzyme to desorb from the support and sensitivity to environmental conditions, such as temperature and iones concentration (Tanaka & Kawamoto 1999). The immobilised β-galactosidase particles (size 1–2 mm) prepared by physical adsorption of the enzyme on the porous ceramic support and intermolecular cross-linking with glutaraldehyde reached binding efficiency 80% and good operational stability. After 135 days in continuous operation, no activity loss was detected (Papayannakos & Markas 1993). Commercially available β-galactosidases were adsorbed on phenol-formaldehyde resins of the Duolite type and applied in galactoside synthesis. The immobilisation yields varied from 23% for *K. fragilis* β-galactosidase adsorbed on Duolite A-7 to 54% for *A. oryzae* β-galactosidase adsorbed on Duolite S-761 (Woudenberg-van Oosterom *et al*. 1998). Numbers of other supports for adsorption of β-galactosidase were tested; e.g. adsorption on celite (Gaur *et al*. 2006), on zeolite pellets (Poletto *et al*. 2005), on hydrophobic cotton cloth (Sharma & Yamazaki 1984), on chromosorb W (Bodalo *et al*. 1991), etc.

Cross-linking. The cross-linking method utilises a bi- or multifunctional compounds, which serve as the reagent for intermolecular cross-linking of the biocatalyst (Tanaka & Kawamoto 1999). In case of β-galactosidase immobilisation, cross-linking is often used in combination with other immobilisation method, mainly with adsorption and entrapment (described below).

Drawbacks of immobilisation

The immobilisation process is associated with some disadvantages, such as drop of enzyme activity after immobilisation, leakage and desorption of the biocatalyst from the matrix. The drop of activity after immobilisation of β-galactosidase ranges from 0.01% to 90%, what depends on the immobilisation method and the source of the enzyme (Table 2). Despite of this, the repeated use of immobilisates can compensate it. For example, although the yield of immobilised β-galactosidase by adsorption onto bone powder was high (83%), the immobilised enzyme lost its activity continuously and only 24% of the initial activity remained after four batch reactions (Carpio *et al.* 2000). On the other hand, β-galactosidase immobilised in fibres composed of alginate and gelatin cross-linked with glutaraldehyde retained only 56% of its activity, but the immobilised enzyme was active for 35 days without any decrease in its activity (Tanriseven & Dogan 2002). Szczodrak (2000) reported more than 90% of initial enzyme activity after covalent binding of the enzyme on porous silanised glass without any notable decrease in enzyme activity during 5 repeated batch conversions. Similar result of good operational stability of immobilised enzyme was obtained with β-galactosidase immobilised in/on poly(2-hydroxyethyl methacrylate) (pHEMA) membranes by two different methods: adsorption on Cibacron F3GA derivatised pHEMA membranes (pHEMA-CB), and entrapment in the bulk of the pHEMA membranes. After 15 repeated batch conversions the retained activity of the adsorbed and the entrapped enzymes was 80% and 95%, respectively (Baran *et al*. 1997).

The problems of desorption of β-galactosidase from immobilisation matrix and the leakage of the entrapped enzyme due to a small molecular weight compared to poruses of gel in matrices can be overcome by cross-linking using bifunctional or multifunctional reagents. Cross-linking can be realised by intermolecular cross-linking

of the protein, either to other protein molecules or to functional groups on an insoluble support matrix using the functional groups of cross linking reagents (Tanaka & Kawamoto 1999). Several studies have described various cross linking reagents used for improvement of β-galactosidase stability in immobilised state (Table 3). These reagents form covalent bonds using their reactive functional groups, such as carbonyl groups of glutaraldehyde (Szczodrak 2000), imidoester groups of dimethyladipimidate (Khare & Gupta 1988), carbodiimide group of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Dominguez *et al*. 1988), etc. Among these, glutaraldehyde, which interacts with the amino groups through a base reaction, has been the most extensively used in the view of its GRAS status, low cost, high efficiency, and stability (Nakajima *et al*. 1993). For example, in β-galactosidase immobilisation in fibres composed of alginate and gelatin, glutaraldehyde cross-links the enzyme and gelatin forming an insoluble structure and also stabilises the alginate gel, helping in the prevent of the leakage of the enzyme (Tanriseven & Dogan 2002). Beneficial effect of glutaraldehyde as a cross-linker was also shown in immobilisation of β-galactosidase from *A. oryzae* by entrapment in cobalt alginate beads. Relative activity of the entrapped enzyme without cross-linking was 83%. After the first usage the relative activity dropped to 67.5% as a result of leakage. Relative activity of the entrapped enzyme cross-linked by glutaraldehyde was unchanged (83%) and stable even after the eighth use. However, the leakage of cobalt into the reaction mixture during lactose hydrolysis limits the use of this method in the food industry (Ates & Mehmetoglu 1997). Rogalski *et al*. (1994) compared the effect of two cross-linking reagents: glutaraldehyde and bis-oxirane on the pH and thermal stability of immobilised β-galactosidase on controlled porous glass (CPG). In the case of glutaraldehyde cross-linking, relatively high thermostability was observed (retaining 70% of the initial activity at 70°C). The enzyme immobilised on CPG and cross-linked with oxirane lost 50% of the initial activity at 70°C, but these immobilisates showed a very high stability at pH 8 (more than 80% of relative activity), compared to 20% of relative activity of glutaraldehyde cross-linked enzyme at the same pH value. Transglutaminase has been used to stabilise immobilisates formed with β-galactosidase and acidic-processed gelatins of different qualities. N epsilon-(γ-L-glutamyl)-L-lysine bonds formed by transaminase were used for transformation of gelatin into an insoluble protein. Consequently, the enzyme was entrapped in the gelatin matrices with a yield of 8–46% (Fuchsbauer *et al*. 1996).

The other problem associated with the immobilised enzyme system is microbial contamination. Therefore, using immobilised enzyme in the large-scale continuous processing of milk necessitates the introduction of intermittent sanitation steps, which includes the use of regular sanitation with basic detergent and a dilute protease solution (Novalin *et al*. 2005). Recently, two types of β-galactosidases: thermostable and cold-active enzymes have increasing interest in industrial lactose hydrolysis process to avoid microbial contamination. The thermostable β-galactosidases have the ability to retain their activity at high

Table 3. Different cross-linking reagents used in β-galactosidase immobilisation

Cross-linking reagent	References
Glutaraldehyde	Ates & Mehmetoglu (1997); Szczodrak (2000); Zhou & Chen (2001b); Tanriseven and Dogan (2002)
Chromium (III) acetate	Sungur & Akbulut (1994)
Bisimidoesters Dimethyladipimidate	Khare and Gupta (1988)
Polyethyleneimine	Decleire *et al*. (1987); Albayrak & Yang (2002b)
Carbodiimide	Dominguez *et al*. (1988)
Bis-oxirane	Rogalski *et al*. (1994)
Transglutaminase	Fuchsbauer *et al*. (1996)

temperatures for prolonged period and could be used in the industrial processing of dairy products simultaneously with heat treatment to sterilise the product (Wolosowska & Synowiecki 2004). The cold- active β-galactosidases provide treatment of milk and dairy foods below 5°C so that the taste and nutritional values remain unchanged (Fernandes *et al*. 2002).

Bioreactors for enzymatic hydrolysis of lactose

The choice of suitable reactor system with immobilised biocatalyst depends mainly on the type of immobilisation and the type of process (Roy & Gupta 2003).

Packed bed reactor (PBR). PBR is the most popular of all bioreactors for enzymatic lactose hydrolysis. In a PBR the immobilised enzyme particles are held in a column and substrate is pumped through in plug flow direction. Generally, enzyme is immobilised to fairly rigid matrix with pellets diameter of about 1–3 mm. The PBR permits the use of the biocatalysts at the high density, resulting in high volumetric productivities. These reactors are preferred in the processes involving product inhibition, which occurs in enzymatic hydrolysis of lactose (especially for fungal β-galactosidases). The main disadvantage of PBR is, that temperature or pH is not easily regulated especially in reactors of >15 cm diameter. It is desirable to use pellets of uniform size and an upward flow of substrate stream (Lilly & Dunnill 1976). PBR containing 750 cm^2 graphite slabs (the size of 3 mm × 60 mm × 300 mm) with covalent bounded *K. lactis* β-galactosidase on its surface (0.63–1.3 mg of the enzyme per cm^2 of the graphite surface) was used for continuous hydrolysis of lactose in skim milk solution (lactose app. 20 g/l). At flow rate 7 ml/min the conversion of 90% lactose was achieved within a residence time of 15 min (Zhou & Chen 2001b).

Siso *et al*. (1994) used a recycle batch PBR with recirculation of substrate (milk lactose) at flow rate of 0.8 ml/min. The reactor was filled with 0.19 g of dry weight of yeast β-galactosidase covalently bounded by glutaraldehyde to chemically modified corn grits. This system being used up to 5 times without any significant drop of activity. Hydrolysis rates 50% were obtained within 3 hpors.

The column PBR containing *K. lactis* β-galactosidase immobilised on to activated agarose gel by thiosulfinate was used in lactose hydrolysis of whey permeates (50 g/l of lactose). The enzyme (15.45 mg, 280 U) was immobilised by recirculating through the column of thiosulfinate-agarose gel (5 ml of packed gel) with immobilisation yield of 90%. The mini-reactor was fed continuosly with whey permeate at a flow rate of 7 ml/h. The steady state of lactose hydrolysis maintained at 90% of conversion and remained stable for 12 days (Ovsejevi *et al*. 1998). Similar high lactose conversion (90%) of whey permeate was achieved in a recycle PBR with β-galactosidase from *K. fragilis* immobilised on silanised porous glass. Whey permeate (lactose 5%) was recycled through the column for 48 h at flow rate of 0.3 ml/min and the residence time of 20.6 min (Szczodrak 2000).

Fluidised bed reactor (FBR). In a fluidised bed reactor, the immobilised enzyme particles are fluidised, i.e., the particles become suspended in the substrate stream due to the flow of the substrate stream. The immobilised enzyme particles are usually quite small; e.g., 20–40 μm in diameter, if their density is sufficiently high, otherwise larger particles have to be used to prevent them from being flown out of the reactor (Roy *et al*. 2000). FBR was the first tested reactor for whey hydrolysis in pilot-plant. The reactor consisted of β-galactosidase from *A. niger* adsorbed on porous alumina and cross-linked with glutaraldehyde (Coughlin *et al*. 1978). The FBR with recirculation of the substrate was used to hydrolyse lactose present in milk whey and whole milk by β-galactosidase immobilised on epichlorohydrin-activated cellulose beads. Milk whey (90 ml) was loaded on a fluidised column of cellulose beads (bed volume 5 ml) with the immobilised enzyme, with an effectiveness factor of 0.5 (ratio between the activity of immobilised enzyme and amount of bounded enzyme to the matrix) at a flow rate of 2 ml/min. The effluent was recirculated through the column. About 94% conversion of the lactose in milk whey could be achieved by about 30 hours. The same reactor was used for lactose hydrolysis in whole milk, but conversion could not proceed beyond the point of 60% due to the presence of fat that impairs the performance of fluidised-bed (Roy & Gupta 2003). The use of fluidised beds, as opposed to packed bed format, allows the use of feed without pretreatment before the use. The main disadvantage of FBR is that they are difficult to scale up and their use is generally restricted to small scale high priced products (Roy *et al*. 2000; Poletto *et al*. 2005).

Membrane reactor (MR). A membrane reactor has a membrane immersed in a stirred tank; e.g., dialysis membrane, which contains the enzyme (usually in free form) in a chamber where the substrate moves in and the product moves out. The main advantages of this process are the continuous operation of the reactor at low pressure and high enzyme concentration. On the other hand, compared to the PBRs, enzyme has less stability due to wash out effects. Further disadvantages of MRs are the need of regular replacement of membranes and diffusion limitation through the membrane (Rios *et al*. 2004).

One type of the MRs used for lactose hydrolysis is hollow fibre MR. The reactor contains hundreds of fibres into which β-galactosidase is retained. This system provides direct lactose hydrolysis in skim milk without any previous ultrafiltration step. Novalin *et al*. (2005) used hollow fibre MR (area of 4.9 m^2) for lactose hydrolysis in skim milk. *K. lactis* β-galactosidase were circulated abluminally during luminal flow of the substrate. When skim milk was pumped through MR with flow rate of 10.5 l/h and the enzyme solution (activity of 120 U/ml) was circulated at 25 l/h, the measured lactose conversion in skim milk reached 81% after 2 hours. The hollow fiber membrane was also applied in continuous stirred tank MR. This reactor was the combination of a membrane (polysulfone hollow fiber of 30 000 molecular weight cut-off) and a reaction vessel to provide a continuous reaction and simultaneous separation of the product from the reaction mixture. The enzyme was in the system recycled and reused. The reactor offers an effective lactose hydrolysis (> 90% of conversion) in cheese whey permeates with residence time of about 1 h, at flow rate of 5 ml/min and at a substrate/enzyme ratio of 2.5 or less. The productivity of the continuous stirred tank MR was 6 times higher than a comparable batch process, even after just 10 h of operation (Mehaia *et al*. 1993).

Besides above mentioned reactors with immobilised β-galactosidase there are a few other reactors, which were employed for hydrolysis of milk and whey lactose; e.g. axial-annular flow reactor with β-galactosidase from *A. oryzae* immobilised on polyvinylchloride and silica gel membrane (Bakken *et al*. 1990), stirred tank reactor with beads of the enzyme from *B. circulans* immobilised onto Duolite ES-762 by adsorption with glutaraldehyde treatment (Nakanishi *et al*. 1983), etc.

Production of galacto-oligosaccharides (GOS) using immobilised technologies

β-Galactosidase catalyses both hydrolysis and transgalactosylation reactions. Compared to the hydrolysis, requirements for GOS synthesis are altogether different. The reaction conditions should be those favouring transgalactosylation, namely high lactose concentration, elevated temperature and low water activity in the reaction medium (Boon *et al*. 2000). Hence, immobilised β-galactosidase should be stable at high temperature, low water content and giving high transgalactosylation activity. Many of the carriers used for immobilisation of β-galactosidases applied in GOS production were some types of microparticles, such as ion-exchange resins (Matsumoto *et al*. 1989), chitosan beads (Sheu *et al*. 1998; Shin *et al*. 1998), cellulose beads (Kmínková *et al*. 1988) and agarose beads (Berger *et al*. 1995). It was observed that the immobilised enzyme in these particle carriers often resulted in 20–30% reduction in GOS yield due to introduction of mass transfer resistance in the system (Sheu *et al*. 1998; Shin *et al*. 1998). An appropriate system of β-galactosidase immobilisation leading to increase its transgalactosylation activity is still in developing process. Gaur *et al*. (2006) compared two different techniques for *A. oryzae* β-galactosidase immobilisation: covalent coupling to chitosan (beads form) and aggregation by cross-linking (using glutaraldehyde) in terms of stability and efficiency in GOS synthesis. Using 20% (w/v) of lactose, the chitosan-immobilised β-galactosidase gave maximum trisaccharides yield (17.3% of the total sugar) within 2 h as compared to 10% obtained with free enzyme and 4.6% obtained with cross-linked aggregates.

The main reactor systems for GOS production by immobilised β-galactosidase are PBRs. Using a continuous PBR with β-galactosidase from *Bullera singularis* ATTC 24193 immobilised in chitosan beads, 55% (w/w) of GOS was produced continuously with a productivity of 4.4 g/l·h for over 15 days. The substrate (100 g/l of lactose solution) was fed at flow rate 80 ml/h into a reactor (100 ml of bed volume), in which 970 GU/g (GU-galactosidase unit defined as the amount of enzyme which liberated 10^9 mol of *o*-nitrophenol per min at 40°C) enzyme was immobilised (Shin *et al*. 1998). The PBR (60 ml) filled with 90 g of immobilised recombinant β-galactosidase from *Aspergillus candidus* CGMCC3.2919 (on

adsorptive resin D113) was used for continuous production of GOS. The maximum productivity 87 g/l·h was reached when 400 g/l lactose was fed at dilution rate of 0.8/h. The maximum GOS yield reached 37% at dilution rate of 0.5/h (Zheng *et al.* 2006). Stable continuous production of GOS was also demonstrated in fibrous bed reactor (the bed volume was 37 ml) with β-galactosidase from *A. oryzae* immobilised on cotton cloth. Pieces of cotton cloth (together 20 g) were tightly rolled into a cylinder and then packed in the reactor. The high porosity, low pressure drop, and high mechanical strength of cotton cloth allowed the enzyme reactor to operate with a concentrated lactose feed (400 g/l) at flow rate 37 ml/h. At this conditions the maximum GOS production was 26% (w/w) of total sugars and corresponding volumetric productivity was 106 g/l·h (Albayrak & Yang 2002a). Using polyethyleneimine (PEI) multilayered β-galactosidase immobilisation on cotton cloth, several-hundred-fold higher productivity (6 kg/l·h) was obtained in the same reaction conditions. PEI was used in such a way that the exterior surface of the cotton fibrils in the knitted form was coated with large PEI-enzyme aggregates of high activity. With the enzyme loading of 250 mg/g cotton cloth and 95% immobilisation yield, the multilayered polyethyleneimine method is among the most successful ever reported in the literature (Albayrak & Yang 2002b). A comparison of above mentioned continuous GOS productions using PBRs is listed in Table 4.

Continuous UF-hollow fiber membrane reactor (area of 0.5 m^2) was also applied for GOS production from whey as a substrate. The enzyme (*K. lactis* β-galactosidase) was kept in the ultrafiltration unit while the sugars (including GOS) permeated the membrane and were collected outside the vessel. The highest production of GOS obtained was 31% for whey UF permeate with initial 20% (w/v) lactose and 0.5% (v/v) initial enzyme concentration (flow rate of 2.75 l/h). Corresponding productivity was 13.7 g/l·h (Foda & Lopez-Leiva 2000).

Industrial applications of immobilised β-galactosidases

Numerous immobilisation systems for lactose hydrolysis have been investigated, but only few of them were scaled up with success and even few applied at an industrial or pilot scale. It is mainly because the materials and methods used for enzyme immobilisation are either too expensive or difficult to use in industrial scale (Albayrak & Yang 2002c).

The first industrial application of immobilised β-galactosidase in food industry was realised by SnamProgetti (Italy) and by Sumitomo Chemicals (Japan) in 1970s (Gekas & Lopez-Leiva 1985). SnamProgetti used *K. lactis* β-galactosidase entrapped in cellulose triacetate fibres by fibre wet spinning. The fibres were cut up and used for hydrolysis of lactose in milk (Marconi & Morisi 1978). Sumitomo Chemical has developed high-

Table 4. Continuous GOS production in the packed bed reactors

Source of enzyme	Immobilisation method	Reaction conditions			Max GOS (wt%)	Productivity (g/l·h)	Operation period (h)	References
		lactose conc. (g/l)	*T* (°C)	pH				
B. singularis	immobilised in chitosan beads	100	45	4.8	55.0	4.4	360	Shin *et al.* (1998)
A. oryzae	immobilised on cotton cloth	400	40	4.5	26.6	106	400	Albayrak & Yang (2002a)
A. oryzae	polyethyleneimine multilayered immobilisation on cotton cloth	400	40	4.5	26.0	6000	400	Albayrak & Yang (2002b)
A. candidus	immobilised on resin D113	400	40	6.5	37.1	87.1	> 480	Zheng *et al.* (2006)

Max GOS – weight percent of GOS based on the total sugars in the reaction mixture; GOS – content also includes disaccharides

purity immobilised β-galactosidase from *A. oryzae* covalently bounded to macroporous amphoteric ion exchange resin of phenol formaldehyde polymer. This technology was used for producing market milk and hydrolysed whey (Katchalski 1993). Nowadays, the development of immobilised β-galactosidase research and applications are typical of the development of enzyme engineering and reflect generally the basic trend of industrial biotechnology, that is formation of joint venture companies. For example Specialist Dairy Ingredients, a joint venture between the Milk Marketing Board of England and Wales and Corning Glass Works, have set up an immobilised β-galactosidase plant in North Wales for the production of lactose-hydrolysed whey with enzyme covalently bound to silica beads.

Gist Brocades (Holland) and Centrale del Latte (Italy) have used SnamProgetti immobilisation technology for the commercial process of lactose hydrolysis in milk. Rohm GmbH (Germany) developed a pilot plant for processing of whole milk using the PBR with fungal β-galactosidase covalently bound to macroporous beads made of plexiglas-like material. In several cases low-lactose milk, obtained by β-galactosidase covalently bound to silica beads, has been used in order to accelerate the ripening of cheddar cheese (Milk Marketing Board, UK, Union Laitierre Normande, France).

Since 1977 Valio laboratory in Finland has used fungal β-galactosidase adsorbed to phenol-formaldehyde resin Duolite ES-762 for whey processing. In this process whey and whey permeate are hydrolysed continuously by pumping through the column (Marconi & Morisi 1978; Gekas & Lopez-Leiva 1985; Tosa & Shibatani 1995; Schmid *et al*. 2001).

It can be seen from the foregoing that the immobilisation technology plays a great role in milk and whey processing. The use of immobilised β-galactosidase in hydrolysis of lactose is a topic of considerable scientific and technological interest.

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