International Journal of Biological Macromolecules 45 (2009) 437–447

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Review

Use of immobilised biocatalysts in the processing of cheese whey

Maria R. Kosseva[a,*], Parmjit S. Panesar[b], Gurpreet Kaur[b], John F. Kennedy[c]

[a] UCD School of Chemical & Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, Ireland
[b] Department of Food Engineering & Technology, Sant Longowal Institute of Engineering & Technology, Longowal 148106, Punjab, India
[c] Advanced Science and Technology, 5 The Croft, Buntsford Drive, Stoke Heath, Bromsgrove, Worcestershire B60 4JE, UK

ARTICLE INFO

Article history:
Received 19 May 2009
Received in revised form 4 August 2009
Accepted 11 September 2009
Available online 18 September 2009

Keywords:
Immobilisation techniques
Immobilised cells
Immobilised enzymes
Cheese whey
Lactose hydrolysis
Ethanol
Lactic acid

ABSTRACT

Food processing industry operations need to comply with increasingly more stringent environmental regulations related to the disposal or utilisation of by-products and wastes. These include growing restrictions on land spraying with agro-industrial wastes, and on disposal within landfill operations, and the requirements to produce end products that are stabilised and hygienic. Much of the material generated as wastes by the dairy processing industries contains components that could be utilised as substrates and nutrients in a variety of microbial/enzymatic processes, to give rise to added-value products. A good example of a waste that has received considerable attention as a source of added-value products is cheese whey. The carbohydrate reservoir of lactose (4–5%) in whey and the presence of other essential nutrients make it a good natural medium for the growth of microorganisms and a potential substrate for bioprocessing through microbial fermentation. Immobilised cell and enzyme technology has also been applied to whey bioconversion processes to improve the economics of such processes. This review focuses upon the elaboration of a range of immobilisation techniques that have been applied to produce valuable whey-based products. A comprehensive literature survey is also provided to illustrate numerous immobilisation procedures with particular emphasis upon lactose hydrolysis, and ethanol and lactic acid production using immobilised biocatalysts.

Contents

1. Introduction 437
2. Whey types and composition 438
3. Whey utilisation 439
4. Methods of immobilisation 439
   4.1. Immobilisation of β-galactosidase for lactose hydrolysis in whey 440
   4.2. Immobilised cell systems used in ethanol production 442
   4.3. Immobilised cell systems in lactic acid production 442
5. Bioreactor systems with immobilised biocatalyst 445
   5.1. Packed-bed reactors (PBRs) 445
   5.2. Fluidised-bed reactors (FBRs) 445
   5.3. Continuous flow stirred tank reactors (CSTR) 445
   5.4. Membrane reactors (MRs) 445
6. Conclusions and future prospects 446
   References 446

1. Introduction

Every year, the food processing industry produces vast volumes of aqueous wastes. Most nations have large dairy and cheese production operations. A major by-product is whey, a complex mix of many components. On the basis of cheese consumption [1] and production details, it is estimated that approximately 9 million tonnes of cheese *per annum* is produced within the EU, giving rise to an annual whey production figure of the order of 50 million m^3. Whereas proportions of this are used within food and even pharmaceutical formulations, often after drying or fractionation, significant volumes remain surplus to requirements and must be disposed

* Corresponding author.
E-mail address: Maria_Kosseva@yahoo.com (M.R. Kosseva).

0141-8130/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijbiomac.2009.09.005

Table 1
Whey utilisation or disposal [4].

Processing scheme	Added-value product	Pollutant
Condensed + dry whole whey	• Human food • Animal feed	
Demineralised whey	• Baby food	
Refined lactose	• Edible lactose • Animal feed	
Ultrafiltration: whey protein concentrate	• Edible protein	
Ultrafiltration: whey permeate	• Refined lactose • Lactose-hydrolysed products • Fermentation products	• Disposed as waste (see below)
Unutilised whey: for disposal		• Disposal on land • Into inland surface water • Into a common sewer • U/F whey permeate disposed of as waste

of or further processed. Its disposal as waste poses serious pollution problems for the surrounding environment [2]. However, the situation could be seen instead as an opportunity to make better use of by-products that are potential resources of energy and added-value products, given the development of appropriate and economic conversion technologies. Much of the material generated as wastes by the dairy processing industries contains components that could be utilised as substrates and nutrients in a variety of microbial/enzymic processes, to give rise to added-value products. Added-value products actually produced from dairy industry wastes, or potentially so, include animal feed, single-cell protein and other fermented edible products, baker's yeast, organic acids, amino acids, enzymes, flavours and pigments, the biopreservative bacteriocin, and microbial gums and polysaccharides. Although, these processes have been proven to be technically feasible, they are still far from being economically viable [3]. Marwaha and Arora [4] have tracked the products currently produced from whey, and the main destinations of unutilised whey for disposal: these are summarised in Table 1.

However, land disposal of whey as a waste product has been practiced not only in Europe but also in both the United States of America and Canada over the past 50 years. Muller [5] reported that of the 1.2 billion kg of liquid whey produced in 1979 in Canada, 43% was disposed of as a waste product (17% was dumped into sewers and 26% was disposed of on land). In the United States, of the 13.4 billion kg produced in the same year, 42% was disposed of as a waste product. Although whey production has increased over the past 28 years by 165% in both countries, the utilisation and disposal practices have remained essentially the same [6]. However, because of its high biochemical oxygen demand (40,000–60,000 mg/L), whey disrupts the biological process of conventional sewage treatment plants and its disposal into these plants has, therefore, been banned by many municipalities [7]. Whey disposal as waste poses serious pollution problems for the surrounding environment [2]. It affects the physical and chemical structure of soil resulting in a decrease in crop yield and when released in water bodies, reduces the aquatic life by depleting the dissolved oxygen. Thus, whey poses a major threat to the environment and human health [3], which is heavily affected by its biodegradability. Biodegradability evaluation of dairy effluents was studied by Janczukowicz et al. [8]. The results obtained proved that all dairy production effluents can be treated together, with the exception of whey, whose complex biodegradation demands may cause too much burden to any wastewater treatment technological system and thus should be managed within a separate installation. The pollutants in cheese and cottage cheese whey proved to be the most resistant to biodegradation.

Recently, demand for whey started to increase with news of the benefits that the high-quality proteins found in whey provide children, adults, and the elderly. Increased pharmaceutical applications of protein fractions for the control of blood pressure and for inducing sleep might further enlarge the market. *The World Market for Whey and Lactose Products 2006–2010—From Commodities to Value Added Ingredients* [9] clearly demonstrates how whey continues to show significant growth rate both in volume terms and particularly in value terms. There has been a significant increase in consumer product launches containing whey protein concentrate (WPC) from 2001–2003 to 2004–2006, corresponding to approximately 60%.

Peters [10] evaluated economic consequences of the cheese making process through several example calculations concerning processing of whey in relation to cheese making throughput and several whey processing alternatives. All value added enhancement by conversion of whey into whey protein concentrates creates a larger stream of an aqueous lactose fraction, with the exception of lactoferrin extraction. This means that the high price which can be obtained for whey protein isolate products has to take into account the large quantity of lactose permeate that will necessarily be created in parallel. The most beneficial step in increasing value for whey products would be to add more value to the lactose fraction. The production of galacto-oligosaccharides for the displacement of antibiotics in animal feeding is promising to influence the lactose market. It was calculated that the price of edible lactose has a greater influence on the economics than the price of whey protein.

The overall aim of this review is to emphasise trends in cheese whey utilisation by the use of immobilised biocatalysts focusing on the processes of lactose hydrolysis, and ethanol and lactic acid production from whey lactose. Choice of support and method of enzyme and viable cell immobilisation as well as the type of bioreactor in which the immobilised biocatalyst is subsequently used is also addressed.

2. Whey types and composition

Whey may be defined broadly, as the serum or watery part of milk remaining after separation of the curd that results from the coagulation of milk by acid or proteolytic enzymes. The type and composition of whey at dairy plants mainly depends upon the processing technique resulting in casein removal from fluid milk (Table 2). The most often encountered type of whey originates from the manufacture of cheese or certain casein cheese products, where processing is based on coagulating the casein by rennet, an industrial casein-clotting preparation containing chymosin or other casein-coagulating enzymes. Since the rennet-induced coagulation of casein occurs at approximately pH 6.5, this type of whey

Table 2
Typical composition of sweet and acid whey [2].

Components	Sweet whey ($g\,L^{-1}$)	Acid whey ($g\,L^{-1}$)
Total solids	63.0–70.0	63.0–70.0
Lactose	46.0–52.0	44.0–46.0
Protein	6.0–10.0	6.0–8.0
Calcium	0.4–0.6	1.2–1.6
Phosphate	1.0–3.0	2.0–4.5
Lactate	2.0	6.4
Chloride	1.1	1.1

is referred to as sweet whey. The second type of whey, acid whey (pH <5), results from processes using fermentation or addition of organic or mineral acids to coagulate the casein as in the manufacture of fresh cheeses or most industrial casein [11]. The main components of both sweet and acid whey, after water are lactose (approximately 70–72% of the total solids), whey proteins (approximately 8–10%) and minerals (approximately 12–15%). The main differences between the two whey types are in mineral content, the acidity and the composition of the whey protein fraction.

3. Whey utilisation

The dairy industry all over the world generates ample amounts of whey per litre of milk processed, depending upon the process employed, product manufactured and house keeping exercised. About 50% of total world cheese-whey production is treated and transformed into various food products, of which about 45% is used directly in liquid form, 30% in the form of powdered cheese whey, 15% as lactose and de-lactosed by-products, and the rest as cheese-whey-protein concentrates [2]. Since lactose is the major component of whey solids in addition to water soluble vitamins, minerals and proteins, numerous biotechnological processes using different microorganisms/enzymes have been developed to utilise whey for the production of some useful products of industrial importance such as ethyl alcohol (ethanol), lactic acid, citric acid, single-cell proteins, biogas, vitamins and fermented beverages, etc. The use of immobilisation technology can further improve the economics of the above processes due to different advantages over free cell/enzyme systems.

4. Methods of immobilisation

The immobilised enzymes/cells are defined as "the enzymes/microbial cells physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" [12]. Immobilisation technology has several advantages; it permits higher cell densities in bioreactors, improves stability, makes reutilisation and continuous operation possible, and precludes the need to separate the cells from the substrate products following processing. Adsorption, gel entrapment, and covalent binding are the popular methods of immobilisation used in various bioprocesses. In adsorption, the biocatalysts are held to the surface of the carriers by physical (Van-der-Waals forces) or electrostatic forces (Fig. 1). The advantages of adsorption are that it is simple to carry out and has little influence on conformation of the biocatalyst [13]. However, a major disadvantage of this technique is the relative weakness of the adsorptive binding forces. The physical entrapment method is extremely popular for the immobilisation of whole cells. The major advantages of the entrapment technique is the simplicity by which spherical particles can be obtained by dripping a polymer-cell suspension into a medium containing precipitate-forming counter ions or through thermal polymerisation. The major limitation of this technique is the possible slow leakage of cells during continuous long-term operation. However, improvements can be made by using suitable cross-linking procedures. Mass-transfer limitations are a significant drawback for many immobilised cell techniques.

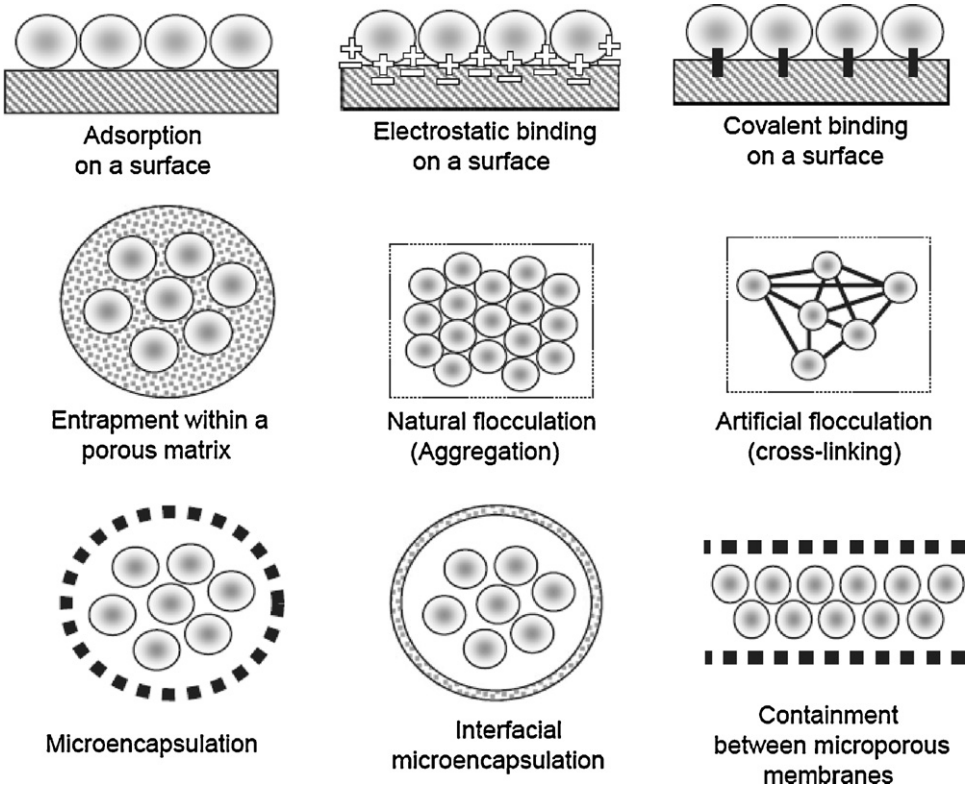

Fig. 1. Basic methods of biocatalyst immobilisation (adapted from Ref. [15]).

Table 3
Immobilised β-galactosidase.

Source of enzyme	Method of immobilisation	Supports used	Equipment/bioreactor	Productivity/conversion (%)	References
Saccharomyces fragies	Covalent coupling	Corn grits	Packed-bed reactor	Hydrolysis rates (50% within 3 h)	[22]
Aspergillus oryzae	Cross-linking with glutaraldehyde	Commercial chitosan beads (Chitopearl BCW-3007) from Fujibo	Plug reactor	~15% production of GOS	[80]
Kluyveromyces fragilis	Covalent coupling using glutaraldehyde	Silanized controlled porous glass (CPG)	Recycling packed-bed reactor lab scale	86–90% saccharification of whey permeate	[21]
Kluyveromyces fragilis	Silanised with a 10% g-APTES solution in water	Commercial silica-alumina (KA-3, from Südchemie)	Erlenmeyer flasks	ND	[81]
Kluyveromyces lactis	Entrapment	Calcium alginate beads	Laboratory scale bioreactor with recirculation Tubular plug-flow bioreactor	99.5% of hydrolysis (30 h) 50% (24h)	[82]
Aspergillus oryzae	Cross-linking	Poly(vinyl alcohol) chitosan	Fixed-bed reactor	~95% hydrolysis of lactose	[83]
Aspergillus oryzae	Enzyme coupling to cotton fibres activated with *p*-toluenesulfonyl chloride (tosyl chloride)	Cotton cloth	Plug-flow reactor	>70% GOS production	[23]
Kluyveromyces fragilis	Covalent coupling activated by epichlorohydrin	Cellulose beads	Fluidised-bed reactor	>90% conversion in 5 h	[84]
β-Galactosidase	Core-shell microcapsulation	Alginate–chitosan alginate core cross-linked with Ca^{2+} and Ba^{2+} ions	ND	–	[85]
Kluyveromyces lactis	Covalent coupling using glutaraldehyde as activating agent	Polysiloxane–polyvinyl alcohol magnetic (mPOS–PVA) composite	Eppendorfs (V = 1 ml)	–	[86]
β-Galactosidase	Membrane retention	–	CSTMR	90% with 1 h residence time	[89]
Aspergillus oryzae	Entrapment	Polyvinylalcohol hydrogel capsules LentiKat®	Batch runs and 530 h of continuous hydrolysis	–	[25]

The immobilisation of enzymes/cells on solid supports by covalent coupling and metal coordination usually leads to very stable preparations with extended active life when compared with other methods of immobilisation. Generally, it involves two steps, first, activation of the support and second coupling of enzyme to the activated support. The wide variety of binding reactions and of supports with functional groups capable of covalent coupling, or susceptible to being activated to give such groups, makes it a generally applicable method of immobilisation. The coupling of protein molecules to solid supports involves mild reactions between amino acid residues of the protein and several groups of functionalised carriers. The support materials most commonly used do not possess reactive groups but rather hydroxyl, amino, amide, and carboxy groups, which have to be activated for immobilisation of proteins. There are many reaction procedures for coupling enzymes/cells and a support via a covalent bond [14]. Some classes of coupling reaction used for the immobilisation of proteins are:

- Diazotisation.
- Amide (peptide) bond formation.
- Alkylation and arylation.
- Schiff's base formation.
- Ugi reaction.

4.1. Immobilisation of β-galactosidase for lactose hydrolysis in whey

Soluble β-D-Galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23) most commonly known as lactase is normally used for lactose hydrolysis to obtain glucose and galactose [16]. The hydrolysis of the lactose present in whey converts it into very useful sweet syrup, which can be used in the dairy, confectionary, baking and soft drink industries. Hydrolysed lactose solutions possess greater sweetening power than lactose and have applications in the confectionery and ice cream industries, replacing saccharose or starch syrup. Sweetness can be further increased through bioconversion of glucose present in the lactose-hydrolysed whey to fructose with immobilised glucose isomerase [17].

β-D-Galactosidase is one of the most studied enzymes in term of its immobilisation. Currently, GRAS status is valid for *Aspergillus niger*, *Aspergillus oryzae*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, which are the main producers of β-galactosidase used in the food industry. Fungal β-D-galactosidases are more suited for acidic whey hydrolysis, compared to yeast enzymes, since they are more thermostable, but they are more sensitive to product inhibition, mainly by galactose [18]. β-D-Galactosidases have been immobilised by several methods onto a variety of matrices, including entrapment, cross-linking, adsorption, covalent binding or a combination of these methods (Table 3). Since each method has its own advantages and drawbacks, the selection of a suitable immobilisation method depends on the enzyme (different properties of various β-D-galactosidases, such as molecular weight, protein chain length, and position of the active site), matrix, reaction conditions, reactor, etc. [19].

Covalent binding of an enzyme to a support is the most interesting method of immobilisation from an industrial point of view. Compared to other techniques this method has the following advantages: enzymes do not leak or detach from the carrier, the biocatalyst can easily interact with the substrate, since it is 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 [19]. Several matrices have been used for β-D-galactosidase immobilisation. Oxide materials such as alumina, silica, and silicated alumina were used for covalent binding of β-galactosidase from *Kluyveromyces*

Table 4
Comparison of immobilised systems proposed for ethanol production.

Microorganism	Method	Matrix	Bioreactor	Conversion (%)	References
Kluyveromyces marxianus	Entrapment	Alginate beads	Packed-bed bioreactor	84–88%	[37]
Kluyveromyces fragilis	Adsorption	Shell side of an industrial size hollow fibre module	Hollow fibre reactor	30–60 g L^{-1} h^{-1}	[39]
Kefir yeast	Adsorption	Delignified cellulosic material	Static fermentation	~90%; 5.9% (v/v) ethanol	[40]
Kluyveromyces marxianus	Adsorption	Delignified cellulosic material	0.500 L shaking flask (150 rpm)	9.3 g L^{-1}	[41]
Saccharomyces cerevisiae	Entrapment	Ca alginate beads	Packed-bed reactor	–	[42]
Recombinant *Saccharomyces cerevisiae*	Aggregation (natural flocculation)	Yeast flocs-flocculent strain	0.600 L bubble column	7% (v/v) ethanol; 53% theoretical; ~90%	[43]
Saccharomyces cerevisiae	Co-immobilised yeast cells with enzyme β-galactosidase	Ca alginate beads cross-linked with GA	5L PBR with circulation	15.6% (m/v)	[44]
Kluyveromyces marxianus	Adsorption	Olive pits	Continuous packed column bioreactor	~95%	[45]

marxianus and applied in lactose hydrolysis processes. In spite of the fact that immobilisates showed good stability, the immobilisation yields were less than 5% [20]. 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 [21]. This example of high lactose conversion (90%) of whey permeate was achieved in a recycle packed-bed reactor (PBR). Whey permeate (lactose 5%) was recycled through the column for 48 h at a flow rate of 0.3 ml/min and a residence time of 20.6 min. Siso et al. [22] used a recycle batch PBR with recirculation of substrate (milk lactose) at a flow rate of 0.8 ml/min. Yeast β-D-galactosidase was covalently linked by glutaraldehyde to chemically modified corn grits. The immobilisation material showed great characteristics: inexpensive, highly stable, good mechanical properties. This system was used up to 5 times without any significant drop of activity. Hydrolysis rate of 50% was obtained within 3 h.

Among different fibrous matrices tested (non-woven polyester fabric, cotton wool, terry cloth, rayon non-woven cloth, etc.), β-D-galactosidase (from *A. oryzae*) covalently bound 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 1 year at 40 °C [23]. Glutaraldehyde, which interacts with the amino groups through a base reaction, has been the most extensively used cross-linking agent in view of its 'generally regarded as safe' GRAS status, low cost, high efficiency, and stability [24].

In the case of β-D-galactosidase immobilisation, cross-linking is often used in combination with other immobilisation methods, mainly with adsorption and entrapment. β-D-Galactosidase isolated from *A. oryzae* was entrapped in lens-shaped polyvinylalcohol capsules (with activity 25 U g^{-1}) giving 32% of its original activity [25]. No decrease of activity was observed after 35 repeated batch runs and during 530 h of continuous hydrolysis of lactose (10%, w/v) at 45 °C. The immobilised enzyme was stable for 14 months without any change of activity during storage at 4 °C and pH 4.5. For example, in β-D-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 prevention of leakage of the enzyme [26]. The beneficial effect of glutaraldehyde as a cross-linker was also shown in immobilisation of β-D-galactosidase from *A. oryzae* by entrapment in cobalt alginate beads.

Mineral support can be added to the biopolymer materials, which, apart from improving their mechanical and barrier properties, have proved to be very efficient in enzyme binding. Silica has been widely used as an inert and stable matrix for enzyme immobilisation owing to its high specific surface area and controllable pore diameter, which can be tailored to the dimension of a specific enzyme. Initial studies focused on the immobilisation of enzymes within biosilica nanoparticles that were formed by reaction of a silicate precursor with a silica-precipitating peptide (R5) [27]. The R5 peptide is a synthetic derivative of a naturally occurring silaffin protein that is found in the silica skeleton of the marine diatom *Cylindrotheca fusiformis* [28,29]. The reaction rapidly forms a network of fused silica nanospheres with a diameter of ~500 nm that entraps the scaffold peptide and any other material that is contained within the reaction mixture. β-D-Galactosidase, for example, was encapsulated directly onto a silicon wafer by entrapment in silica particles [30]. The silica was formed by R5 peptide, directly at the amino-activated surface of a silicon wafer. Amino groups are known to be critical in biological silicification reactions and, as such, interact with the silica particles as they form and cova-

lently associate the resulting silica-immobilised enzymes directly at the silicon surface [31]. This silica encapsulation method provided a significant increase in enzyme loading capacity relative to immobilisation by alternative methods [30]. The morphology of the inorganic matrix can also be varied to create more functionalised and three-dimensional structures [32]. Controlling specific morphology provides the opportunity to modify the surface area for catalysis and potentially control the mass-transfer properties of the matrix for specific substrates and products.

4.2. Immobilised cell systems used in ethanol production

Ethyl alcohol is one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as solvent, germicide, beverage, antifreeze, fuel, depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals. Among the several microorganisms (e.g. *K. lactis* or *K. marxianus*, *Candida pseudotropicalis*) evaluated for direct production of ethanol, *Kluyveromyces fragilis* is the yeast of choice for most commercial plants. In batch fermentation *K. fragilis* utilises more than 95% of the lactose of unconcentrated whey with a conversion efficiency of 80–85% of the theoretical value [33]. Lactose fermenting yeast strains are more sensitive to high ethanol concentrations. Fewer than 10 commercial dairies worldwide ferment lactose in whey permeate directly into ethanol [33,34]. Different strains of *K. marxianus* have been used for alcoholic fermentation from deproteinised whey [35,36].

Cell immobilisation technology, applied to ethanol fermentation, has been shown to offer many advantages for biomass and metabolite productions such as: high cell density and very high volumetric productivity, reuse of biocatalysts, high process stability (physical and biological) over long fermentation periods, retention of plasmid-bearing cells, improved resistance to contamination, uncoupling of biomass and metabolite productions, stimulation of production and secretion of secondary metabolites and physical and chemical protection of the cells [37,38]. Immobilisation of microbial cells for fermentation has been developed to eliminate inhibition caused by high concentration of substrate and product, also to enhance productivity and yield of ethanol production. A hollow fibre bioreactor has been used for ethanol production from lactose solution by *K. fragilis* [39]. The yeast cells were immobilised in the shell side and the synthetic feed media containing 50 g L^{-1} lactose were pumped through the tube side. Ethanol productivities between 30 and 60 g L^{-1} h^{-1} were obtained with dilution rates of 1–4 h^{-1}. The ethanol productivity decreased when cheese whey permeate containing 45 g L^{-1} lactose was used instead of synthetic media (Table 4).

Kefir granular biomass used in the fermentation of sweet whey proved to be more effective as compared to single-cell biomass of kefir yeast, and ethanol productivity levels reached 2.57 g L^{-1} h^{-1}, whereas the yield was 0.45 g g^{-1} [40]. However, it is preferable to ferment mixtures of whey-molasses by adding molasses in whey after the completion of whey fermentation. The delignified cellulosic-supported biocatalyst, prepared by immobilisation of kefir yeast on delignified cellulosic material, was suitable for continuous, modified whey (whey containing 1% raisin extract and molasses) fermentation [41]. Ethanol productivities ranged from 3.6 to 8.3 g L^{-1} day^{-1} and there is the possibility of using such a process for the production of potable alcohol or a novel, low-alcohol content drink.

The morphology and physiology of *Saccharomyces cerevisiae* SL 100 cells entrapped in Ca alginate gel beads for a long-term period (up to 5 years) were studied [42]. During long-term continuous anaerobic process (1100 h cultivation) performed in a packed-bed reactor on complete medium at 30 °C, immobilised cells retained high metabolic activity. Furthermore, beads may be stored in 4 °C water as non-growing and starving immobilised culture for a long time (>1 year) before application or between cultivations without loosing glycolytic activity and viability.

Alcoholic fermentation of cheese whey permeate using a recombinant flocculating *S. cerevisiae*, expressing the LAC4 (coding for β-D-galactosidase) and LAC12 (coding for lactose permease) genes of *K. marxianus* using a continuously operating bioreactor resulted in ethanol productivity near 10 g L^{-1} h^{-1} (corresponding to 0.45 h^{-1} dilution rate), which raises new perspectives for the economic feasibility of whey alcoholic fermentation [43].

Another practical and economic approach for ethanol production from whey is co-immobilisation of enzyme and yeast. A study [44] was carried out to improve the effectiveness of a semi-continuous ethanol fermentation of lactose mash combined with a pervaporation module. The fermentation was conducted with a biocatalyst immobilised in calcium alginate and consisted of the yeast *S. cerevisiae* co-immobilised with β-galactosidase cross-linked with glutaraldehyde. A 5 L fermenter, with a water jacket operated in circulation with mash feeding through the biocatalyst layer packed in a perforated cylinder, was used in this study. The productivity of ethanol calculated for 24 h of process was in the range of 1.58–2.38 g L^{-1} h^{-1}, and mean ethanol concentration in the received permeate of 44% (w/w) was obtained in such a system.

Cheese whey powder (CWP) solution containing 50 g L^{-1} total sugar was fermented to ethanol in a continuously operated packed column bioreactor (PCBR) using olive pits as support particles for cell attachment [45]. Pure culture of *K. marxianus* was used in the PCBR for ethanol formation from lactose content of CWP solution. Sugar utilisation and ethanol formation were investigated as a function of the hydraulic residence time (HRT) between 17.6 and 64.4 h. The ethanol yield coefficient also increased with increasing HRT and reached the highest level of 0.54 g E g^{-1} S at an HRT of 50 h.

4.3. Immobilised cell systems in lactic acid production

Lactic acid has widespread applications in food, pharmaceutical, textile and leather industries, and the use of whey as a cheap substrate for lactic acid bioproduction has proved to be more attractive economically than its organic synthesis. Lactic acid bacteria (LAB) are the main organisms involved in the bioconversion of lactose to lactic acid. These bacteria are recognised as GRAS bacteria. The genera that comprise LAB are at its core *Lactobacillus* (*L.*), *Lactococcus* (*Lc.*), *Leuconostoc* (*Ln.*), *Pediococcus* (*P.*), and *Streptococcus* (*S.*) as well as the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Teragenococcus*, *Vagococcus*, and *Weisella*. *Lactobacillus* is by far the largest genus included in LAB, and more than 125 species and subspecies names are currently recognised [46–48]. The key property in defining LAB is that these bacteria produce lactic acid as the major or sole fermentation product. Some of the useful applications of LAB are found in biopreservation and aroma development of food. Because of their ability to produce organic acids (mainly lactic and acetic acids) from lactose and other inhibitory products such as bacteriocins, LAB are used to inhibit unwanted bacteria in food processing increasing the shelf-life of products. Some strains, including *Leuconostoc* sp. and *Lactococcus lactis* ssp. *lactis* participate in the formation of the aroma of fermented milk products by metabolising citrate to produce C_4 compounds (diacetyl, acetoin, acetaldehyde and 2-3-butanediol) [49]. Exopolysaccharide (EPS)-producing lactic cultures have been used to modify the textural and functional properties of fermented dairy products, mainly cheese [50]. EPS provides functions that benefit reduced-fat cheeses. They bind water and increase the moisture in the nonfat portion, interfere with protein–protein interactions and reduce the rigidity of the protein network, and increase viscosity of the serum phase. Understanding of the structure–function relationship of EPS would allow chemical, enzymatic, or genetic

Table 5
Comparison of immobilised systems proposed for lactic acid production.

Microorganism	Method	Matrix	Bioreactor	Productivity/conversion (%)	References
Lactobacillus casei	Entrapment	Agar Polyacrylamide	Stationary conditions batch	~90%	[70]
Lactobacillus delbrueckii subsp. *bulgaricus*	Adsorption	Hollow fibre ultrafiltration module	Membrane Bioreactor with cell recycle	35 and 80 g L^{-1} h^{-1}	[53]
Streptococcus thermophilus, *Lactobacillus bulgaricus* *S. lactis*	Entrapment	κ-Carrageenan/locust bean gum beads	0.5 L STR	1.5–2% ~1%	[54]
L. helveticus	Entrapment	κ-Carrageenan-locust bean gum beads	1 L stirred tank reactor (STR)	75–85% at pH 4.7–6.3 28.5 g L^{-1} h^{-1} 90% at pH 4.3	[55]
L. casei and *L. lactis* cells	Entrapment	Ca alginate beads	6 L stirred tank reactor fed-batch	85.5%	[71]
Lactobacillus casei subsp. *casei*	Adsorption	Porous sintered glass beads	Continuous stirred tank and fluidized bed reactors	100% 93%	[61]
Lactobacillus casei	Adsorption	PUF cubes	0.5 L STR with circulation	99%	[60]
Lactobacillus casei subsp. *rhamnosus*	Adsorption	PEI-coated foam glass Pora-bact A beads	Packed-bed column (V = 1.8L)	~90%	[56]
Lactobacillus casei	Adsorption	Poraver® beadsPostbauer-Heng, Germany	Recycle packed-bed column (V_m = 0.7 L)	90–95% 93 g L^{-1}	[57]
L. helveticus	Adsorption	Activated alumina sphinx adsorbents	1 L packed-bed reactor + STR (PBR/CSTR)	96%	[66]
Lactobacillus brevis	Adsorption	Delignified cellulosics support	Batch stationary condition	100%	[62]
Lactobacillus casei	Adsorption	Fruit (apple and quince) pieces	Ferm. under stationary conditions	~40%	[63]
Bifidobacterium longum *L. helveticus*	Entrapment	Sodium alginate beads	Spiral-sheet bioreactor	69% 79%	[73]
Lactobacillus casei	Entrapment	Ca alginate–chitosan beads	0.1 L shaking flasks	90%	[74]
L. helveticus	Entrapment	κ-Carrageenan/locust bean gum	Two-stage batch and continuous	19–22 g L^{-1} h^{-1}	[72]
Lactobacillus casei	Entrapment	Pectate beads	0.1 L shaking flasks	94.4%	[75]

modification of the polysaccharide to obtain tailored characteristics in fermented dairy products [51].

LAB have been immobilised by several methods on different supports (Table 5) and the immobilised systems have been investigated for lactic acid production from whey [52–57]. Generally, covalent binding is not the preferred method for immobilisation of lactic acid bacteria due to the use of chemicals, which may be harmful to the cells. In the search for economical immobilisation supports, wood chips, brick particles and porous glass and egg shell have been tested for immobilisation of *Lactobacillus casei*. Out of these, wood chips showed the highest adsorption [58,59]. This immobilised preparation displayed a high rate of production of lactic acid 16 g L^{-1} from whey in a batch system, and a lactic acid production rate of 14.8 g L^{-1} with a dilution rate 0.2 h^{-1} was observed in a continuous packed-bed reactor after 5 days.

A porous polyurethane foam (PUF) was chosen as a support for immobilisation of *L. casei* cells *via* adsorption [60]. The rate of reaction obtained with immobilised cells was higher than that obtained with free cells. The microbial biocatalyst was stable and used repeatedly in a batch stirred bioreactor with circulation for a month. A mathematical model, which took into account product inhibition, internal diffusion in the biocatalyst particles, external mass transfer and biomass growth rate was developed. A satisfactory agreement between the model predictions and the experimental data was achieved. Kinetic constants were also estimated from the model parameters. It was found that the bacterial growth was not substrate-inhibited, particularly at higher substrate concentrations (>33 g L^{-1}). There was good agreement between this data, reported for the specific growth rate of immobilised biomass (μ_{max} = 0.33 h^{-1}), and those of Krischke et al. (μ_{max} = 0.3 h^{-1}) obtained for *L. casei* cells immobilised on sintered glass beads [61].

Lactobacillus brevis cells immobilised by adsorption on delignified cellulosic (DC) material resulted in 70% yield, whereas the remaining lactose in whey was converted to alcohol by-product, leading to 90% lactose exploitation [62]. The system showed high operational stability without any loss in cell activity with 10 repeated batch fermentations. *L. casei* cells immobilised by adsorption on fruit (apple and quince) pieces have been used for 15 successive fermentation batches of whey and milk [63]. These immobilised biocatalysts proved to be very effective and suitable for food grade lactic acid production.

Fluidised-bed reactors with *L. casei* subsp. *casei* immobilised by adsorption on sintered glass beads showed higher productivities of lactic acid than conventional stirred tank reactors in a continuous lactic acid production [61]. A fibrous-bed bioreactor has also been tested for continuous lactic acid production from un-supplemented acid whey using adsorption-biofilm immobilised cells of *L. helveticus* [64]. Reactor performance was stable for continuous, long-term operation for both sterile and non-sterile whey feeds over a 6-month period. The chemostat system in salt whey permeate fermentation with *Lactobacillus* cells immobilised in agarose beads displayed a steady lactic acid concentration of 33.4 g L^{-1} [65]. In a packed-bed bioreactor, a high lactic acid production rate of 3.90 g L^{-1} h^{-1} was obtained with an initial lactose concentration of 100 g L^{-1} and a hydraulic retention time of 18 h [66].

In lactic acid production, entrapment is the most common technique used by researchers. The performance of an immobilised cell biocatalyst, in which viable cells are entrapped in a gel matrix, depends on the coupled phenomena of cellular reaction kinetics, external and internal mass transfer of solutes, and cell release from the surface of the bead. LAB are sensitive to their micro-environment, they undergo substrate and product inhibitions. Usually substrate and product concentrations as well as pH profiles play a major role in immobilised cell productivity and growth. This competitive diffusion-reaction phenomenon explains the non-uniform cell growth in the colonised gel that results in the formation of high density cell regions near the bead surface [67]. The high entrapped biomass concentration or cell density at steady state corresponds to a high cell density reactor. It was illustrated previously [49] that the maximum cell densities reported in batch fermentation are achieved within κ-carrageenan–locust bean gum gel (5.1×10^{11} CFU/ml) for *L. casei* subsp. *casei*, followed by calcium alginate (3.8×10^{11} CFU/ml) for *L. lactis* [68] and finally, 3.0×10^{11} CFU/ml in pectate gel for *L. delbrueckii* [69].

Among the two matrices assessed, agar was better than polyacrylamide in its effectiveness to carry out batch fermentation in whey permeate medium for up to three repeated runs [70]. The supplementation of Mg^{2+} and agricultural by-products (mustard oil cake) in whey permeate medium further improved the acid production ability of the immobilised cells. Among different matrices (calcium alginate, κ-carrageenan, agar, and polyacrylamide gels) tested for co-immobilisation of *L. casei* and *Lc. lactis* cells, alginate proved to be a better matrix for the production of lactic acid from deproteinised whey [71]. The polyacrylamide was polymerised in situ and this could cause significant cell death due to toxicity of the monomer and activator present. The immobilisation process protected the cells from adverse conditions and improved the yields of lactic acid.

A two-stage process was used for continuous fermentation of whey permeate medium with *L. helveticus* immobilised by entrapment in κ-carrageenan/locust bean gum, which resulted in high lactic acid productivity (19–22 g L^{-1} h^{-1}) and low residual sugar [72]. However, after continuous culture operation with very low or no residual sugar for several days, loss of productivity was observed in the second reactor due to loss of biomass activity and cell death by starvation.

Immobilised *Bifidobacterium longum* in sodium alginate beads and on a spiral-sheet bioreactor has also been evaluated for the production of lactic acid from cheese whey [73]. *B. longum* immobilised in sodium alginate beads showed better performance in lactose utilisation and lactic acid yield than *L. helveticus*. In producing lactic acid, *L. helveticus* performed better when using the spiral-sheet bioreactor and *B. longum* showed better performance with gel bead immobilisation. Response surface methodology was used to investigate the effects of initial sugar, yeast extract and calcium carbonate concentrations on the lactic acid production from whey by immobilised *L. casei* NRRL B-441 [74]. Higher lactic acid production and lower cell leakage were observed with *L. casei* cells immobilised in alginate–chitosan beads compared with Ca alginate beads and these gel beads were used for five consecutive batch fermentations without any marked activity loss and deformation.

Recently, process conditions for the immobilisation of *L. casei* using Ca pectate gel have been optimised, and the developed cell system was found highly stable during whey fermentation to lactic acid [75]. A high lactose conversion (94.37%) to lactic acid (32.95 g L^{-1}) was achieved with the developed immobilised system. The long-term viability of the pectate entrapped bacterial cells was tested by re-using the immobilised bacterial biomass, and the entrapped bacterial cells showed no decrease in lactose conversion to lactic acid up to 16 batches, which proved its high stability and potential for commercial application.

Harel [76] investigated the rheological properties/mechanical behaviour of the citrus pectate and algal alginate gel membranes, and reported that citrus pectate gels behave as a viscoelastic material whereas alginate gels show an elastic behaviour, which results in different fracture properties of these materials. Citrus pectate gel (2%, w/v) membranes, used in this study, were less resistant to stress than algal alginate membranes, with stresses at fracture of 4.83×10^5 N m^{-2} as compared to 2.44×10^6 N m^{-2} for alginate. The degree of esterification and distribution of methyl groups, as well as the pH, the molecular weight and the presence of counter

Table 6
Factors influencing choice of bioreactor type for immobilised system.

1	Type of support matrix and method of immobilisation
2	Enzyme activity/cell viability requirements
3	Nature of substrate
4	Kinetics of reactions involved
5	Operational requirements of the process
6	Ease of catalyst replacement and regeneration
7	Hydraulic considerations
8	Ease of design, fabrication, and process scale-up
9	Reactor cost

ions in the solution, strongly influence solubility, gel forming ability, conditions required for gelation, gelling temperature and gel properties of the pectin preparation [77,78]. Application of pectate gel as support for LAB entrapment has very good prospects for lactic acid production due to its good stability at low pH values and also biocompatibility and acceptability in the food industry.

5. Bioreactor systems with immobilised biocatalyst

The choice of suitable reactor systems with immobilised biocatalyst depends on the process requirements and conditions. The following factors, summarised in Table 6, influence the choice of reactor type [79].

5.1. Packed-bed reactors (PBRs)

This is the most often employed reactor type for immobilised systems. When the biocatalyst is in the form of spheres, chips, disks, sheets, beads, or pellets, it can be packed readily into a column. In a PBR, there is a steady movement of the substrate across a bed of immobilised biocatalyst in a chosen special direction. If the fluid velocity is perfectly flat over the cross section, the reactor is said to operate as a plug-flow reactor (PFR). An example of PFR was used by Sheu et al. [80] for the production of galacto-oligosaccharides (GOS) by β-galactosidase immobilised on glutaraldehyde-treated chitosan beads. Maximum yield of GOS was about 18%, 21%, and 26% of the total saccharides on a dry weight basis for the initial lactose concentrations of 100, 200, and 300 g L^{-1}.

PBRs have the advantage of simplicity of operation, high mass-transfer rates, and high reaction rates (for non-substrate-inhibited kinetics). For immobilised cells oxygen transfer can pose a serious problem in the scale-up of these reactors, unless they are staged or segmented. Another problem in such systems is the periodic fluctuation in the viable cell population due to nutrient depletion along the reactor length. Examples of PBR applied in ethanol and lactic acid production are given in Tables 4 and 5.

PBRs are also the most popular of all bioreactors for enzymatic lactose hydrolysis [21,22,82,83]. Generally, enzyme is immobilised to a fairly rigid matrix with pellet diameters of about 1–3 mm. The PBR permits the use of the biocatalysts at high density, resulting in high volumetric productivities. These reactors are preferred in processes involving product inhibition, which occurs in enzymatic hydrolysis of lactose (especially for fungal β-D-galactosidases). The main disadvantage of PBRs is that temperature or pH is not easily regulated [25].

5.2. Fluidised-bed reactors (FBRs)

In a FBR, the individual catalyst particles are kept in motion by a continuous flow of the substrate. The pressure drop of the fluid flow effectively supports the weight of the bed. The reactor thus provides for free movement of the catalyst particles throughout the bed. The fluidisation may be carried out either by liquid or by gas (e.g. air when oxygenation is necessary for immobilised cells). FBRs offer the advantage of good solid–fluid mixing and minimal pressure drops.

A FBR with recirculation of the substrate was used to hydrolyse lactose present in milk whey by β-galactosidase immobilised on epichlorohydrin-activated cellulose beads. Milk whey (90 ml) was loaded into 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 bound enzyme to the matrix) at a flow rate of 2 ml/min. The effluent was re-circulated through the column. About 94% conversion of the lactose in milk whey could be achieved by about 30 h [84]. The use of fluidised-beds, as opposed to packed-beds, allows the use of feed without pre-treatment before use. The main disadvantage of FBRs is that they are difficult to scale-up and their use is generally restricted to small scale high priced products [87].

5.3. Continuous flow stirred tank reactors (CSTR)

In an ideal CSTR, the contents of the reactor are perfectly mixed. Consequently, all elements of the reactor have essentially the same concentration, which is the same as the concentration of the outflow. The reaction rate is determined by the composition of the exit stream from the reactor. Whereas in a PFR, the substrate concentration is maximised with respect to final concentration at every point of the reactor. Thus, in CSTR the average reaction rate is lower. Hence, this reactor configuration may be more suitable for substrate-inhibited reaction kinetics. The open construction of the CSTR permits ready replacement of the immobilised biocatalyst. It also facilitates easy control of temperature and pH. Thus the system may be suitable where substrate costs are not very important and where a stable productivity is essential. Applications of these type bioreactors for the production of lactic acid are given in Table 5 [60,61].

5.4. Membrane reactors (MRs)

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 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 [88]. A hollow fibre membranes has been applied in a continuous stirred tank MR. This reactor was the combination of a membrane (polysulfone hollow fibre 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 in the system was recycled and reused. The reactor offers an effective lactose hydrolysis (>90% of conversion) in cheese whey permeates with a residence time of about 1 h, at a flow rate of 5 ml/min and at a substrate/enzyme ratio of 2.5 or less. The productivity of a continuous stirred tank MR (CSTMR) was 6 times higher than a comparable batch process, even after just 10 h of operation [89].

Numerous immobilisation systems for lactose hydrolysis have been investigated, but only a few of them have been scaled up with success, and even fewer 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 on an industrial scale [23]. 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-D-galactosidase plant in North Wales for the production of lactose-hydrolysed whey

with enzyme covalently bound to silica beads. Since 1977 Valio Laboratory in Finland has used fungal β-D-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 [90].

6. Conclusions and future prospects

While the first definition of immobilised enzymes was available 30 years ago, the topic of immobilised biocatalysts is still of considerable scientific and technological interest. Immobilised biocatalysts have also found important applications in the processing of milk and whey. The most common methods used for enzyme immobilisation are covalent binding and cross-linking with glutaraldehyde which can also be used as a surface activating agent. As enzyme immobilisation supports, chitin and chitosan-based materials are used in the form of powders, flakes and gels of different geometrical configuration. Chitin/chitosan-based powders and flakes are available as commercial products among others from Sigma–Aldrich and chitosan gel beads (Chitopearls) from Fuji Spinning Co. Ltd. (Japan). As functional materials, chitin and chitosan offer a unique set of characteristics: biocompatibility, biodegradability to harmless products, non-toxicity, physiological inertness, antibacterial properties, heavy metal ion chelation, gel forming properties and hydrophilicity, and remarkable affinity to proteins. Owing to these characteristics, chitin- and chitosan-based materials, are predicted to be widely exploited in the near future especially in environmentally kind applications in systems working in biological environments, among others as enzyme immobilisation supports [91]. Recent developments in stabilising enzymes within bio-inspired inorganic matrices have substantially extended the range of operational stabilities of immobilised enzymes. The resulting nano-sized materials offer several intrinsic advantages, such as larger surface areas that allow higher loading capacities [30].

Among the supports and methods for cell immobilisation, the most widely used is entrapment using natural polymers like alginate, agarose, carrageenan, chitosan and pectin. Such natural gelling polysaccharides represent an emerging group due to their advantage of being non-toxic, biocompatible, cheap, offering a versatile technique for biocatalyst preparation. Bioconversion of whey to bio-products also reduces the pollution load of whey and its subsequent safer disposal. Thus, the processing of whey into any form of useful product would serve a dual purpose, i.e. reduction in environmental pollution as well as preparation of valuable products. The whey products manufactured have a very wide range of uses, such as food ingredients with unique functional and nutritional properties. Whey is also a renewable source of bioenergy: processing of cheese whey residues leads to the production of ethanol and valuable organic acids, namely, acetic, citric, gluconic and lactic acids.

References

[1] British Cheese Board. http://www.britishcheese.com/new/cheese/cheesefacts.htm, 2004.
[2] S.S. Marwaha, J.F. Kennedy, Int. J. Food Sci. Technol. 23 (1988) 323–336.
[3] A.E. Ghaly, N.S. Mahmoud, D.G. Rushton, F. Arab, Am. J. Agric. Biol. Sci. 2 (2007) 106–117.
[4] S.S. Marwaha, J.K. Arora, Food Processing: Biotechnological Applications, Asiatech Publishers Inc., New Delhi, India, 2000.
[5] P.G. Muller, Economic evaluation of feeding liquid whey to livestock, Technical Report, Food Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario, 1979.
[6] J.P. Singh, A.E. Ghaly, Bioproc. Biosyst. Eng. 24 (2006) 269–281.
[7] A.E. Ghaly, D.R. Ramkumar, S.S. Sadaka, J.P. Rochon, Can. Agric. Eng. 42 (1999) 173–183.
[8] W. Janczukowicz, M. Zielinski, M. Debowski, Bioresour. Technol. 99 (2008) 4199–4205.
[9] The World Market for Whey and Lactose Products 2006–2010—From Commodities to Value Added Ingredients. http://www.3abc.dk/Report%20information%202007.pdf.
[10] R.H. Peters, Int. Dairy J. 15 (2005) 537–545.
[11] P. Jelen, in: H. Roginski, J.W. Fuquay, P.F. Fox (Eds.), Encylcopedia of Dairy Sciences, vol. 4, Academic Press, London, 2003, p. 2739.
[12] I. Chibata, Immobilised Enzymes—Research and Development, John Wiley and Sons, Inc., New York, USA, 1978.
[13] S.S. Marwaha, J.F. Kennedy, Proc. Biochem. 19 (1984) 79.
[14] J.M.S. Cabral, J.F. Kennedy, Protein Immobilization—Fundamentals and Applications, Marcel Dekker Inc., New York, USA, 1991, pp. 73–138.
[15] Y. Kourkoutas, A. Bekatorou, I.M. Banat, R. Marchant, A.A. Koutinas, Food Microbiol. 21 (2004) 377–397.
[16] T.P. Shukla, Crit. Rev. Food Technol. 5 (1975) 325–356.
[17] T. Sienkiewicz, C.L. Riedel, Whey and Whey Utilization: Possibilities for Utilization in Agriculture and Food Stuffs Production, Th. Mann Verlag, Germany, 1990.
[18] M.A. Boon, A.E.M. Janssen, K. van't Riet, Enzyme Microb. Technol. 26 (2000) 271–281.
[19] A. Tanaka, T. Kawamoto, Cell and Enzyme Immobilization, vol. 94, American Society for Microbiology, Washington, USA, 1999.
[20] M. Di Serio, C. Maturo, E. De Alteriis, P. Parascandola, R. Tesser, E. Santacesaria, Catal. Today 79–80 (2003) 333–339.
[21] J. Szczodrak, J. Mol. Catal. B: Enzym. 10 (2000) 631–637.
[22] M.I.G. Siso, A. Freire, E. Ramil, E.R. Belmonte, A.R. Torres, E. Cerdan, Proc. Biochem. 29 (1994) 7–12.
[23] N. Albayrak, S.T. Yang, Enzyme Microb. Technol. 31 (2002) 371–383.
[24] M. Nakajima, K. Nishizawa, H.A. Nabetani, Bioproc. Biosyst. Eng. 9 (1993) 31–35.
[25] Z. Grosová, M. Rosenberg, M. Rebroš, Czech J. Food Sci. 26 (2008) 1–14.
[26] A. Tanriseven, S. Dogan, Proc. Biochem. 38 (2002) 27–30.
[27] H.R. Luckarift, Nat. Biotechnol. 22 (2004) 211–213.
[28] N. Kroger, Curr. Opin. Chem. Biol. 11 (2007) 662–669.
[29] N. Poulsen, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 12075–12080.
[30] L. Betanco, Biotechnol. Bioeng. 99 (2008) 261–267.
[31] E.A. Coffman, A.L. Melechko, D.P. Allison, M.L. Simpson, M.J. Doktycz, Langmuir 20 (2004) 8431–8436.
[32] R.R. Naik, Chem. Commun. (2003) 238–239.
[33] A.J. Mawson, Bioresour. Technol. 47 (1994) 195–203.
[34] J.E. Murtagh, T.P. Lyons, D.R. Kelsall, The Alcohol Textbook, Nottingham University Press, Nottingham, UK, 1995.
[35] S. Grba, V.S. Tomas, D. Stanzer, N. Vahcic, A. Skrlin, Chem. Biochem. Eng. Q 16 (2002) 13–16.
[36] L.G.S. Longhi, D.J. Luvizetto, L.S. Ferreira, R. Rech, M.A.Z. Ayub, A.R. Secchi, J. Ind. Microbiol. Biotechnol. 31 (2004) 35–40.
[37] S.S. Marwaha, J.F. Kennedy, Br. Polym. J. 17 (1984) 60–63.
[38] C. Lacroix, F. Grattepanche, Y. Doleyres, D. Bergmaier, Applications of Cell Immobilisation Biotechnology, Springer Verlag, New York, USA, 2005, pp. 295.
[39] M.A. Mehaia, M. Cheryan, Enzyme Microb. Technol. 3 (1984) 117–120.
[40] I. Athanasiadis, D. Boskou, M. Kanellaki, V. Kiosseoglou, A.A. Koutinas, J. Agric. Food Chem. 50 (2002) 7231–7234.
[41] Y. Kourkoutas, S. Dimitropoulou, M. Kanellaki, R. Marchant, P. Nigam, I.M. Banat, Bioresour. Technol. 82 (2002) 177–181.
[42] K. Melzoch, M. Rychtera, V. Habova, J. Biotechnol. 32 (1994) 59–65.
[43] L. Domingues, N. Lima, J.A. Teixeira, Biotechnol. Bioeng. 72 (2001) 507–514.
[44] M. Lewandowska, M. Kujawski, J. Food Eng. 79 (2007) 430–437.
[45] S. Ozmihci, F. Kargi, Biochem. Eng. J. 42 (2008) 180–185.
[46] J.P. Euzeby, List of bacterial names with standing in nomenclature. http://www.bacterio.cict.fr/.
[47] G.K.Y. Limsowtin, M.C. Broome, I.B. Powell, in: H. Roginski, J.W. Fuquay, P.F. Fox (Eds.), Encylcopedia of Dairy Sciences, vol. 3, Academic Press, London, 2003, p. 2739.
[48] L. Axelsson, Lactic Acid Bacteria: Microbiological and Functional Aspects, Marcel Dekker, Inc., New York, USA, 2004.
[49] C.P. Champagne, C. Lacroix, I. Sodini-Gallot, Crit. Rev. Biotechnol. 14 (1994) 109–134.
[50] A.N. Hassan, J. Dairy Sci. 91 (2008) 1282–1298.
[51] L. De Vuyst, F. De Vin, F. Vaningelgem, B. Degeest, Int. Dairy J. 11 (2001) 687–707.
[52] D. Roy, J. Goulet, A. LeDuy, Appl. Microbiol. Biotechnol. 24 (1986) 206–213.
[53] M.A. Mehaia, M. Cheryan, Appl. Biochem. Biotechnol. 14 (1987) 21–27.
[54] P. Audet, C. Paquin, C. Lacroix, Appl. Microbiol. Biotechnol. 29 (1988) 11–18.
[55] S. Norton, C. Lacroix, J.C. Vuillemard, Enzyme Microb. Technol. 16 (1994) 457–466.
[56] A. Senthuran, V. Senthuran, B. Mattiasson, R. Kaul, Biotechnol. Bioeng. 55 (1997) 841–853.
[57] A. Senthuran, V. Senthuran, B. Mattiasson, R. Kaul, J. Biotechnol. 73 (1999) 61–70.
[58] A. Kazemi, P. Baniardalan, Sci. Iran. 8 (2001) 218–222.
[59] B. Nabi, R. Gh, P. Baniardalan, J. Environ. Stud. 30 (2004) 47–60.
[60] M.R. Kosseva, V.N. Beschkov, E.I. Pilafova, Bulg. Chem. Commun. 28 (1995) 690–702.
[61] W. Krischke, M. Schroder, W. Trosch, Appl. Microbiol. Biotechnol. 34 (1991) 573–578.
[62] O. Elezi, Y. Kourkoutas, A.A. Koutinas, M. Kanellaki, E. Bezirtzoglou, Y.A. Barnett, P. Nigam, J. Agric. Food Chem. 51 (2003) 5285–5289.
[63] Y. Kourkoutas, V. Xolias, M. Kallis, E. Bezirtzoglou, M. Kanellaki, Proc. Biochem. 40 (2005) 411–416.

[64] E.M. Silva, S.T. Yang, J. Biotechnol. 41 (1995) 59–70.
[65] G. Zayed, J. Winter, Appl. Microbiol. Biotechnol. 44 (1995) 362–366.
[66] M.S.A. Tango, A.E. Ghaly, Appl. Microbiol. Biotechnol. 58 (2002) 712–720.
[67] J.P. Arnaud, C. Lacroix, L. Choplin, Biotechnol. Bioeng. 38 (1991) 1041–1049.
[68] H. Prevost, C. Divies, Biotechnol. Lett. 14 (1992) 583.
[69] K. Richter, I. Ruehlemann, R. Berger, Acta Biotechnol. 12 (1992) 229–241.
[70] A. Tuli, R.P. Sethi, P.K. Khanna, S.S. Marwaha, J.F. Kennedy, Enzyme Microb. Technol. 7 (1985) 164–168.
[71] T. Roukas, P. Kotzekidou, Enzyme Microb. Technol. 13 (1991) 33–38.
[72] A.W. Schepers, J. Thibault, C. Lacroix, Enzyme Microb. Technol. 38 (2006) 324–337.
[73] Y. Li, A. Shahbazi, S. Coulibaly, AIChE Annu. Conf. (2005) 2207–2216.
[74] Y. Göksungur, M. Gunduz, S. Harsa, J. Chem. Technol. Biotechnol. 80 (2005) 1282–1290.
[75] P.S. Panesar, J.F. Kennedy, C.J. Knill, M.R. Kosseva, Appl. Microbiol. Biotechnol. 74 (2007) 35–42.
[76] P. Harel, Ind. Crop. Prod. 11 (2000) 259–264.
[77] M.R. Kosseva, J.F. Kennedy, C.R. Acad. Bulg. Sci. 50 (1997) 33–36.
[78] E. Murano, Minerva Biotechnol. 12 (2000) 213–222.
[79] K. Venkatasubramanian, S.B. Karkare, W.R. Vieth, Immobilised Microbial Cells, Academic Press, New York, USA, 1983, pp. 311.
[80] D.C. Sheu, S.Y. Li, K.J. Duan, C.W. Chen, Biotechnol. Tech. 12 (1998) 273–276.
[81] M. Ladero, A. Santos, J.L. Garcia, F. Garcia-Ochoa, Enzyme Microb. Technol. 27 (2000) 583–629.
[82] M. Becerra, B. Baroli, A.M. Fadda, J.B. Méndez, M.I.G. Siso, Enzyme Microb. Technol. 29 (2001) 506–512.
[83] S. Rejikumar, S. Devi, Int. J. Food Sci. Technol. 36 (2001) 91–98.
[84] I. Roy, M.N. Gupta, Proc. Biochem. 39 (2003) 325–332.
[85] E. Taqieddin, M. Amiji, Biomaterials 25 (2004) 1937–1945.
[86] D.F.M. Neri, V.M. Balcão, M.G. Carneiro-da-Cunha, L.B. Carvalho, J.A. Teixeira, Catal. Commun. 9 (2008) 2334–2339.
[87] M. Poletto, P. Parascandola, I. Saracino, G. Cifarelli, Int. J. Chem. React. Eng. 3 (2005) A43.
[88] G.M. Rios, M.P. Beelleville, D. Paolucci, J. Sanchez, J. Membr. Sci. 242 (2004) 186–196.
[89] M.A. Mehaia, J. Alvarez, M. Cheryan, Int. Dairy J. 3 (1993) 179192.
[90] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, Nature 409 (2001) 258–268.
[91] B. Krajewska, Enzyme Microb. Technol. 35 (2004) 126–139.