Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol14-issue6-fulltext-11 *REVIEW ARTICLE*

Whey upgrading by enzyme biocatalysis

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Received June 3, 2011 / Accepted August 19, 2011
Published online: November 15, 2011

Abstract Whey is a co-product of processes for the production of cheese and casein that retains most of the lactose content in milk. World production of whey is estimated around 200 million tons per year with an increase rate of about 2%/per year. Milk production is seasonal, so surplus whey is unavoidable. Traditionally, whey producers have considered it as a nuisance and strategies of whey handling have been mostly oriented to their more convenient disposal. This vision has been steadily evolving because of the upgrading potential of whey major components (lactose and whey proteins), but also because of more stringent regulations of waste disposal. Only the big cheese manufacturing companies are in the position of implementing technologies for their recovery and upgrading, so there is a major challenge in incorporating medium and small size producers to a platform of whey utilization, conciliating industrial interest with environmental protection within the framework of sustainable development. Within this context, among the many technological options for whey upgrading, transformation of whey components by enzyme biocatalysis appears as prominent. In fact, enzymes are green catalysts that can perform a myriad of transformation reactions under mild conditions and with strict specificity, so reducing production costs and environmental burden. This review pretends to highlight the impact of biocatalysis within a platform of whey upgrading. Technological options are shortly reviewed and then an in-depth and critical appraisal of enzyme technologies for whey upgrading is presented, with a special focus on newly developed enzymatic processes of organic synthesis, where the added value is high, being then a powerful driving force for industrial implementation.

Keywords: β-galactosidase, enzyme catalysis, lactose, oligosaccharides, prebiotics, whey

WHEY AS RAW MATERIAL FOR UPGRADING

Whey is a major co-product of cheese and casein industries. Cheese whey, or milk whey (from now on, simply whey), is the yellowish residual liquid remaining after casein is precipitated out from milk using enzymes or acids (Green, 1977). Whey can then be considered as an aqueous solution of lactose containing mineral salts and some residual protein. Mineral or organic acids are preferably used in the production of casein and derivatives (Early, 1998), while enzymes are mostly used as coagulants in cheese production (Britz and Robinson, 2008). Rennin or chymosin, a protease extracted from the abomasum of suckling calves, has been the traditional milk coagulating enzyme (Duxbury, 1989); however, it has been progressively replaced by microbial variants (Neelakantan et al. 1999) and recombinant chymosins, the latter being produced in fungal and yeast hosts (Johnson and Lucey, 2006). The ones from *Aspergillus oryzae* and *Kluyveromyces lactis* are GRAS enzymes that have been in the market for more than 20 years now, being the ones mostly used for cheese production (Tramper, 1994; van Dijck, 1999). Whey contains all the lactose, a high proportion of mineral salts, a small amount of casein and most of the other proteins in milk; such proteins are collectively referred as whey proteins. Whey represents almost 90% of the milk volume and retains about 55% of their nutrients (González-Siso, 1996). Representative composition of bovine milk and whey is presented in Table 1.

Table 1. Proximal analysis of bovine milk and whey (Smithers et al. 1996).

Component	Milk		Whey	
	(% wet basis)	(% dry basis)	(% wet basis)	(% dry basis)
Casein	2.8	21.9	0.1	1.6
Whey proteins	0.7	5.5	0.7	11.1
Lactose	4.9	38.3	4.9	77.8
Fat	3.7	28.9	0.1	1.6
Ash	0.7	5.4	0.5	7.9
Total solids	12.8	100	6.3	100

Lactose (β-D-galactopyranosyl-α-D-glucopyranose) is a slightly sweet disaccharide, significantly less soluble than their monosaccharide constituents, which is present in the milk of all mammals, being its only natural source. Lactose varies from 2 to 10% (d.w.) in the milk of terrestrial mammals, being quite less in the milk of marine mammals (Schaafsma, 2008); content in human milk is around 7%, while in bovine milk is somewhat lower than 5% (Gänzle et al. 2008). Lactose represents the major caloric output in milk and dairy products but, due to intestinal lactase deficiency, lactose is poorly digested and absorbed in a substantial part of the world population (Schukla and Wierzbicki, 1975). Lactose intolerance is ethnic associated, being frequent among American people, with an estimate close to 50% in South American population (Vesa et al. 2000) and around 40% for Chilean population (Lacassie et al. 1978). Intestinal lactase activity tends to disappear with age, lactose intolerance being prevalent in the elderly (Obermayer-Pietsch et al. 2004). This problem represents a major restriction for the use of whey in food formulation, making its processing necessary, as will be analyzed in the next section.

Whey proteins are composed mainly by β-lactoglobulin (approx. 50%) and α-lactalbumin (approx. 20%), with minor contents of immunoglobulins, lactoferrin, lactoperoxidase, whey albumin, lysozyme and several peptides (Zadow, 1994). They have a high biological value and are used not only as food ingredients (Huffman, 1996; de Wit, 1998), but also in cosmetics (Cotte, 1991) and pharmaceutical products (Audic et al. 2003). Among the latter, bioactive peptides obtained by hydrolysis of whey proteins are outstanding (Meisel and Schlimme, 1990).

Cheese and casein producers have traditionally considered whey as a waste, being its handling oriented towards its rational disposal (Calli and Yukselen, 2004). This view has evolved because of the upgrading potential of major whey components (lactose an whey proteins) and also because of increasingly stringent regulations for waste disposal, who have forced the development of adequate handling strategies. In fact, whey is a potent potential contaminant with a biochemical oxygen demand (BDO) between 30,000 and 50,000 ppm, representing about 0.2 kg of BDO per kg of cheese, being its direct disposal into water courses forbidden (Marwaha and Kennedy, 1988). Chemical oxygen demand (CDO) is between 60,000 and 80,000 ppm, meaning that most of that demand corresponds to biodegradable organic matter, being lactose its main contributor, so the recovery of whey proteins (with a DQO of approximately 10,000 ppm) does not help much in terms of waste treatment (Mawson, 1994). Being highly perishable, whey drying is the usual way of recovery. However, liquid whey surplus is inevitable because milk production is mostly seasonal and often the drying capacity is used in full for producing milk powder during peak production. Drying cost is high (whey has about 6% solids) and, depending on the market price, drying whey may be non profitable, especially for medium and small size producers.

Worldwide annual whey production is estimated between 150 and 200 million tons, with an increase rate of 2% per year (Smithers, 2008). Whey market is quite unstable, as illustrated by the situation Chile, where in recent years, owing to the high international price of whey, most of it was dried for export. In 2007, close to 70% of the 35,000 tons of whey produced by the main cheese companies was dried and exported (http://www.odepa.gob.cl). However, the situation changed in 2008 when the international price dropped and liquid whey surplus increased over 250 million liters generating a serious problem of handling for the producers. This is but one small example that illustrates the convenience of establishing a technological platform for whey upgrading by developing processes

leading to products of high added value, conciliating profitability with environmental care and compliance with environmental regulations. Whey is increasingly being considered as a co-product of cheese or casein production and its potential as raw material exploited. Under this scenario, the development of a platform for whey upgrading becomes an opportunity for their producers to campaign for sustainable development. This potential will be now analyzed.

TECHNOLOGICAL PLATFORM FOR THE UPGRADING OF WHEY AND THEIR DERIVATIVES

Whole whey

Whey, despite having been considered as a nuisance, is a valuable material with respect to its main components. As such, whole dried whey can be directly used in food and feed formulation and also as culture medium for different fermentation processes. Liquid whey can be added to the drinking water of farm animals, representing a caloric supply (lactose) and also providing high quality proteins, calcium, phosphate, and water soluble vitamins; however, its supply under acceptable sanitary conditions must be granted. It has been used also as soil fertilizer, although its high salt content might be a problem. Liquid whey has been evaluated also as a culture medium ingredient for the production of biofuels: Biogas (Ergüder et al. 2001), bioethanol (Ghaly and El-Taweel, 1994; Zafar and Owais, 2006; Ozmihci and Kargi, 2007) and biohydrogen (Ferchichi et al. 2005; Davila-Vazquez et al. 2009), potable ethanol (Athanasiadis et al. 2002), microbial protein (Mawson, 1994; Ferrari et al. 2001), lactic acid starters (Koutinas et al. 2009), enzymes (Bajpai et al. 1992; Rech et al. 1999) and other microbial metabolites, like lactic acid (Tejayadi and Cheryan, 1995; Kourkutas et al. 2005), citric acid (El-Samragy et al. 1996), glycerol (Rapin et al. 1994), polyhydroxyalkanoates (Koller et al. 2008) and biosurfactants (Rodrigues et al. 2006). However, use of liquid whey is restricted for being a highly perishable material whose transport to the processing factories is cumbersome (Kosikowsky, 1979).

Spray drying is the usual operation for whey preservation for further use, rendering a non hygroscopic product of low moisture content and well defined particle size (Galsmar and Bergmann, 1967). Market for dry whey is big but fluctuating, being mostly used as animal feed ingredient mixed with molasses or soy flour (Guy et al. 1969), even though it is also used in the formulation of institutional foods (Jelen, 1979) and as antioxidant in edible oils (Browdy and Harris, 1997). Whey levels in foods are restricted by its high lactose and minerals content. Whey can be demineralized by ionic exchange or electrodialysis (Greiter et al. 2002) so increasing its spectrum of use, but the high lactose content still imposes restrictions for food formulation.

Whey requires to be fractionated to fully exploit its potential. Lactose and whey proteins are its major components, representing about 80 and 10% of its dry weight respectively (Table 1). Whey fractionation involves the separation of proteins, which can be done my membrane ultrafiltration and, since whey proteins are high quality proteins, this is a conventional operation in large cheese producing facilities (Gardner, 1989). A protein retentate is produced, named whey protein concentrate (WPC), and a filtrate, known as whey permeate. Depending on the saline content of whey and their use, desalting may be required. Desalting can be accomplished by nanofiltration (Kelly and Kelly, 1995), ion exchange (Safari and Shahnazari, 2001) or electrodialysis (Hoeting, 1970). Because of the advances in membrane technology, nanofiltration represents now the best option for whey demineralization (Suárez et al. 2006). Technological alternatives for the upgrading of whey and their derived products (whey permeate and whey proteins) will now be analyzed and discussed.

Whey proteins

Whey processing for protein recovery has been benefited by the developments in membrane technology so that now whey ultrafiltration and diafiltration are standard operations in the dairy industry (Pouliot, 2008) that allow their recovery without significant loss of their functional properties and with a low salt content, making it apt for human consumption (Kosikowsky, 1979). Whey proteins represent between 15 and 22% of the proteins in milk, the rest being mostly the α, β and κ-casein forming the curd; its average composition is presented in Table 2. The product (WPC) contains between 50 and 85% protein on a dry basis, while a more refined product, known as whey protein isolate (WPI) contains between 90 and 98% protein and quite small amounts of lactose and fat. Whey proteins are high quality proteins with a protein efficiency ratio (PER) of 3.4, higher than casein (2.8) and similar to egg albumin (González-Siso, 1996).

Table 2. Protein distribution in bovine whey serum (Audic et al. 2003).

Component	(% of total protein)
β-lactoglobulin	50
α-lactalbumin	22
serum albumin	12
peptides	10
immunoglobulins	5

The concentration of essential amino acids in whey proteins exceeds 400 mg/g, being higher than in casein and egg, meat and soy proteins. Even though the level of sulfur amino acids in whey proteins is lower than in casein and meat proteins, the contents of methionine and cysteine are still high (between 15 and 25 mg/g), making whey a good source of these essential amino acids whose metabolic role is very important, acting as precursor of glutathione, a potent antioxidant (Shoveller et al. 2005). The content of branched amino acids (isoleucine, leucine and valine) is also high, representing 20% in total; these amino acids are regulators of cell metabolism, being considered important in corporal weight control (Zemel, 2004). Its lysine content is also considerable, being then adequate to balance a cereal based diet whose proteins are lysine deficient.

Whey proteins are used not only as nutritional supplement but also as functional ingredients in foods (Morr and Foegeding, 1990). The use of WPC has been restricted by the inconsistency and lack of validation of its performance in foods; however, this problem has been solved by purification techniques that allow to produce WPI of high purity whose performance is consistent (Smithers, 2008). Beyond nutrition and food, whey proteins are used as surfactants in different cosmetic applications (Audic et al. 2003) and also as plastic films for coatings foods, drugs and special papers (McHugh et al. 1994; Han and Krochta, 1999).

The enzymatic hydrolysis of whey proteins increases their solubility in water and modify their functional properties (Gauthier et al. 1993), these hydrolyzates being used as protein supplements for infants, senescents, athletes and bodybuilders (Sousa Jr et al. 2004). Resulting peptides are more easily absorbed but the level of hydrolysis has to be carefully controlled to avoid the formation of bitter peptides (Mann, 2000). The increasing knowledge of the physicochemical and functional properties of whey protein components and the advances in protein fractionation, mostly molecular membrane fractionation, are on the basis for the development of new applications (Etzel, 2004). Pure fractions of β-lactoglobulin and α-lactalbumin are now produced at industrial scale (Smithers, 2008), while the fractionation of whey protein hydrolyzates opens up interesting options for the production of bioactive peptides (Fitzgerald and Murray, 2006), like the angiotensin converting enzyme and other antihypertensive peptides (Abubakar et al. 1998; Pihlanto-Leppälä et al. 1998; Murakami et al. 2004; Welderufael and Jauregui, 2010).

Lactose

Mammals' milk is the only natural source of this disaccharide. Industrial production of lactose began more than a century ago being produced from whey or, more recently, from whey permeate. Basically, the process consists in whey (or permeate) concentration up to 50% (w/w) lactose, followed by crystallization by controlled cooling for a period of time long enough to allow for nucleation and adequate growth of lactose crystals, that are recovered from the mother liquor, washed and flash dried at temperatures from 120 to 180ºC. Crystals may be subsequently dried in a fluidized bed chamber to further crystallize the amorphous lactose formed at the crystal surface during flash drying. Lactose so produced, be it anhydrous or lactose monohydrate, is considered food-grade and should be at least 99% pure. Lactose for pharmaceutical use should comply with the quality standards of the United States Pharmacopeia (USP) and requires additional purification steps to remove traces of riboflavin, proteins, phosphates and lactic acid (Evans and Young, 1982; Paterson, 2009).

Lactose uses in the food and pharmaceutical sectors are many. Lactose is used as supplement in baby formulas, since cow's milk contains 30% less lactose than human milk. It is also used as a building agent in confectionary due to its low sweetness as compared to sucrose or glucose. These two

applications represent more than 80% of lactose consumption in the USA, but only 35% in the European Union, where other significant applications exist in bakery and meat products. However, the use of lactose in foods is restricted by its low solubility and intolerance. In the pharmaceutical industry, lactose is used as excipient for most tablet drugs because it is inert, innocuous, non-hygroscopic, available with high purity and having good binding properties (Fox, 2009). Pharmaceutical applications in the USA represent only 5% of total lactose consumption, while in the European Union represent more than 25%. Lactose price is always fluctuating, and values for food-grade lactose as low as US$ 500/ton have been observed. Current international price can be estimated around US$ 2000/ton (Paterson, 2009). Bulk selling price of food-grade anhydrous lactose in Chile is now US$ 3000/ton.

Products derived from lactose

Beyond its direct applications in the food and pharma sector, lactose, either as such or contained in whey or whey permeate, is a valuable raw material for upgrading by fermentation or chemical transformation. Some of these processes employ purified lactose while others use whey or whey permeate as source of lactose. The decision of whether using already purified lactose, whey permeate or whey as raw material will depend on the desired product and also on economic considerations that will vary according to different places and scenarios. Use of lactose as culture medium ingredient for fermentation was already mentioned in section Whole whey. In some cases, lactose is previously hydrolyzed, chemically or enzymatically (Tin and Mawson, 1993; Dlamini and Peiris, 1997; Roukas, 1999), to improve cell metabolism or making it possible for those microorganism that do not synthesize β-galactosidase (Mehaia and Cheryan, 1990; Domingues et al. 1999).

Chemicals derived from lactose can be their products of hydrolysis (glucose and galactose) or products synthesized from it by different chemical reactions like oxidation, reduction, isomerization and esterification. The enzymatic hydrolysis of lactose is technologically important and applies to milk and dairy products, as well as to whey and whey permeate. This aspect will be thoroughly reviewed in the next section. Commercial products derived by chemical transformation of lactose are listed in Table 3. Some of them are synthesized by chemical catalysis and some by enzyme biocatalysis; the latter will be analyzed in detail in the next section.

Table 3. Chemical products derived from lactose.

Product	Process of synthesis	Use	Reference
Galactooligosaccharides	Enzyme catalysis	Prebiotic	Sako et al. 1999 Torres et al. 2010
Lactulose	Chemical catalysis	Laxative, prebiotic	Zokaee et al. 2002 Aider and de Halleux, 2007
Lactitol	Chemical catalysis	Low-calorie sweetener	van Velthuijsen, 1979 Harju, 1993
Lactosucrose	Enzyme catalysis	Prebiotic (?)	Kawase et al. 2001 Park et al. 2005
Lactobionic acid	Chemical catalysis	Preserver, chelating agent	Yang and Silva, 1995 Dhariwal et al. 2006
Gluconic acid	Chemical catalysis	Food acidulant	Zadow, 1984
Lactosyl urea	Chemical catalysis	Animal feed	Yang and Silva, 1995
Lactosyl monolaurate	Enzyme catalysis	Cosmetics, foods, drugs	Walsh et al. 2009
Tagatose	Enzyme catalysis	Low-calorie sweetener Prebiotic (?)	Bertelsen et al. 1999 Kim, 2004

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) has been traditionally used as a laxative in the treatment of acute and chronic constipation (Tamura et al. 1993), and also in the treatment of hyperammonemia and chronic hepatic encephalopathy (Als-Nielsen et al. 2004). Lactulose is sweeter and more soluble than lactose, which makes it interesting as an ingredient in baking and confectionery (Mizota et al. 1987). More recently, it has been considered as prebiotic (Mizota et al. 2002). Even though lactulose is produced exclusively by chemical catalysis, enzymatic synthesis is an alternative that will be analyzed in detail in the forthcoming section.

Lactitol (4-β-galactopyranosyl-sorbitol) is a sugar alcohol produced by chemical hydrogenation of lactose (Young, 2006). It is readily metabolized to short chain fatty acids by the colonic microbiota (Dills, 1989), being therefore considered as a potential prebiotic. It has been used also as a lactulose substitute in the treatment of hepatic encephalopathy and also as a low-calorie sweetener for diabetics (Monsan and Paul, 1995). Aside from the food sector, lactitol has been used as moisturizing agent, plasticizer and adhesive (Audic et al. 2003). Even though the enzymatic synthesis of lactitol has been reported, it is produced exclusively by chemical catalysis.

Lactobionic acid (4-O-β-galactopyranosyl-D-gluconato) is produced industrially by chemical oxidation of lactose (Playne and Crittenden, 2009) even though the enzymatic synthesis has been reported with glucose-fructose oxidoreductase (Satory et al. 1997) and also with cellobiose dehydrogenase in a dual enzymatic-electrochemical process (Dhariwal et al. 2006) and in a fully enzymatic process with cellobiose dehydrogenase and laccase for electron replenishment (van Hecke et al. 2009). It is a powerful chelating agent used in calcium supplement tablets, as sequestrant in detergents and also in organ preservation for transplants (Gänzle et al. 2008). It is considered a potential prebiotic but its action as such has not yet been proven conclusively (Saarela et al. 2003).

Gluconic acid has been produced from lactose by chemical oxidation at acidic conditions but the process is not competitive (Ramachandran et al. 2006) with the current process of fermentation with *Aspergillus niger* and *Gluconobactaer suboxydans* (Anastassiadis et al. 2003). In some cases, whey has been used as culture medium for gluconic acid production (Mukhopadhyay et al. 2005). Gluconic acid is a highly demanded product which is used in the food and pharmaceutical sector as acidulant and also to increase the resistance of cement (Singh and Kumar, 2007).

Lactosyl urea is produced chemically and used as a non-protein nitrogen vehicle in animal feed for ruminants (Zadow, 1984; Audic et al. 2003); other synthetic lactosyl derivatives have been tested in the amelioration of burn injuries and the control of blood pressure (Zhao et al. 2009).

Enzyme catalyzed lactose derived products will be analyzed in detail in section Upgrading by enzymatic transgalactosylation.

WHEY UPGRADING BY ENZYME BIOCATALYSIS

Whey and its derivatives (permeate and lactose) have been considered as raw materials of several enzymatic processes mostly using β-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23). β-galactosidase catalyzes the hydrolysis of the β1-4' linkage in lactose to yield D-glucose and D-galactose and several applications based in such hydrolytic reaction have been developed, mostly aimed to reduce the lactose content in different products, as it will be analyzed in section Upgrading by enzymatic hydrolysis. However, β-galactosidase has the potential of catalyzing the formation of β1-4 type bonds by a transgalactosylation reaction in which the galactose moiety, stemming from the hydrolysis of lactose, is transferred to the hydroxyl group of the galactose residue in the molecule of lactose, producing compounds collectively termed galacto-oligosaccharides (Jørgensen et al. 2001; Plou et al. 2007).

Upgrading by enzymatic hydrolysis

β-galactosidase has been used for decades as a catalyst for breaking down lactose in its monosaccharide components: Glucose and galactose, with the purpose of reducing the lactose content, where lactose is a nuisance. Lactose, despite its nutritional value as calorie supplier, has

severe restrictions as a food ingredient being it poorly soluble, poorly sweet and of unpleasant taste to many, and hardly metabolized by a substantial part of the world population.

Lactose exists in two anomeric forms: α and β, from which the former is less water soluble, being the solubility of lactose a complex function of temperature since both anomers are in mutarotational equilibrium which is temperature dependent (Roos, 2009). In practical terms, lactose is a rather poorly water soluble sugar, with a solubility of around 180 g/L at room temperature, so that it will tend to precipitate at lower temperatures and higher concentrations affecting the organoleptic and functional properties of products like ice-cream (Livney et al. 1995), condensed sweet milk (Guu and Zall, 1991; Dagbagli and Goksungur, 2008) and "dulce de leche" (Hough et al. 1990; Garitta et al. 2004). Lactose is considerably less sweet than sucrose or glucose, having a sweetening power of 20 and 30% respectively (Schaafsma, 2008; Fox, 2009), so its contribution to sweetness is irrelevant and, on the contrary, it may impart an unpleasant taste to the foods containing high amounts of it (Illanes et al. 1990a) and also present problems due to intolerance (Shaukat et al. 2010). Whey and their derivatives are adequate culture media for industrial fermentations as mentioned in section Products derived from lactose. Such applications are, however, restricted to those microorganisms bearing the β-galactosidase gene, be it a part of their genetic make-up (Rajoka et al. 2003) or acquired by genetic recombination (Guimarães et al. 2008), which is also a limitation. The above mentioned facts highlight the importance of hydrolyzing lactose into its monosaccharide components. In fact, glucose and galactose are much more water soluble than lactose, the mixture is sweeter and there are no intolerance problems associated, even though it is contraindicated for persons suffering galactosemia, who may suffer hepatic damage and galactose cataract (Kalderon et al. 1992).

Hydrolysis of lactose with β-galactosidase found many applications in the dairy industry, which have been thoroughly analyzed (Gekas and López-Leiva, 1985; Rehman, 2009). Main interest is in the production of low or non lactose milks for lactose intolerant people (Grano et al. 2004; Neuhaus et al. 2006), and concentrated and frozen dairy products (Sabioni et al. 1984; Lindamood et al. 1989; Dagbagli and Goksungur, 2008). Hydrolysis of milk has been carried out in membrane reactors that allow the free passage of milk microsolutes (lactose and dissolved salts) to the compartment where the enzyme is, but not of its macrocomponents (proteins and fat); in this way, lactose is hydrolyzed without alteration of the remaining components of milk (Neuhaus et al. 2006). Despite the obvious advantage of this type of process, the preferred method for producing delactosed milk consists in hydrolyzing the milk already packed in the carton and let it react during storage until enzyme deactivation (Gänzle et al. 2008). β-galactosidase has also been used in the upgrading of whey (and permeate) as ingredients of food and feed and in the production of sugar substitutes from permeate (Knopf et al. 1979, Illanes et al. 1990b). The production of high-fructose syrups by a sequential enzymatic process of hydrolysis and isomerization of whey permeate with immobilized β-galactosidase and glucose isomerase has been developed, obtaining a product of similar sweetening power than sucrose (Illanes et al. 1999), which represents an option for sugar-importing countries (Marwaha and Kennedy, 1988). Hydrolysis of whey and their derivative expands their use as fermentation medium for the production of fodder yeast, (Mawson, 1988; Boze et al. 1992), bioethanol (Coté et al. 2004) and other commercially important microbial metabolites (Degeest et al. 2001, Audic et al. 2003; Virtanen et al. 2007).

β-galactosidase is quite ubiquitous so many microorganisms have been evaluated as their source for the hydrolysis of lactose (Panesar et al. 2006). Even though β-galactosidases from thermophilic (Dąabrowsky et al. 2000; Ladero et al. 2002) and psychrophilic organisms (Cavicchioli et al. 2002; Nakagawa et al. 2006) are attractive, the mesophilic enzymes from *Aspergillus* and *Kluyveromyces*, both having GRAS (generally recognized as safe) status, are the most adequate for industrial use (Illanes et al. 1993). The former are thermostable and have a pH optimum between 3.5 and 5.5 (Yang et al. 1994), being well suited for the hydrolysis of acid whey and whey permeate; the latter, less stable and active at neutral pH, are preferred for the hydrolysis of sweet whey and milk (van Griethuysen-Dilber et al. 1988). Operational stability of the enzyme is critical for the process of hydrolysis of lactose in whey or permeate and robust catalysts have been produced by immobilization of β-galactosidase to different carriers (Greenberg and Mahoney, 1981; Bodalo et al. 1991; Guisan, 2006; Grosová et al. 2008; Haider and Husain, 2009a; Haider and Husain, 2009b; Sun and Zhang, 2009; Zhang et al. 2010). Stabilization factors (ratio of half-life of the biocatalyst with respect to the free enzyme at the same conditions) higher than 10 have been reported, allowing the development of continuous processes with high efficiency of catalyst use (Illanes et al. 1988). The use of whole whey produces considerable fouling, which is particularly critical if packed-bed reactors are used, being then necessary to pretreat it by ultrafiltration or microfiltration (Mariotti et al. 2008). The operational stability of β-galactosidase is increased by the presence of the competitive inhibitory product galactose and

decreased by the substrate lactose, glucose having no noticeable effect on enzyme stabilization (Illanes et al. 1998); temperature explicit functions of all kinetic and stability parameters have been determined and use for the temperature optimization of continuously operated multiple staggered bed reactors with chitin-immobilized β-galactosidase from *Kluyveromyces marxianus* (Illanes et al. 2000).

Upgrading by enzymatic transgalactosylation

As described in the previous section, β-galactosidase is a commodity enzyme widely used in the food industry in its hydrolytic capacity (Ogawa and Shimizu, 2002). However, under proper conditions, the enzyme can also catalyze transgalactosylation reactions (Planas and Faijes, 2002; Plou et al. 2007; Husain, 2010). Formation of oligosaccharides was early detected during the hydrolysis of lactose with β-galactosidase and was considered detrimental for such process (Roberts and McFarren, 1953; Asp et al. 1980). However, the potential of such activity for the production of valuable oligosaccharides was soon realized (López-Leiva and Guzmán, 1995).

Chemical synthesis of oligosaccharides is complex since the monosaccharide units contain several hydroxyl groups of similar reactivity, allowing indiscriminate bond formation and, as a consequence, the synthesis of highly branched compounds (Barreteau et al. 2006). On the contrary, the enzymatic synthesis of oligosaccharides is regio and stereospecific, being these major advantages (Bucke, 1996; Crout and Vic, 1998). Enzymes that perform such reactions within the cell metabolism are glycosyl transferases; however, they are not readily available, have stoichiometric coenzyme requirements and their performance *in-vitro* is poor, precluding their use as process biocatalysts. Glycosidases are, however, primarily hydrolytic enzymes, robust and without coenzyme requirements that under proper conditions can act in reverse, this is, catalyzing the formation of a glycosidic bond instead of their hydrolysis (Scigelova et al. 1999), being then quite attractive process biocatalysts for the synthesis of oligosaccharides, as already mentioned for the case of β-galactosidase in the synthesis of galacto-oligosaccharides.

The enzymatic reaction of synthesis can be conducted by simply displacing the equilibrium of the reaction towards glycosydic bond formation (thermodynamically controlled synthesis, TCS) or by using activated glycosyl donors (kinetically controlled synthesis, KCS). In TCS, reduction of water activity is essential to displace the equilibrium towards synthesis, which may be obtained by using high concentrations of substrates (Stevenson et al. 1996), ionic liquids (Kaftzik et al. 2002; Lang et al. 2006) or organic solvents (Shin and Yang, 1994; Chen et al. 2001; del-Val and Otero, 2003; Kwon et al. 2007; Srisimarat and Pongsawasdi, 2008), even though this latter strategy is in most cases inadequate due to the low solubility of oligosaccharides in such media. High temperatures will favor the reaction of synthesis by increasing substrates solubility, making the use of enzymes from thermophilic organisms quite appealing (Nakkharat and Haltrich, 2006; Chen et al. 2008). Although TCS is simple, greater versatility can be obtained by KCS where the activated glycosyl donor (lactose in the case of the synthesis of galacto-oligosaccharides), forms an active glycosyl-enzyme complex that can be nucleophilically attacked either by a water molecule, leading to the hydrolysis products, or by a nucleophile acceptor other than water to yield the oligosaccharide by the formation of a new glycosidic bond. In KCS, substrate conversion is not limited by the equilibrium of the reaction, but instead determined by the relative rate of the transglycosylation reaction with respect to the reaction of hydrolysis with which it competes. In this case conversion is dependent on the properties of the enzyme which makes it a more flexible and productive strategy (Plou et al. 2007). In KCS is also essential to reduce the hydrolytic potential of the enzyme, which can be achieved by using very active glycosyl donors at very high concentrations and also by continuous removal of the oligosaccharide formed (Torres et al. 2010). Conversion into oligosaccharides can be increased by biocatalyst engineering strategies, *i.e.* site-directed mutagenesis (Faijes and Planas, 2007; Hu et al. 2010) and immobilization (Matella et al. 2006; Pan et al. 2009), and also by engineering the reaction medium, mostly by using very high substrates concentrations (Malá et al. 1999).

Different oligosaccharides derived from lactose can be synthesized by enzymatic transgalactosylation using β-galactosidase as catalyst, as shown in Table 3. All of them are considered non-digestible oligosaccharides (NDO), having some of them functional properties as prebiotics and others as non-caloric sweeteners (Delzenne, 2003). Prebiotics have been defined as selectively fermented ingredient that allows specific changes, both in composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health (Roberfroid, 2007). The criteria accepted for an ingredient to be considered as a prebiotic are: Resistance to degradation by acids, enzymes, no absorption through the gastrointestinal passage, and selective stimulation of the growth and/or activity

of colonic bacteria associated with health and well-being. NDO are those that more closely meet all requirements to be properly considered as prebiotics. Galacto-oligosaccharides (GOS) and lactulose are lactose derived NDO now confirmed as prebiotics, while lactosucrose and tagatose comply with most of the attributes of a prebiotic being considered beneficial for bowel functioning (Wang, 2009). Synthesis by enzyme biocatalysis, its present situation and future perspective is described in detail for each of them. In all cases, there is a significant added value with respect to the raw material (whey or whey derivatives) that originates them, which represents a quite different scenario of the one shown in section Upgrading by enzymatic hydrolysis, where added value was modest and the focus was more on the adequate treatment of an otherwise contaminant effluent, envisaging a strategy of upgrading conciliating economic profitability with environmental protection (Mawson, 1994; González-Siso, 1996). In the present case, the focus is primarily on the products of synthesis which find a very favorable scenario considering the present tendency to a healthier food intake. In fact, the global market for functional foods has grown at an annual rate between 10 and 15% during the last decade. The European market was close to 10 million Euros in 2005 with a projected figure of 15 million Euros for 2015 (RTS Resources Ltd.). The market in the USA was US$ 70 million in 2007 with a projected figure of US$ 200 million for 2015 (http.//www.reuters.com/article/pressRelease). Japan was the first country to introduce NDO into healthy foods (FOSHU) and is the world leader in the production of prebiotics; their market was US$ 125 million in 2001 and the forecast was a 100% increase by the end of that decade (Nakakuki, 2002; Tanigushi, 2005).

Enzymatic synthesis of galacto-oligosacaccharides (GOS)

GOS are NDOs whose prebiotic condition is beyond any doubt, its beneficial effects being proven consistently *in-vitro* and also *in-vivo* in trials with model animals (Djouzi and Andrieux, 1997), human adults (Bouhnik et al. 1997) and infants (Moro et al. 2003), although there is some controversy about the *in-vivo* effect (Alander et al. 2001). GOS are particularly suited as a prebiotic supplement in milk and dairy products: GOS are natural components of human breast milk so their addition to infant formula (Savino et al. 2003; Bakker-Zierikzee et al. 2005; Miñana, 2007; Gosling et al. 2011) and to yogurts as a prebiotic or as symbiotic mixture with probiotics is in practice both in Japan and in Europe (Lamoureux et al. 2002; Nakakuki, 2002; Sairanen et al. 2007). Their use is quite extended in the Japanese food industry where it is included in a variety of products like bread, jams, sport drinks, confectionery and desserts (Rastall, 2006). GOS are slightly sweet (about 40% relative to sucrose) and quite stable, even at high temperatures and low pHs (Voragen, 1998). GOS are also being used in milk products for senescent persons (Macfarlane et al. 2008). Beyond their prebiotic condition, GOS are interesting functional food ingredients (Sangwan et al. 2011): They are non-cariogenic and their excellent taste, acid resistance and low sweetness make them appealing as functional sweeteners (Sako et al. 1999; Splechtna et al. 2006). We have recently reported a strong cryoprotective effect produced by GOS addition in the preservation of prebiotic *Lactobacillus* strains (Tymcszysin et al. 2011), stronger than with conventional protectants like sucrose (Golowczyk et al. 2011), which opens up perspectives for their use in the formulation of self-protected symbiotic dairy products, acting the prebiotic also as a probiotic protectant.

GOS are oligosaccharides composed by a variable number of galactose units (from one to ten, usually from two to four) and a terminal glucose unit, linked mostly by β1-4 and β1-6 bonds (Casci and Rastall, 2006), even though some polygalactose oligosaccharides not containing glucose can also be formed at low levels during synthesis (Sanz et al. 2002; Vera et al. 2011a). Disaccharides (mostly galactose-glucose) are refereed as GOS-2, trisaccharides (mostly galactose-galactose-glucose) as GOS-3, tetrasaccharides (mostly galactose-galactose-galactose-glucose) as GOS-4 and so on. Prebiotic action is mostly assigned to GOS-3 and GOS-4 (Gopal et al. 2001). Different from other lactose derived NDO, GOS are produced industrially by enzyme biocatalysis with microbial β-galactosidases (Gänzle et al. 2008), chemical synthesis having been displaced for being more complex and environmentally objectionable (Monsan and Paul, 1995; Sears and Wong, 2001). The commercial products now available are rather impure preparations, with total GOS amounting about 50% of solids and significant variations, both in the kind of linkage and molecular size distribution, according to the origin of the enzyme used in their synthesis (Rastall, 2006; Crittenden and Playne, 2009). Main GOS producers are the Dutch company Friesland Foods Domo and the Japanese companies Nissin Sugar Manufacturing Company and Yakult Honsha Company (Playne and Crittenden, 2009); there are also GOS producers in China, like Qingdao FTZ United International Inc.

GOS are produced by a kinetically controlled reaction of synthesis from lactose, catalyzed by microbial β-galactosidases, among which those from the molds *Aspergillus niger* and *Aspergillus oryzae*, the

yeasts *Kluyveromyces marxianus, Kluyveromyces fragilis* and *Kluyveromyces lactis* and the bacterium *Bacillus circulans* outstand (Husain, 2010; Park and Oh, 2010). However, β-galactosidases have been selected mostly for hydrolytic processes, being their performance rather poor for GOS synthesis with respect to conversion yield and productivity. Therefore, a considerable number of microbial strains have been evaluated as catalysts for GOS synthesis, like the thermophiles *Pyrococcus furiosus* (Hansson and Adlercreutz, 2001; Splechtna et al. 2002), *Talaromyces thermophilus* (Nakkharat and Haltrich, 2006), *Bacillus stearothermophilus* (Chen et al. 2008) and *Sulfolobus solfataricus* (Petzelbauer et al. 2002), the prebiotic bacteria *Bifidobacterium bifidum* (Depeint et al. 2008), *Bifidobacterium lomgum* (Hsu et al. 2007), *Bifidobacterium infantis* (Roy et al. 2002), *Lactobacillus reuteri* (Splechtna et al. 2006) and *Lactibacillis sakei* (Iqbal et al. 2011), the molds *Penicillium simplicissimum* (Cruz et al. 1999) and *Auerobasidium pullulans* (Chi et al. 2009), and the yeasts *Bullera singularis* (Shin et al. 1998; Cho et al. 2003) and *Sterigmatomyces elviae* (Onishi et al. 1995). Genetic engineering (Zheng et al. 2006; Park et al. 2008; Chen et al. 2009) and protein engineering strategies (Hansson et al. 2001; Jørgensen et al. 2001) have been used to improve β-galactosidase performance in the synthesis of GOS. Despite these advances, most β-galactosidase producing strains for the synthesis of GOS are those from *Aspergillus oryzae* (Iwasaki et al. 1996; Gaur et al. 2006; Panesar et al. 2006; Vera et al. 2011b), *Kluyveromyces marxianus* (var. *lactis* and var. *marxianus*) (Jurado et al. 2002; Martínez-Villaluenga et al. 2008a) and *Bacillus circulans* (Fujimoto et al. 1998; Playne and Crittenden, 2009), all of them enzymes primarily used for lactose hydrolysis. Product distribution is quite different in GOS synthesis according to the β-galactosidase source (Nakayama and Amachi, 1999): The enzyme from *Aspergillus oryzae* produces GOS-3 as the main product component, a lesser proportion of GOS-4 and quite insignificant level of GOS-2, GOS-5 and higher; the one from *Kluyveromyces marxianus* produces significant levels of GOS-2 and few GOS-5 or higher; the one from *Bacillus circulans* produces a wider spectrum with a more even distribution between GOS-2 and GOS-5 (Panesar et al. 2006). From that perspective, the enzyme from *Aspergillus oryzae* is the most attractive for the production of GOS as prebiotic (prebiotic potential is mainly associated with GOS-3 and GOS-4), being in addition thermosable, readily available at reasonable price and bearing GRAS status (Albayrak and Yang, 2002a). A thorough comparison of the differences in chain length and type of glycosidic bonds among the GOS produced by *Bacillus circulans*, *Aspergillus oryzae* and *Kluyveromyces marxianus* β-galactosidases has been reported by Boon et al. (2000a).

β-galactosidase immobilization, though initially developed for the hydrolysis of whey and its derivatives (Greenberg and Mahoney, 1981; Husain, 2010), has been intensively studied to improve GOS production (Panesar et al. 2006). The most striking results reported up to know refers to the immobilization of *Aspergillus oryzae* β-galactosidase in cotton cloth, obtaining a half-life of 48 days at 50ºC, 25 times higher than for the soluble counterpart (Albayrak and Yang, 2002a); results were later improved in terms of specific activity of the biocatalyst by aggregation of the enzyme with polyethyleneimine prior to immobilization (Albayrak and Yang, 2002b). β-galactosidases from *Aspergillus oryzae* have been also immobilized on chitosan, with a moderate increase in stability (twofold at 60ºC) and in lactose conversion (Gaur et al. 2006), but with a slight decrease in GOS yield (Sheu et al. 1998); by covalent multi-point attachments to glyoxyl agarose, a 7-fold increase being obtained with respect to the soluble counterpart at 50ºC and similar lactose conversion (Huerta et al. 2011); by covalent immobilization onto magnetic Fe_3O_4-chitosan nanoparticles, retaining more than 90% of activity after 15 production cycles (Pan et al. 2009). β-galactosidase from *Kluyveromyces marxianus* was immobilized in anionic exchange membranes to develop a continuous process: Hydrodynamic behavior was very good but conversion yields were about the same as with the non-supported enzyme (Engel et al. 2007). The enzyme from *Kluyveromyces lactis* was immobilized in a polysiloxane-polyvinyl alcohol magnetic composite obtaining a biocatalyst essentially free of diffusional restrictions and improved stability (Neri et al. 2008), which was subsequently tested in the synthesis of GOS, obtaining a kinetic behavior similar to the free enzyme (Neri et al. 2009a); the same authors immobilized the same enzyme in another magnetic support based on polysiloxane-polyaniline particles obtaining a behavior similar than the free enzyme, being the most attractive issue the easiness of biocatalyst recovery and reuse (Neri et al. 2009b). β-galactosidase containment in ultrafiltration membranes is a strategy of immobilization that allows a more efficient use of the biocatalyst, avoiding the problems associated to the immobilization in solid matrices; several studies have been reported for the synthesis of GOS in membrane reactors allowing continuous operation and increase in productivity, though not in yield (Petzelbauer et al. 2002; Chockchaisawasdee et al. 2004; Ebrahimi et al. 2010; Güleç et al. 2010; Pocedičová et al. 2010). To sum up, even though β-galactosidase immobilization has been thoroughly studied, protocols have been mostly developed with respect to their use as catalyst for the hydrolysis of lactose; therefore, some of them are inadequate to perform under the conditions required for GOS synthesis: High temperatures, very high substrate concentrations and low water activity. In addition, no significant increase in lactose to GOS conversion yield has been obtained

by immobilization, being similar or even lower than those obtained with the free enzyme, so the bonus lies exclusively in the increased stability and recoverability of the biocatalyst leading to its more efficient use. However, being β-galactosidase a commodity enzyme, this aspect is not critical for process economics, being downstream processing of the product a more critical issue, as will be analyzed below. Since immobilization does not solve the problem of product purity, synthesis with immobilized β-galactosidase does not represent for the moment an alternative to the more conventional use of the enzyme dissolved in the reaction medium. However, there is still room for biocatalyst improvement, mostly referred to thermostability and selectivity (Irazoqui et al. 2002; Gaur et al. 2006).

Synthesis of GOS with immobilized whole cells containing β-galactosidase activity has been proposed as an alternative to enzyme biocatalysis, so avoiding the costs associated with the recovery and purification of the enzyme. One potential advantage is related to product purification, since lactose, glucose and galactose are metabolized to certain extent, while GOS are not, so their relative content in the product increases; besides product inhibition is relieved (Torres et al. 2010). However, the reaction becomes less specific and yield of product is significantly reduced because lactose and their hydrolysis products are further metabolized. In addition, high temperatures are precluded limiting its applicability (Sakai et al. 2008; Osman et al. 2010). Synthesis of GOS with permeabilized cells of *Kluyveromyces marxianus* has been reported, which makes sense for intracellular β-galactosidases; however, no significant improvement in yield or operational stability has been obtained (Manera et al. 2010).

Enzymatic synthesis of GOS is a kinetically controlled reaction that has been modeled based on a mechanism that considers the formation of a galactosyl enzyme complex that can be nucleophilically attacked either by lactose to produce GOS or by water to produce glucose and galactose, including also inhibition by galactose (Boon et al. 1999). However, such mechanism considers only the production of GOS-3 which in the case of synthesis with *Aspergillus oryzae* β-galactosidase represents no more than 65% of total GOS, being therefore inadequate to describe such process. Variants of this model have been proposed aiming to a better description of the reaction system (Kim et al. 2004; Neri et al. 2009a) but they contain too many kinetic parameters that are difficult to estimate. We have constructed a simplified model that accounts for the synthesis of GOS-2, GOS-3, GOS-4 and GOS-5 containing a reduced number of kinetic parameters that can be determined by non-linear multi-response fitting to experimental data (Vera et al. 2011a).

Enzymatic synthesis of GOS has been conducted in different operation modes and reactor configurations. The usual way, and most probably the one used at industrial scale, is batch operation in stirred tank reactors with β-galactosidase dissolved in the reaction medium (Boon et al. 2000a; Martínez-Villaluenga et al. 2008a). However, synthesis of GOS with immobilized β-galactosidases has been evaluated in repeated batch operation with biocatalyst recovery (Gaur et al. 2006; Sakai et al. 2008; Huerta et al. 2011), in recycle batch reactors where the reaction medium is circulated throughout a biocatalyst module until the maximum conversion is obtained and a new cycle is initiated (Albayrak and Yang, 2002a; Albayrak and Yang, 2002b; Matella et al. 2006), in continuous operation in packed bed reactors with immobilized enzyme (Shin et al. 1998; Nakkharat and Haltrich, 2007), and in membrane reactors to alleviate enzyme inhibition by monosaccharides (Chockchaisawasdee et al. 2004; Engel et al. 2007; Splechtna et al. 2007). To the same purpose, reactors have been operated with continuous removal of inhibitory products by adsorption in activated carbon (Boon et al. 2000b) and biphasic partition in a polyethylene glycol-salt system (del-Val and Otero, 2003), obtaining an increase in lactose conversion into GOS using β-galactosidase from *Bacillus circulans* and *Kluyveromyces lactis* respectively. The latter enzyme, immobilized in ionic-exchange resins, has been also evaluated in the synthesis of GOS in organic medium in a micro-wave irradiated reactor, obtaining a significant increase in selectivity (transgalactosylation to hydrolysis ratio), although the separate effect of microwave heating and organic cosolvent was not reported (Maugard et al. 2003).

One of the key aspects in GOS production is the quality of the product. Up to date conversion of lactose into GOS rarely exceeded 40%, meaning that a substantial fraction of the product is residual lactose, glucose and some galactose, which may be a restriction according to the intended use for the product (Crittenden and Playne, 2002). Purification at large-scale can be done by adsorption into activated charcoal, but only partial removal of monosaccharides is attainable (Wang et al. 2005). Best results have been obtained with molecular exclusion chromatography, achieving high levels of purity (Hernández et al. 2009), but cost may be too high for industrial operation. Simulated bed chromatography has been developed as a high throughput operation (Schulte et al. 2000) and has been recently applied at productive scale in the synthesis of fructo-oligosaccharides (FOS) (Vaňková et al. 2008) and GOS. Separation of monosaccharides is also feasible by nanofiltration in polymeric and

ceramic membranes (Goulas et al. 2003; Martínez-Ferez et al. 2006; Feng et al. 2009; Hernández et al. 2009; Botelho-Cunha et al. 2010), but differences in molecular size between GOS-3 and di and monosaccharides are rather small, so that a high efficiency of separation is not attainable. A plausible strategy for GOS purification is the selective fermentation of the reacted mixture, taking advantage of the non-metabolizable character of GOS. A reacted mixture of GOS produced by lactose transgalactosylation with *Penicillium expansum* β-galactosidase was fermented by *Saccharomyces cerevisiae*, obtaining a 98% removal of monosaccharides, but only 9% of residual lactose; the same level of monosaccharide removal was obtained by fermentation with *Kluyveromyces lactis*, but in this case lactose was removed completely as a consequence of the very high β-galactosidase activity in *Kluyveromyces lactis* strains (Li et al. 2008). Similar results were obtained in the purification of the commercial product Vivinal-GOS™ with *Saccharomyces cerevisiae*, where almost all monosaccharides, but none of the residual lactose, were removed (Hernández et al. 2009). GOS mixtures produced by lactose transgalactosylation with β-galactosidases from *Aspergillus oryzae*, *Kluyveromyces lactis* and *Bacillus* sp. were fermented with a strain of *Kluyveromyces marxianus*, obtaining a GOS product with 95% purity (Cheng et al. 2006). We have recently used a suspension of *Kluyveromyces marxianus* cells grown in induced lactose media to purify a GOS mixture produced with *Aspergillus oryzae* β-galactosidase. Removal of monosaccharides and residual lactose was complete and cells were easily removed to yield a high purity GOS preparation (unpublished results).

A comprehensive review on the structure-function analysis of β-galactosidases with respect to the synthesis of GOS has been recently published, suggesting interesting strategies for the improvement of the existing biocatalysts for the synthesis of GOS of high prebiotic efficiency (Gosling et al. 2010).

Enzymatic synthesis of lactulose

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a non-digestible disaccharide not present in nature, although it is produced by isomerization of lactose during heat treatment of foods containing it (Marconi et al. 2003). Even though it has been used as a laxative for decades (Sahota et al. 1982; Tamura et al. 1993) it is now properly considered as a prebiotic (Rycroft et al. 2001; Mizota et al. 2002; Schumann, 2002; Bouhnik et al. 2004a), even though of slightly lower index than GOS and FOS (Bouhnik et al. 2004b; Gänzle et al. 2008). Its accepted prebiotic condition has expanded its market considerably (Olano and Corzo, 2009). Lactulose is exclusively produced by lactose isomerization with chemical catalysts, mainly calcium and sodium hydroxides, but sulfites, phosphates, borates, silicates and ion-exchange resins have also been used (Aider and de Halleux, 2007). Main lactulose producers are Solvay Pharmaceuticals and Morinaga Milk Industry (Playne and Crittenden, 2009). The mechanism of synthesis involves the formation of an enolic intermediate by a Lobry de Bruyn-van Ekenstein reaction, followed by the transformation of the glucose residue into fructose. Chemical processes have, however, several drawbacks stemming from the low specificity of the reaction leading to low yields and undesirable side products. Lactose conversion yield into lactulose hardly exceeds 30% and even though it can be increased by *in-situ* removal of product that drives the equilibrium into lactulose formation, complexing agents are inadequate for use at productive scale (Zokaee et al. 2002; Schuster-Wolff-Bühring et al. 2010). Therefore, intense purification is required, increasing operation costs (Dendene et al. 1994, Zokaee et al. 2002).

Enzymatic synthesis represents then a technological option which is interesting both from process (higher conversion, less downstream processing, less energy consumption) and environmental (less offensive waste streams to be treated) considerations that have triggered recent studies exploring biocatalytic processes for lactulose synthesis. However, studies are few so that for the moment it remains non competitive with the chemical process. Isomerization with glucose isomerase (xylose isomerase) that converts glucose into fructose appears in principle as a logical choice for the isomerization of the glucose residue in lactose. High-fructose syrup is the biggest industrial enzymatic process where glucose isomerase plays a central role (Crabb and Mitchinson, 1997; Olsen 2002), so the enzyme is produced massively and is now readily available at commodity price. Unfortunately, the enzyme cannot catalyze the isomerization of the glucose residue in lactose (Bhosale et al. 1996) and the existence of a reputedly lactose isomerase has not been reported yet. Lactulose can be produced enzymatically by the kinetically controlled transgalactosylation of lactose, using fructose as galactosyl acceptor and β-galactosidase (Mayer et al. 2004; Tang et al. 2011) or β-glycosidase (Mayer et al. 2010) as catalysts. Under appropriate conditions, β-galactosidase can catalyze the glycosyl transfer to a receptor molecule bearing hydroxyl groups different from water (*i.e.* fructose) so that synthesis instead of hydrolysis can occur (Schuster-Wolff-Bühring et al. 2010). Since lactose and fructose are present in the reaction medium, both sugars can act as acceptors producing GOS and lactulose

respectively (Kim et al. 2006a). This may represent a disadvantage in terms of product purity, but GOS, as well as lactulose, are prebiotics and mixtures of both may produce a NDO mix of high prebiotic index (Palframan et al. 2003; Sanz et al. 2005).

Enzymatic synthesis of lactulose has been reported with β-galactosidases from *Aspergillus* (Mayer et al. 2004), *Kluyveromyces* (Martínez-Villaluenga et al. 2008b), *Arthrobacter* (Tang et al. 2011) and from thermophilic organisms like *Pyrococcus furiosus* (Mayer et al. 2004) and *Sulfolobus solfataricus* (Kim et al. 2006a). In a recent comparative study with β-galactosidases from *Aspergillus oryzae*, *Kluyveromyces lactis* and *Kluyveromyces fragilis*, the effect of lactose-fructose ratio on lactose conversion into lactulose was evaluated and significant differences were observed, being the best results obtained with the enzyme from *Aspergillus oryzae*, with which a product was obtained where lactulose represented 60% of total sugars (Adamczak et al. 2009). This later enzyme is particularly well suited for lactulose synthesis, because of its high transgalactosylation activity (Huerta et al. 2011; Vera et al. 2011b), being a GRAS enzyme readily available as a commodity. Despite this, it has been reported that bacterial and yeasts β-galactosidases, both as isolated enzymes or whole cells are also good lactulose producers (Lee et al. 2004a; Kim et al. 2006a; Tang et al. 2011). Enzymatic synthesis of lactulose is a newly emerged strategy that, for the moment, is not competitive with ongoing chemical synthesis. To date, only two reports exist about process considerations (Adamczak et al. 2009; Mayer et al. 2010), so more research and development is needed. Control of product distribution in lactulose production by reaction medium engineering is an unexplored area which is important in defining the quality of lactulose-GOS mixtures. Very few information has been gathered about lactulose synthesis with immobilized β-galactosidases; there is one report with a recombinant β-galactosidase from *Pyrococcus furiosus* expressed in *Escherichia coli*, partially purified and immobilized in Eupergit™ (Mayer et al. 2004; Mayer et al. 2010). However, advances in biocatalyst engineering for GOS synthesis are in principle applicable for lactulose synthesis, so new outcomes in the near future are to be expected. New generation oligosaccharides have been recently synthesized by further transglycosylation reactions from lactulose, using pectinase and β-galactosidase commercial preparations (Rodriguez-Fernandez et al. 2011).

Other non-digestible oligosaccharides derived from lactose by enzyme conversion

Lactosucrose. It is a trisaccharide (β-D-galactopyranosyl-(1-4)-α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside) that can be synthesized by transfructosylation of lactose with sucrose using β-fructofuranosidase (Pilgrim et al. 2001) or β-galactosidase (Li et al. 2009) as catalysts, and also using levansucrase or cells containing it (Choi et al. 2004). Synthesis at high temperatures, favoring the solubility of sugars, has also been reported with thermophilic enzymes (Petzelbauer et al. 2000). Lactosucrose is a slightly sweet NDO which is not properly considered as prebiotic (Gänzle et al. 2008), even though its stimulating effect on bifidobacteria and depressing effect on clostridia is documented (Park et al. 2005). It is currently produced in Japan by an enzymatic process using β-fructofuranosidase from *Arthrobacter* sp. and sold as healthy food. Main lactosucrose producers are the Japanese companies Ensuiko Sugar Refining Company and Hayashibara Shoji Inc. from Japan (Playne and Crittenden, 2009).

Tagatose. D-tagatose is a galactose isomer that, as lactulose, is not present in nature though it is produced in small quantities during heat processing of dairy products (Mendoza et al. 2005). Initially, it was produced by chemical isomerization of galactose using calcium catalysts (Beadle et al. 1991; Beadle et al. 1992). Low specificity of the chemical reaction made necessary a complex purification process and waste treatment of offensive compounds was also a problem (Oh, 2007). D-tagatose has been also produced by microbial bioconversion of galactitol with cells of different microorganisms like *Mycobacterium smegmatis* (Izumori and Tsuzaki, 1988), *Enterobacter agglomerans* (Muniruzzaman et al. 1994), *Klebsiella pneumoniae* (Shimonishi et al. 1995) and *Gluconobacter oxydans* (Rollini and Manzoni, 2005). Likewise, chemo-enzymatic synthesis of tagatose has been proposed where galactose (coming from lactose hydrolysis) is oxidized with pyranose oxidase to galactosone, which is then chemically reduced to tagatose (Freimund et al. 1996). The chemical process has been gradually substituted by an enzyme process, more specific and environmentally sound (Bertelsen et al. 2006), based on the isomerization of galactose into tagatose with L-arabinosa isomerase (EC 5.3.1.4) (Kim et al. 2001). The enzyme catalyzes the isomerization of L-arabinose into L-ribulose, but it is also active over D-galactose owing to the similar structure of both substrates (Cheetham and Wootton, 1993; Lee et al. 2004b). Such reaction is coupled to the conventional process of lactose hydrolysis with β-galactosidase, being the glucose separated from the galactose by chromatography or by selective glucose fermentation with *Saccharomyces cerevisiae* prior to isomerization (Ibrahim and Spradley,

2000). Several arabinose isomerases have been evaluated for the synthesis of D-tagatose, like the ones produced by *Escherichia coli* (Roh et al. 2000), *Thermotoga marítima* (Lee et al. 2004b), *Geobacillus stearothermophilus* (Kim et al. 2006b) and *Lactobacillus planctarum* (Zhang et al. 2007), and recombinant enzymes produced by heterologous expression of the genes from *Thermotoga napolitana* and *Thermoanaerobacter mathranii* in *Escherichia coli* as host (Kim et al. 2002; Jørgensen et al. 2004). Immobilized arabinose isomerases have been used in the synthesis of D-tagatose to increase operational stability and allow biocatalyst recovery (Oh et al. 2001; Kim et al. 2003; Ryu et al. 2003; Zhang et al. 2009); also immobilized cells containing arabinose isomerase activity have been used (Jung et al. 2005; Hong et al. 2007). Tagatose was awarded GRAS status in 2001 and began to be marketed in 2003 as a low-calorie sweetener, with a sweetening power similar to sucrose and 70% less caloric content (Levin, 2002). Tagatose does not increase glucose blood level, being acceptable for diabetics and it does not contribute to dental plaque formation. It has also been considered as a drug for diabetes and obesity control (Lu et al. 2008) and, being not digested at the small intestine level, it is considered as a potentially prebiotic NDO (Bertelsen et al. 2001). Main tagatose producer is at present Nutrilab, a subsidiary of the Belgian company Damhert. Production is carried out by enzyme biocatalysis with an estimated output of 10,000 ton/yr (Playne and Crittenden, 2009).

Upgrading by enzymatic esterification

Sugar esters are biodegradable surfactants that find assorted applications in medical and personal care products, detergents and foods (Devulapalle et al. 2004). It has been recently proved that some of these esters can act as antimicrobial and antiviral agents and also as plant growth promoters (Chang and Shaw, 2009), which highlights their technological potential. The lipase-catalyzed enzymatic synthesis of sugar esters has been studied in the last two decades as an alternative to conventional chemical synthesis, offering the usual advantages of enzyme biocatalysis, namely high specificity and moderate reaction conditions (Ferrer et al. 2005). The low solubility of sugar esters in water and the requirement of low water activity to drive the reaction towards synthesis makes necessary the use of non-conventional reaction media (Ballesteros et al. 1995; Davis and Boyer, 2001): Organic solvents (Chamouleau et al. 2001; Reyes-Duarte et al. 2005), supercritical fluids (Habulin et al. 2008) and ionic liquids (Lee et al. 2008; Roosen et al. 2008). Lipases are structurally well conditioned to perform in such media and are the most used enzymes for sugar ester synthesis. Proteases have been used as well (Park et al. 1999; Tokiwa et al. 1999; Kennedy et al. 2006), being in this case mandatory to increase the stabilization of the enzyme to withstand the harsh conditions of reaction in non-conventional media; therefore proteases for sugar esters are usually used in immobilized form (Ferreira et al. 2002; Plou et al. 2002; Pedersen et al. 2003).

Transesterification is a complex reaction due to the different nature of the substrates involved, being the sugar polar while the esterifying agent (acyl donor) apolar. This problem has been approached by the use of solvents capable of dissolving both substrates, which is not always feasible. The use of soft solvents, like tertiary alcohols, where the sugar dissolves readily have been reported as successful; however, this strategy is only applicable to monosaccharides since oligosaccharides, such as lactose, are poorly soluble in such solvents. Another approach is the hydrophobization of the sugar by derivatization with apolar molecules, and the use of mixtures of miscible solvents in one of which the sugar is dissolved prior to reaction. The type of fatty acid moieties in the esters used as reactants vary in chain length from 4 to 18 carbon atoms and it has been reported that this is a key variable in the enzymatic transesterification reaction, conversion yields generally increasing with chain length (Plou et al. 2002).

The synthesis of lactose esters has been thoroughly studied (Drummond and Wells, 1998) and several products have been enzymatically synthesized, among which the synthesis of lactose monostearate with *Mucor miehei* lipase (Sarney et al. 1994) and lactose monolaurate with *Candida antarctica*, *Pseudomonas cepacia* and *Mucor miehei* lipases (Walsh et al. 2009) are worth mentioning The synthesis of lactose esters has been considerably more difficult to achieve than monosaccharide and sucrose esters, low conversion yields being obtained in most cases (Ku and Hang, 1995; Cao et al. 1999; Degn and Zimmermann, 2001). Likewise, low yields have been obtained in the synthesis of lactose esters when using proteases as catalysts (Park et al. 1999; Wu et al. 2004). Further developments in biocatalyst and medium engineering are still required for making the enzymatic synthesis of lactose esters commercially viable.

CONCLUDING REMARKS

Whey is a plentiful material that has been traditionally considered as a residue of cheese production. The magnitude of the dairy industry makes whey production volumes enormous. From that volume, a significant portion is underutilized or discarded, increasing the production costs as a consequence of waste management and treatment to comply with environmental regulations or, even worse, disposing it without treatment when such regulations are soft, not enforced, or inexistent. Only big companies are in the capacity of implementing technologies of efficient recovery and upgrading of whey, so that a challenge exists in incorporating medium and small size whey producers to strategies for its management that include recollection, processing and upgrading. Recovery, fractionation and transformation of whey may then acquire a significant economic value for their producers, conciliating the interest of the productive sector with the social demands of environmental protection, within the framework of sustainable development.

The potential of whey as a valuable industrial output has been analyzed with a special focus on those applications representing a high added value and a technological challenge. Among them, enzyme biocatalysis has been highlighted as a powerful technological tool for the upgrading of whey and its derivatives, with a special emphasis in the production of functional food components according to the increasing tendency to health-promoting food consumption. In this context, there are still important technological challenges with respect to conversion and productivity of the processes of synthesis that are successfully being tackled by combining strategies of biocatalyst and medium engineering. The impressive advances in biotechnology experienced in recent decades forecast a growing impact of bioprocesses in whey upgrading. We claim that an industrial platform considering the various options of recovery, fractionation and upgrading of whey will necessarily be developed driven by the imperative of sustainable development.

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How to reference this article:

ILLANES, A. (2011). Whey upgrading by enzyme biocatalysis. *Electronic Journal of Biotechnology*, vol. 14, no. 6. http://dx.doi.org/10.2225/vol14-issue6-fulltext-11