Chapter 9

PRODUCTION AND BIOACTIVITY OF OLIGOSACCHARIDES DERIVED FROM LACTOSE

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Abstract

In an attempt to offer a broad overview on lactose applications as a source of bioactive carbohydrates, in this chapter the authors have collected the most recent investigations on methods to produce these compounds, which have a positive effect on gut microbiota. Chemical and biological productions of tagatose, lactulose, lactosucrose, lactulosucrose and galactooligosaccharides, among others, have been described. Moreover, their main uses, based on their bioactivity and techno-functional properties, are also shown together with a brief description of purification methods of the mixtures obtained. Particular attention has been paid to a new generation of oligosaccharides derived from lactulose with a potential prebiotic effect, which is even better than that of this recognised functional ingredient. These novel oligosaccharides, mainly composed of 6'-galactosyl lactulose and 1-galactosyl lactulose can be obtained with good yields either by transgalactosylation of lactulose or by chemical isomerisation under basic media of galactooligosaccharides; the latter constitutes a good and economic option.

Key words: lactose derivatives, chemical and enzymatic isomerisation, transglycosylation, galactooligosaccharides, 6'-galactosyl lactulose, 1-galactosyl lactulose, lactulosucrose, properties.

9.1. Introduction

Lactose, a disaccharide formed by the condensation of the monosaccharides glucose and galactose to give **β-D-Gal-(1→4)-D-Glc**, is the major constituent in most mammalian milks. The lactose that is manufactured on an industrial scale is mainly produced from the whey obtained as a by-product of cheese making industries, which represents a major disposal problem. Proteins can be recovered by ultrafiltration, a standard operation in cheese plants. Ultrafiltration of whey generates large amounts of permeate that contains, on a dry weight basis, 80–85% lactose, 8–10% ash and some vitamins (Shay & Wegner 1986). Even though the industry has developed many uses for permeate, a huge surplus still exists (Guimarães *et al.* 2010).

Although lactose is useful in the pharmaceutical and food industries, due to its unique physicochemical properties, the demand is insufficient to absorb all of the actual whey lactose available. Thus, different industrial processes have been developed to convert lactose into value added products. Among the many industrial applications of lactose, the synthesis of its derivatives has been used for many years, lactose may be chemically or enzymatically hydrolysed or subjected to oxidation, reduction, isomerisation or esterification and compounds such as lactulose, lactitol, lactosucrose, galactooligosaccharides (GOS) and lactobionic acid are currently on the market. The enzymatic hydrolysis of lactose by the action of β-galactosidase has been used for many years to produce hydrolysed lactose that may be used as an alternative for people with lactose intolerance and it is sweeter and more soluble in water that lactose. Once the concept of prebiotics was introduced, the development of new prebiotics derived from the action of β-galactosidase on lactose, and the study of their bioactivity has currently begun to attract great interest.

9.2. Mono- and disaccharides

9.2.1. Tagatose

Tagatose is a keto-hexose isomer of galactose and although rarely found in nature, it can be formed in appreciable amounts from lactose during heat treatment of milk under sterilisation conditions. Since the concentration of tagatose increases with the severity of heat treatment, its presence in commercial milk may be an indication of the processing intensity (Troyano *et al.* 1992; 1994), especially in lactose-free milk (Rada-Mendoza *et al.* 2005; Ruiz-Matute *et al.* 2012).

9.2.1.1. Chemical isomerisation

Tagatose may be obtained from galactose using chemical methods involving isomerisation of galactose in the presence of a basic catalyst such as calcium hydroxide (Beadle *et al.* 1992) and aluminates (Ekeberg *et al.* 2002). The best source of galactose is lactose from cheese whey permeate (WP) by the hydrolysis of lactose using immobilised lactase, yielding together with galactose, glucose as an economic by-product. The current commercial production of tagatose is based on chemical isomerisation with calcium hydroxide (Beadle *et al.* 1992), which is the original method used by Spherix Inc., a Japanese company. This catalyst shifts the isomerisation equilibrium between galactose and tagatose in the direction of the latter because it forms an insoluble complex with tagatose at an elevated pH. Treatment of the complex with acid, preferably carbon dioxide, liberates tagatose by neutralising the mixture and precipitating calcium carbonate. The tagatose is further purified, crystallised from water and dried (Lu *et al.* 2008).

9.2.1.2. Enzymatic synthesis

Since the chemical process has some drawbacks, such as the formation of large amounts of chemical waste and complicated separation and purification of the product, the biological production of tagatose has been explored for many years. Izumori *et al.* (1984) developed a process for the bacterial oxidation of galactitol to D-tagatose using the strain *Arthrobacterglobiformis*ST48 isolated from soil. The yield of D-tagatose accumulated in the medium from galactitol was as high as 85%. They subsequently isolated two more bacterial strains, namely *Mycobacterium smegmatis* strain SMDU (Izumori & Tsuzaki 1988) and *Enterobacter agglomerans* strain 221e (Muniruzzaman *et al.* 1994), which are potent producers of D-tagatose from galactitol. Maximum production from these methods was 18.4 g/L tagatose from 20 g/L galactitol (92% yield). However, since the cost of raw material (galactitol) is relatively high, this process is not considered for bulk scale production (Kim 2004).

In 1993, Cheetham & Wootton reported that tagatose can be produced from galactose by *Lactobacillus gayonii*, probably due to the activity of the L-pentose isomerase (L-arabinose-ketol-isomerase, EC 5.3.1.4). This new line of research was initiated to investigate the possibilities of L-arabinose isomerase (AI) in the isomerisation of galactose to tagatose (Kim *et al.* 2002). Homologous AI has been obtained by cloning and subsequent characterisation from almost 30 different bacterial species (Jorgensen *et al.* 2004; Oh 2007; Liang *et al.* 2012). The resultant syrup of this reaction was then incubated with *Saccharomyces cerevisiae* L1 cells and the selective degradation of D-galactose was achieved. D-tagatose with purity above 95% (Liang *et al.* 2012) was obtained. By using commercial β-galactosidase and immobilised *Lactobacillus fermentum* cells producing AI, D-tagatose was successfully obtained from lactose after a two-step biotransformation; the conversion rate was 60% and

productivity from D-galactose to D-tagatose was 11.1 g/Lh in a packed-bed bioreactor (Xu *et al.* 2012).

Lim *et al.* (2007) used an AI mutant enzyme from *Geobacillus thermodenitrificants* to catalyse the isomerisation of galactose to tagatose in the presence of boric acid as a complexing agent for ketoses and, under optimum conditions; they converted 300 g/L galactose to 230 g/L tagatose. In spite of the number of studies, before the commercial production of tagatose using AI can become economically feasible, there are some technical issues to be resolved, such as enzyme yield, activity, immobilisation and shelf-life (Lu *et al.* 2008).

9.2.1.3. Uses of tagatose

D-tagatose received Generally Recognized as Safe (GRAS) status by the Food and Drug Administration (FDA) in 2001 and entered the US market as a sweetener in 2003 (Donner *et al.* 2010). It was formally approved in 2005 as a "novel food ingredient" in the European Union without any restriction on usages (Lu *et al.* 2008).

The world production of tagatose in 2010 was 3000 tons (Halliday 2010). Its main market appears to be in foods for diabetic people, since it does not increase blood glucose or insulin levels, presenting a glycaemic index of only 3 compared to 100 for glucose (Donner *et al.* 1999). In a subsequent 14-month trial, its potential for treating type 2 diabetes was confirmed and, moreover, the intake of tagatose induced weight loss and raised high-density lipoprotein cholesterol, both important for controlling diabetes (Donner *et al.* 2010).

Furthermore, tagatose has an emerging interest due to the fact that its sweetness is 90% compared to sucrose over a wide range of concentrations (Fujimaru *et al.* 2012). Moreover, it has a sucrose-like taste with no cooling effect or aftertaste, so that it can be

used in a wide variety of foods and dietary supplements. Recently, the experts of the European Food Safety Authority (EFSA) (2010) have recognised tagatose as a sugar replacer, which induces lower blood glucose rise as compared to sugar-containing foods/drinks. Moreover, its caloric value (1.5 kcal/g) is only 38% that of sucrose since it is poorly absorbed in the small intestine (approximately 25%) and the remaining is fermented by the microbiota of the colon favouring growth of lactic acid bacteria and production of short chain fatty acids (SCFA), especially butyrate (Bertelsen *et al.* 1999; Lærke & Jensen 1999; Lærke *et al.* 2000; Topping & Clifton 2001). The SCFA are absorbed almost completely and metabolised. During fermentation, there is relatively low energy recovery, because of an increase of biomass excretion (Bertelsen*et al.* 2001). For this reason, it may also be useful in weight loss diets to help overcome widespread obesity in Western human populations.

In biscuits, tagatose appears to be suitable as a partial replacer (50%) for sucrose on the basis of their similar scores (Taylor *et al.* 2008); these bakery products were indistinguishable when 6% of sucrose was replaced (Armstrong *et al.* 2009). Bell & Luecke (2012) showed that tagatose can be formulated into beverages such as milk and lemonade with minimal degradation.

D-Tagatose can be also used as an additive in detergent, cosmetics, and pharmaceutical formulations. Other characteristics are that it is non-cariogenic and non-plaque forming (Oh 2007); in this respect, tagatose contributes to the maintenance of tooth mineralisation (EFSA 2010).

A consideration that should be taken into account is the fact that the powder formulation is susceptible to chemical and physical deterioration during storage. To prevent this, bulk tagatose powder should be stored in cool and dry environments (Grant & Bell 2012).

9.2.2. Lactulose

Lactulose (β-D-Gal-(1→4)-D-Fru) is a synthetic sugar that does not occur naturally and is produced from isomerisation of lactose by chemical or enzymatic reactions (Table 9.1) and by transgalactosylation of lactose using mainly β-galactosydases (Table 9.2).

9.2.2.1. Isomerisation of lactose

Lactulose is produced from lactose by isomerisation in basic media using different types of catalysts. The preparation of lactulose was first described by Montgomery & Hudson (1930). Isomerisation occurs via the Lobry de Bruyn-Alberda van Ekenstein rearrangement of aldoses to ketoses in alkaline solutions at 35 ºC for 36 h using a pH from neutral to basic (pH 14). The glucose moiety of lactose is isomerised to fructose leading to the formation of lactulose. This reaction is followed by the rapid degradation of lactulose to α- and β-isosaccharinic acids and galactose; the latter is gradually degraded to acidic products and coloured products difficult to separate. Also, lactose isomerisation produces epilactose (Figure 9.1). Industrial production of lactulose is exclusively performed by chemical isomerisation of lactose using WP (see Chapter 26 for more details on the industrial product).

An important number of studies on the synthesis of lactulose have been conducted since the first reported preparation of lactulose. In most of them, the method is based on that of Montgomery & Hudson (1930), the only difference being the replacement of calcium hydroxide by other basic catalysts (Table 9.1). Initially, simple bases such as sodium and calcium hydroxide were used. Other compounds have been employed such as complexation reagents (aluminates and borates); zeolites, sepiolites,

egg and oyster shell (Mendez & Olano 1979; Montilla *el al.* 2005a; Aider & Halleux 2007; Olano & Corzo 2009; Corzo-Martínez *et al.* 2013; Seki & Saito 2012). Aluminates and borates shift the pseudo-equilibrium established during base-catalysed isomerisation in favour of the ketose and prevent degradative side-reactions. The use of these reagents permits the formation of lactulose from lactose with yields as high as 70-80% (Hicks & Parrish 1980; Zokaee *et al.* 2002). However, these catalysts are removed from the final product with great difficulty. Methods to remove these chemicals, including chromatographic purification systems (Kozempel *et al.* 1995) and nanofiltration (Zhang *et al.* 2011) have been successfully applied.

In order to develop feasible methods to produce lactulose for human consumption in a safe and effective way, Kulkarni *et al.* (2012) have described the isomerisation of lactose to lactulose using deep sea water under sub-critical conditions. Moreover, in this study, isomerisation of other disaccharides such as maltose, cellobiose, gentibiose and isomaltose to the corresponding keto-disaccharides has also been studied. This methodology presents several advantages regarding the above-mentioned methods to isomerise lactose, such as the use of water as a benign solvent for organic chemical reactions and the presence of many inorganic ions such as sodium, calcium, potassium, magnesium, borate, etc. in deep sea water, resulting in a pH of 7.5-8.2. Deep sea water is a clean, safe (for food processing) and economical solvent, which fulfils the basic principles of green chemistry. These authors proposed this methodology as an alternative to industrial production of lactulose since it presents interesting economic and environmental perspectives. Despite all of these advantages, further research should be carried out to improve the low yield of lactulose found (30-32% at the optimum reactions conditions, 180 ºC and 5 minutes).

Recently, Aider & Gimenez-Vidal (2012) have proposed electro-isomerisation as a novel methodology to isomerise lactose to lactulose via electro-activation of a lactose aqueous solution. Under the effect of an applied external electric field to an electro-activation reactor, oxido-reduction reactions take place at the anode and cathode, which lead to abrupt changes of the pH of the solution. An acidification and basification process occurs on the anode side and the cathode interface, respectively. The hydroxyl ions produced at the cathode interface are able to create alkaline conditions and can act as proton acceptors in the isomerisation reaction of lactose into lactulose. Furthermore, the effect of different process parameters on the reaction yield, product purity and process efficiency were studied. The most significant operation parameter was the electric field intensity. A 25% lactose isomerisation into lactulose, after 60 min at room temperature, was achieved, whereas the same result is generally obtained at higher temperatures (>70 ºC) with the use of a strong alkali as pH modulator. The isomerisation yield was higher in lactose solutions compared to milk whey permeate with similar lactose concentration and no formation of epilactose was detected. End product purity, excluding lactose, was 96.3%.

Lactulose can also be formed by isomerisation of lactose during heat treatment of milk. This disaccharide is absent in raw milk; however, the dissolved salt system of milk consisting mainly of chlorides, phosphates, citrates, carbonates and bicarbonates of potassium, sodium, calcium and magnesium is a buffered solvent that favours the formation of lactulose from lactose during processing of milk. In 1958 Adachi was the first investigator to detect lactulose in heated milk. Currently the amount of lactulose present in heat-processed milk samples is used as a thermal index to assess the severity of heat treatment undergone by milk (IDF 1993; Montilla *et al.* 2005b; Olano & Corzo 2009).

Obtainment of lactulose via isomerisation of lactose using enzymes can also be feasible, although there is little literature about this subject. The reaction has been proposed as a two-step mechanism employing selective oxidases and reductases. In the first step, a reaction of the hydroxyl group at the C2 position on the glucose-moiety of lactose is oxidised thereby yielding 2-keto-lactose, and the second reaction consists of the reduction of the aldehyde group from glucose, thus converting it to a fructose residue (Schuster-Wolff-Bühring *et al.* 2010).

Regarding the type of enzymes used to produce lactulose through enzymatic isomerisation of lactose, Wang *et al.* (2011) obtained a recombinant glucose isomerase from *Actinoplanesmissouriensis*, which could be used in industrial production of lactulose as a potential economical tool. In addition, Kim & Oh (2012) have reported enzymatic synthesis of lactulose with the single substrate lactose using a thermostable cellobiose 2-epimerase (CE, EC 5.1.3.11) from *Caldicellulosiruptor saccharolyticus*, achieving a yield of a 74% for lactulose and epilactose. The use of thermostable enzymes has some advantages because the substrate solubility and enzyme reaction velocity are increased at high temperatures. Because of the yield, concentration and productivity achieved using this enzyme, these authors proposed this methodology for the industrial production of lactulose from lactose via an enzymatic process. Subsequently, Kim *et al.* (2012b) proposed an improvement of this enzymatic method using borate to increase production of lactulose. Under optimum conditions, the enzyme produced a conversion yield of 88% (w/w) obtaining the best yields of lactulose by comparison with other biological and chemical methods. This method is simpler and more eco-friendly than chemical synthesis under strong alkaline conditions; however, although 99% of borate is eliminated, further research must be carried out to achieve complete borate elimination.

9.2.2.2. Transgalactosylation of lactose

Enzymatic synthesis of lactulose via transgalactosylation of lactose has emerged as an alternative methodology for overcoming the disadvantages of lactulose production via chemical isomerisation. During enzymatic hydrolysis of lactose, using β-galactosidases (EC.3.2.1.23), glucose and galactose are released. However, production of lactulose requires fructose as a co-substrate, which exhibits a low conversion yield (Kim *et al.* 2006). When enzymatic hydrolysis of lactose, using β-galactosidases, is conducted in the presence of fructose, the galactosyl moiety of lactose can be *O*-glycosidically linked with the C4-atom of fructose, resulting in the formation of the corresponding disaccharide lactulose (Schuster-Wolff-Bühring *et al.* 2010). Moreover, transgalactosylation gives rise to the formation of GOS as a kind of side reaction when the galactosyl acceptor is lactose (Torres *et al.* 2010). **Figure 9.2** shows an outline of the transglycosylation pathway of β-galactosidase using lactose and fructose as substrates.

In the last few years, an important number of studies have been carried out in order to optimise enzymatic synthesis conditions (pH and temperature) and lactulose yields. **Table 9.2** shows the substrates and β-galactosidases used from different sources, either in the form of whole cells from *Kluyveromyces lactis* or purified enzymes from *Aspergillus oryzae, Kluyveromyces fragilis, K. lactis*; *Bacillus circulans* or *Sulfolobus solfataricus* or β-glycosidases *(Pyrococcusfuriosus).*

Lee *et al.* (2004) were the first investigators who reported the enzymatic synthesis of lactulose by a bioconversion process using lactose and fructose and permeabilised yeast cells. The enzyme from *K. lactis* exhibited the highest lactulose productivity. It can be concluded from other studies that the most promising enzymes

for lactulose production were *K. lactis, P. furiosus,* and *S. solfataricus,* being 60, 75 and 80 ºC, respectively, the optimal temperatures. Hua *et al.* (2010) proposed an improvement of lactulose yield using a dual enzymatic system (β-galactosidase from *K. lactis* and glucose isomerase) in an organic-aqueous two phase media.

Furthermore, the use of high temperatures and a high concentration of reducing sugars (lactose or fructose) are the best conditions for obtaining high lactulose yields; however, it can promote enzymatic browning. Tang *et al.* (2011) proposed a new β-galactosidase from *Arthrobacter sp.* the optimum temperature of which for hydrolysing lactose was 20 ºC thereby reducing the non-enzymatic browning in biotransformations.

Recently, Hua *et al.* (2013) have studied transgalactosylation of lactose using a commercial β-galactosidase from *K. lactis* (Maxilact) with different fructose concentrations (25-125 g/L) and constant lactose concentration (250 g/L). They detected three transgalactosylation products, lactulose, a novel transgalactosylation product 1-lactulose (β-D-Gal-(1→1)-β-D-Fru; **Figure 9.2**) and an unidentified disaccharide. However, the results of this study showed that the yield ratio of 1-lactulose: lactulose was 3:1 under various reaction conditions. The maximum yield of 1-lactulose and lactulose was approximately 22 and 8 g/L, respectively, when fructose concentration was 100 g/L. Moreover, hydrolysis of lactose was retarded by an increased fructose concentration.

9.2.2.3. Uses of lactulose

Over the past few years the use of non-digestible oligosaccharides as prebiotic ingredients has considerably increased due to the growing interest by consumers in functional foods. In this sense, lactulose offers excellent and scientifically tested functional properties and applications for the development of new functional foods.

Lactulose is resistant to hydrolysis by human small intestinal glycosidases, so that it reaches the colon unaltered where it is selectively metabolised by bifidobacteria and lactobacilli giving rise to the formation of carbon dioxide, hydrogen and short chain fatty acids (SCFA), and, consequently, an increase of faecal biomass and a decreased pH. This acidification favours the conversion of NH_3 to non-absorbable NH_4^+, which is excreted (Tamura *et al.* 1993). These properties are the key to its usefulness not only in nutrition but also for pharmaceutical uses for the treatment of chronic portal systemic encephalopathy (Sharma *et al.* 2008; Schuster-Wolff-Bühring *et al.* 2010) and chronic constipation (Guest *et al.* 2008; Schuster-Wolff-Bühring *et al.* 2010; Panesar & Kumari 2011).

The capacity of lactulose to stimulate bifidobacteria present in the gastrointestinal tract has been well known since the mid-1950s thanks to the work of Petuely (1957) who was the first to use lactulose as an ingredient in infant formulae. Later, Ruttloff *et al.* (1967a, b, c) reported an extensive study on different aspects of the effect of lactulose on faecal flora due to its low absorption in the upper intestinal tract and subsequent assimilation in the lower intestine. Since then, a considerable number of studies on the physiological effects of lactulose as a food ingredient have confirmed that the addition of lactulose to foods (infant formula, yogurt, soymilk) can provide health benefits to the consumer (Mendez & Olano 1979; Schumann 2002, Olano & Corzo 2009; Schuster-Wolff-Bühring *et al.* 2010; Panesar & Kumari 2011) (see chapter 25 for more information on application of lactulose as a food ingredient). In infant formula, lactulose has been added as a bifidus factor and it has been demonstrated that the addition of 0.5% of lactulose is enough to stimulate bifidobacteria flora in non-breast-fed babies; however, the use of 1% may produce a slight laxative effect (Nagendra *et al.* 1995a, b). Studies performed with healthy adults have shown that lactulose is an

effective food-grade prebiotic showing that consumption of 10 g per day of lactulose resulted in a statistically significant increase in the number of bifidobacteria whereas the clostridia population decreased (Tuohy *et al.* 2002). Lactulose can also be used to stimulate calcium and magnesium absorption; thus, a study conducted with healthy adult male volunteers showed an enhancement of absorption of these minerals with intake of foods containing 4 g of lactulose (Seki *et al.* 2007). However, Schuster-Wolff-Bühring *et al.* (2010) indicated that the use of lactulose as a prebiotic nutrient must be restricted to low doses because higher intake would probably cause frequent bowel movements or diarrhoea. Seki & Saito (2012) recommend carrying out more studies using low doses of lactulose as a food ingredient.

Although lactulose is used as a food ingredient, it is important to consider its current regulatory status. In Japan, the Ministry of Health and Welfare has recognised the nutritional benefits of lactulose and it has been catalogued with the official label of FOSHU (Food of Specified Health Use) (Schuster-Wolff-Bühring *et al.* 2010). A panel of experts of the EFSA (2010) have issued a scientific opinion in relation to health claims presented for lactulose, which concludes that a cause-effect relationship between the consumption of lactulose and decreasing of potentially pathogenic gastro-intestinal microorganisms cannot be established. However, a favourable report has been issued establishing a cause-effect relationship between the consumption of lactulose and a reduction in intestinal transit time and experts recommended an intake of at least 10 g in a single serving to obtain the claimed effect.

9.2.3. Epilactose

As shown above, (Figure 9.1), in addition to lactulose, another isomer of lactose, epilactose (β-D-Gal-(1→4)-β-D-Man), is formed in very small amounts during base-

catalysed isomerisation of lactose. However, epilactose can be obtained in considerable amounts by enzymatic epimerisation of lactose with cellobiose 2-epimerase (CE) from different microorganisms such as *Ruminococcus albus* (Ito *et al.* 2008), *Eubacterium cellulosolvens* (Taguchi *et al.* 2008), *Bacteroides fragilis* (Senoura *et al.* 2009), *Rhodothermus marinus* (Ojima *et al.* 2011), *Dictyoglomus turgidum* (Kim *et al.* 2012a) and *Caldicellulosiruptor saccharolyticus* (Kim & Oh 2012). CE catalyses a hydroxyl stereoisomerism at the C-2 position of the glucose moiety of lactose and epilactose is generated. Using CE from *R. albus,* Saburi *et al.* (2010) developed a 5-step process of epilactose obtainment from lactose: epimerisation of lactose, remaining lactose recovery by crystallisation, enzymatic hydrolysis of lactose, removing monosaccharides with yeast and purification with column chromatography. Epilactose thus obtained was 91.1% pure and an 11.3% yield was obtained from the initial lactose.

Epilactose is promising for use as a prebiotic because its resistance to intestinal enzymes. Bifidogenic effects have been demonstrated *in vivo* (Watanabe *et al.* 2008) and *in vitro* assays (Ito *et al.* 2008). Moreover, in rats, epilactose increased mineral absorption (calcium, magnesium and iron), weight of the caecal wall and SCFA levels (Nishimukai *et al.* 2008, Suzuki *et al.* 2010). Furthermore, this carbohydrate inhibited the conversion of primary to secondary bile acids, which have been described as promoters of colon cancer development (Watanabe *et al.* 2008).

9.3. Lactosucrose

Lactosucrose (β-D-Gal-(1→4)-α-D-Glc-(1→2)-β-D-Fru) is a trisaccharide that may be produced from lactose and sucrose by enzymatic transglycosylation.

9.3.1. Enzymatic transfructosylation of lactose

Lactosucrose production by levansucrase (EC 2.4.1.10) from *Aerobacter levanicum* was initially reported by Avigad (1957) using lactose as an acceptor and sucrose as a fructosyl donor. The fructosyl residue is transferred from sucrose to the C-l position of the glucose moiety in the lactose, producing a non-reducing oligosaccharide. In the lactosucrose synthesis, levansucrase from *Bacillus* sp. or whole cells from *Paenibacillus polymyxa* (Choi *et al.* 2004) and *Sterigmatomyces elviae* (Lee *et al.* 2007) have also been used. Important factors in this reaction were fructosyl donor and acceptor molar ratio, enzyme origin, pH, temperature and time of reaction. By selecting an enzyme from *Bacillus subtilis,* lactosucrose production was optimal at pH 6.0 and 55 ºC, where up to 181 g/L of lactosucrose was produced from 225 g/L of lactose and sucrose (ratio 1:1 w/w) in 10 h (Park *et al.* 2005). A mixed enzyme system from *Zymomonas mobilis,* including glucose oxydase and catalase to glucose removal increased the yield from 29% to 43% (Han *et al.* 2009).

Although levansucrase has been widely studied, lactosucrose is industrially produced by means of another fructosyltransferase (EC 3.2.1.26) derived from *Arthrobacter* (Fujita *et al.* 1990, Kawase *et al.* 2001; Pilgrim *et al.* 2001; Fujita 2004; Ruan *et al.* 2012).

9.3.2. Enzymatic transgalactosylation of sucrose

Lactosucrose is also formed by enzymatic hydrolysis of lactose in the presence of sucrose catalysed by the enzyme β-galactosidase (EC 3.2.1.23). When the enzymatic hydrolysis of lactose with β-galactosidase from *B. circulans* is conducted in the presence of sucrose, the released galactosyl moiety can be glycosidically linked to the carbon 4 of the glucose moiety of sucrose, resulting in the formation of lactosucrose

(Farkas *et al.* 2003). In this reaction the ratio of galactosyl-donor to galactosyl-acceptor presents an additional parameter for optimisation (Ganzle 2012). A high yield is theoretically obtained if donor and acceptor are present at a molar ratio of 1:1 to 1:2 (Li *et al.* 2009). High donor (lactose) concentrations favour formation of GOS as by-products, whereas a large excess of the acceptor (sucrose) results in untransformed substrate. However, the yield was lower than that obtained during transfructosylation of lactose, reaching up to 10% of lactosucrose. In this enzymatic reaction, another oligosaccharide analogue to lactosucrose with linkage β-(1→3) was also formed (Li *et al.* 2009).

9.3.3. Uses of lactosucrose

Lactosucrose is a trisaccharide more soluble in water than lactose (up to 3670 g/L at 25 ºC); its sweetness relative to sucrose is 0.3; it is stable for 1 h at pH 4.5 and 120 ºC in powder form, it has high moisture-retaining capacity and is highly hygroscopic (Playne & Crittenden 2009). All these properties make lactosucrose a valuable food ingredient. In Japan, lactosucrose was approved in 2005 as a FOSHU ingredient (a food to modify gastro-intestinal conditions) and it is used as a prebiotic. In spite of rather extensive efforts to purify the reaction mixture, lactosucrose is not sold as a pure compound. A Japanese patent allows obtainment of a product in powder form with high lactosucrose content and a purity of 45-70% w/w (Hara *et al.* 1992). The general characteristics related to health benefits (non-digestibility, non-cariogenicity) are responsible for its usefulness as a functional food ingredient. It is used as a sweetener in a range of beverages, confectionaries, desserts, sweets, bakery products and yoghurts (Playne & Crittenden 2009).

As other non-digestible carbohydrates, lactosucrose is gaining acceptance as a prebiotic, although the evidence is less well established than that for lactulose, GOS and fructooligosaccharides (FOS) (Gibson *et al.* 2004). There is some evidence of prebiotic activity by lactosucrose from human trials (Fujita *et al.* 1991; Kumemura *et al.* 1992; Yoneyama *et al.* 1992; Hara *et al.* 1994; Ohkusa *et al.* 1995). The hydrolysis during passage through the gastrointestinal tract has been determined in *in vitro* model systems of the stomach and small intestine of humans and the tests have shown that lactosucrose, along with FOS, are hydrolysed to a small extent (Playne & Crittenden 2009). The bifidogenic effect of lactosucrose has been recognised since it is selectively fermented by bifidobacteria in the human colon (Kumemura *et al.* 1992; Modler 1994; Ohkusa *et al.* 1995). It is readily metabolised, which in turn produces organic acids and reduces intestinal pH (Modler 1994). The minimum bifidogenic effective dose of lactosucrose is 5 g per day for an adult (Playne & Crittenden 2009). When it is taken in large amounts, lactosucrose may cause a rise in gastrointestinal osmotic pressure and induce diarrhoea.

The possible beneficially modifying faecal flora in patients with chronic inflammatory bowel disease is another effect reported in humans (Teramoto *et al.* 1996). Lactosucrose consumption also inhibits growth of colonic clostridia (Ogata *et al.* 1993). In women, Teramoto *et al.* (2006) determined that lactosucrose intake (6 g twice daily) decreased faecal pH, ammonia, and putrefactive compounds, and faecal SCFA concentration significantly increased during the administration period.

In animal trials, lactosucrose intake has shown different effects. The assays carried out with rats suggested that lactosucrose has some protective effects on indomethacin-induced enteropathy and that this protective effect is, in part, due to the maintenance of intestinal microbiota (Honda *et al.* 1999). The long-term consumption

of a diet containing 5% lactosucrose for 8 weeks significantly decreased the weight of abdominal adipose tissue when compared to that of the control group (Mizote *et al.* 2009). Lactosucrose also enhanced calcium absorption in rats (Kishino *et al.* 2006).

Research has also been oriented to use of lactosucrose as a pet food, particularly for reducing faecal odour and improving bowel consistency. There is increasing evidence that nitrogen excretion is shifted from urea in urine to faeces when carbohydrates as lactosucrose are included in the diet of monogastric animals (broiler-chickens, dogs and cats) thereby decreasing toxin levels and faecal odour. In addition, a statistically significant desirable change to the gut flora, increasing in bifidobacteria and decreasing in clostridia levels, was also observed (Terada *et al.* 1993; 1994; Hussein *et al.* 1999; Nahm 2003).

9.4. Galactooligosaccharides

9.4.1. Enzymatic synthesis from lactose

GOS are oligosaccharides resembling those present in human milk and colostrum and are usually synthesised by transgalactosylation during the hydrolysis of lactose by the enzymatic activity of β-galactosidase. This results in the formation of heterogeneous mixtures of carbohydrates with variable chain length and linkages: mainly chains of 2–8 units of galactose linked by β–(1→4), β–(1→6) and β–(1→3) bonds and a terminal glucose residue (Sako *et al.* 1999; Akiyama *et al.* 2001; Matella *et al.* 2006; Hsu *et al.* 2007). Although the production of GOS during enzymatic hydrolysis of lactose has been known for more than 50 years (Aronson 1952), it has gained renewed interest in recent years due to the recognition of GOS as physiologically functional food ingredients that promote growth of bifidobacteria in the colon;

moreover, a wide variety of health benefits has been related with this effect (Mahoney 1998). See Chapter 26 for industrial applications of this product.

Transgalactosylation is the transfer of the galactosyl moiety, after the cleavage of the β–(1→4) bond of lactose, to an acceptor molecule containing a hydroxyl group. When the acceptor is water free galactose is formed, whereas if the acceptor is a sugar, GOS results (**Figure 9.3**). During this reaction, there is a group acting as a general acid that donates a proton to the glycosidic oxygen and another negatively charged group that stabilises a positively charged carbonium galactosyl intermediate. Finally, there is an enzymatic transfer to a nucleophilic acceptor, which can be all the sugars present in the reaction mix (Mahoney 1998). Therefore, transgalactosylation is enhanced at high lactose concentration and low water content. Thus, initial lactose concentration, independently of the enzyme source, is the most important factor. Concentrations higher than 30% (w/v) are required to favour synthesis over hydrolysis (Torres *et al.* 2010). In addition, transgalactosylation can be highly affected by the source of β-galactosidase and the process conditions; temperature, reaction time, pH and enzyme to substrate ratio are the most influential factors (Boon *et al.* 2000; Martínez-Villaluenga *et al.* 2008a).

In general, the enzymatic reaction can be carried out either with the enzyme in soluble form or immobilised onto some carrier. Panesar *et al.* (2006; 2010), Guidini *et al.* (2010) and Huerta *et al.* (2011) are some examples of application of immobilisation to synthesis of GOS. Some of the benefits of using enzyme immobilisation are: improved enzyme stability, easier separation of end products and reutilisation and continuous operation. However, this procedure has the drawback of the partial loss of enzyme activity; although Lu *et al.* (2012), in a study on immobilisation of β-galactosidase from *Lactobacillus bulgaricus* on cellulose, pointed out that the enzyme retains over 85% activity after twenty batches with GOS yields higher than 40%. In

many cases, the usefulness of immobilised enzymes is commercially limited due to complicated purification procedures of the enzyme together with the fact that sometimes toxic organic compounds are used.

Regarding the source of enzyme, β-galactosidases originating from lactic acid bacteria and bifidobacteria are also of valuable interest for production of GOS with better selectivity for the growth and metabolic activity of these two bacteria genera in the gut, which may lead to an improved prebiotic effect (Rabiu *et al.* 2001; Depeint *et al.* 2008). In this regard, there are several recent works, which address the capability of enzymes derived from these microorganisms for the transgalactosylation reaction (Nguyen *et al.* 2012; Osman *et al.* 2012; Sriphannam *et al.* 2012).

Torres *et al.* (2010) have compiled an exhaustive list of fungal and bacterial sources of glucoside hydrolase enzymes, optimum reaction conditions, and yields for these enzymes. These authors stated that to produce high-GOS-content mixtures, glycoside hydrolases should not only have good ability to catalyse the transgalactosylation reaction relative to hydrolysis, but also have less affinity for the GOS formed than for lactose. Enzymes from species belonging to *Kluyveromyces*, *Aspergillus*, *Bacillus*, *Streptococcus* and *Criptococcus* genera have been used for the synthesis of GOS from lactose and have shown different optimal reaction conditions. Other genera such as *Bullera*, *Sporobolomyces*, *Sulfolobus* and *Thermus* have also been tested (Tzortzis & Vulevic 2009; Otieno 2010; Park & Oh 2010).

Among the different species studied, the β-galactosidase from *A. oryzae*, stands out because of its high specific activity, high thermal stability and low cost. Vera *et al.* (2012) have studied the effect of enzyme to substrate ratio, very high lactose concentration (over 40%, w/w) and temperature on the yield and specific productivity of the synthesis of GOS in a batch with β-galactosidase from *A. oryzae*. The best results in

terms of maximum yield of GOS (290 g/kg lactose) were obtained at 50% (w/w) initial lactose concentration and 47.5 ºC, whereas for specific productivity (0.38 g/h mg of enzyme) they were 40% (w/w) initial lactose concentration and 55 ºC. These results show the complex interplay between temperature and initial lactose concentration on the synthesis reaction.

The effect of temperature (20 to 50 °C) on the enzymatic synthesis of GOS was studied by Boon *et al.* (2000) using β-galactosidases from *B. circulans*, *A. oryzae*, *K. lactis*, and *K. fragilis*. These authors showed that slightly higher oligosaccharide yields were found at higher temperatures but the influence of the initial lactose concentration was much larger. The higher yield at higher temperatures is an additional advantage when operating at high initial lactose concentrations. Clear differences between the β-galactosidases were found concerning amount, degree of polymerisation (DP), and type of oligosaccharides produced. The β-galactosidase from *B. circulans* produced the most abundant amount, the most varied and largest-sized oligosaccharides. The β-galactosidases from *Kluyveromyces* sp. mainly produced trisaccharides.

Chockchaisawasdee *et al.* (2005) found that a transgalactosylation reaction catalysed by β-galactosidase from *K.lactis* of Lactozym 3000 L HP G, a commercial enzymatic preparation widely used to produce lactose-free milk products, mainly gave rise to GOS with linkages β-(1→6). According to Rowland & Tanaka (1993) and Dumortier *et al.* (1994), this type of linkages are quickly cleaved by β-galactosidase from bifidobacteria and, therefore, GOS produced with this enzymatic preparation could exhibit a high prebiotic potential. Mahoney (1998) stated that, with this enzyme, β-(1→6) linkage is preferred for oligosaccharide synthesis during the enzymatic hydrolysis of lactose, followed by β-(1→3) and β-(1→2).

Recently, Martínez-Villaluenga *et al.* (2008a), in a study on the optimisation of GOS synthesis using Lactozym 3000 L HP G, pointed out the different effect of conditions on the formation of di- and trisaccharides. Thus, 50 ºC, pH 6.5 and 300 min were the best conditions to obtain two disaccharides with β-(1→6) linkages, 6-galactobiose and allolactose and the preferred reaction conditions for the production of the trisaccharide 6'-galactosyl lactose (β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Glc) were 40 ºC, pH 7.5 and 120 min. In both cases, 3 U/mL of enzyme and 250 mg/mL of lactose were used. This trisaccharide had also been previously identified in reaction mixtures obtained with β-galactosidases from *Aspergillus aculeatus* (Pectinex-Ultra SP-L) (Del Val *et al.* 2001). Cardelle-Cobas *et al.* (2008a) found that although this commercial enzymatic preparation presents a high specificity for the formation of oligosaccharides with β-(1→6) linkages, GOS with β-(1→3) linkages are also formed in minor amounts. At pH 4.5, disaccharides and, particularly 6-galactobiose, were the main products formed, whereas pH values of 6.5 promoted the formation of 6'-galactosyl lactose.

Mozaffar *et al.* (1984; 1985; 1986), Yanahira *et al.* (1995), and Cheng *et al.* (2006) also investigated the formation of GOS by using β-galactosidase from *B. circulans*. In this case, the β-(1→4) linkage is favoured over others. Very recently, this enzyme has been also assayed by Rodríguez-Colinas *et al.* (2012) for production of GOS. The major transgalactosylation products were the trisaccharide β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc and the tetrasaccharide β-D-Gal-(1→4)-β-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc. The maximum GOS yield (49.4% w/w of total carbohydrates) was found using an enzyme concentration of 15 U/mL and 400 g/L of lactose. When using skim milk (with a lactose concentration of 46 g/L), although the enzyme also exhibited transgalactosylation activity, as expected, the yield was much lower (7.1 g/L).

One of the most promising methods for GOS production is the utilisation of WP, an inexpensive by-product from cheese production, comprising mainly lactose and salts, which can play a certain role during transgalactosylation. Several authors have investigated this topic by using different β-galactosidases (Rustom *et al.* 1998; Goulas *et al.* 2007; Adamczak *et al.* 2009).

Moreover, a very interesting approach to enzymatic synthesis of GOS is the utilisation of recombinant enzymes as very recently reported by Wang *et al.* (2012). These authors used a recombinant glycoside hydrolase (BgaP412) for the enzymatic synthesis of GOS in organic-buffer media, obtaining a maximum GOS yield of 46.6% (w/w) with 75.4% lactose conversion under the optimum reaction conditions (30% (w/v) initial lactose concentration, 50 ºC, pH 7.0, 8 h). According to these authors, the thermodynamic equilibrium can be shifted to the synthetic direction by reversing the normal hydrolysis. In this study, a total quantitation of GOS was carried out by thin layer chromatography (TLC) and no individual characterisation of the formed species was done. In contrast, most of the aforementioned papers on the formation of GOS were carried out by advanced analytical techniques such as high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), among others, which can allow a precise quantitation of individual oligosaccharides formed. The reader will find more information on LC analysis of bioactive oligosaccharides in chapter 19.

9.4.2. Enzymatic synthesis from lactulose

As indicated in Section 9.4.1., the formation of GOS during enzymatic hydrolysis of lactose has been extensively studied in a number of papers. This fact demonstrates the huge interest sparked by the synthesis of compounds that can have a

certain effect on the gut. There is a growing recognition that events taking place in the intestine are influenced by microbiota, which has major consequences for human health. Moreover, it is well known that the chemical structure of formed oligosaccharides may affect the fermentation properties of probiotic microorganisms. Thus, over the last few years, the search for new prebiotic carbohydrates with improved functionality has received increasing interest. One of the strategies is the utilisation of lactulose as substrate for the transgalactosylation reaction, which opens new opportunities for the preparation of new complex prebiotic structures. As is known, lactulose is an excellent prebiotic but its main disadvantage is related to the fact that is consumed by bacteria of the proximal colon giving rise to gas production during its fermentation (Schuster-Wolff-Bühring *et al.* 2010). Therefore, it is reasonable to suppose that oligosaccharides derived from lactulose (OsLu) might be fermented more slowly as they reach the distal portions of the intestine. On the basis of this hypothesis, our research group demonstrated, for the first time, the capability of β-galactosidases from *K. lactis* (Lactozym 3000 L HP G) (Martínez-Villaluenga *et al.* 2008b) and *A. aculeatus* (Pectinex Ultra SP-L) (Cardelle-Cobas *et al.* 2008b) to form OsLu during the hydrolysis of lactulose. Thus, two novel trisaccharides obtained by transgalactosylation of lactulose were isolated and fully characterised by NMR. These trisaccharides presented a galactose unit linked to C-6 of the galactose moiety (β-D-Gal-(1→6)-β-D-Gal-(1→4)-D-Fru; 6'-galactosyl lactulose) (**Figure 9.4**), and the other one has a galactose unit linked to C-1 of the fructose moiety (β-D-Gal-(1→4)-D-Fru-(1→1)-β-D-Gal; 1-galactosyl lactulose) (**Figure 9.5**). Recently, Hernández-Hernández *et al.* (2011) used HPLC-ESI MS to detect OsLu with DP up to 4, 5 and 6 in mixtures obtained with β-galactosidases from *K. lactis*, *A. aculeatus* and *A. oryzae*, respectively. These authors also characterised disaccharides, trisaccharides and galactosyl-glycerols by GC-MS as

trimethylsilyloximes. The formation of glycerol derivatives was due to the presence of glycerol as stabiliser in the commercial preparations of *A. aculeatus* and *K. lactis*.

With the aim of optimising the reaction conditions during hydrolysis and transgalactosylation of lactulose, the effect of time, temperature, pH, and initial lactulose and enzyme concentrations was studied. For the β-galactosidase derived from *K. lactis,* the most favourable conditions for obtaining the highest yields of 6'-galactosyl lactulose (104 g/kg total carbohydrates) and 1-galactosyl lactulose (115 g/kg total carbohydrates) were 50 ºC, pH 6.5, 250 g/L of lactulose, 6 U/mL of enzyme and 2 h of reaction. In the case of the β-galactosidase from *A. aculeatus,* hardly any formation of 1-galactosyl lactulose was detected and the optimal conditions for the maximum formation of 6'-galactosyl lactulose (160 g/kg total carbohydrates) and higher oligosaccharides (120 g/kg total carbohydrates) were 60 ºC, pH 6.5, 450 g/L of lactulose, 16 U/mL enzyme and 7 h of reaction (Cardelle-Cobas *et al.* 20011a).

In general, the complexity of the different reactions involved in oligosaccharide synthesis, which act on different substrates at different rates, is well known. Given that understanding the mechanism of oligosaccharide synthesis could improve the quality of the products of the reaction mixture as well as increase the production efficiency by optimal selecting conditions, there are a number of publications that model GOS formation over time. In general, models can be categorized as either mechanistic or empirical. Particularly interesting is the paper by Gosling *et al.* (2010) who revised the most important works related to GOS synthesis. Taking into account all of these considerations, in the case of OsLu synthesis, a detailed kinetic model using Akaike criterion, robust estimation of the parameters and computation confidence intervals was performed by Rodríguez-Fernández *et al.* (2011) for the first time. The proposed

method describes OsLu synthesis using the β-galactosidases from *A. aculeatus* (Pectinex Ultra SP-L) and *K. lactis* (Lactozym 3000 L HP G) at several temperatures and lactulose concentrations. The kinetic parameters were of different magnitude for both enzymes, giving rise to different amounts and types of oligosaccharides, in agreement with the experimental data. In general, it could be concluded that the formation of trisaccharides was favoured using the β-galactosidases from Pectinex Ultra SP-L, as compared to that of Lactozym 3000 L HP G, which favoured disaccharide formation.

In addition, the synthesis of GOS and OsLu has also been addressed using different *Kluyveromyces strains* isolated from artisanal cheeses belonging to *K. lactis* and *K. marxianus* species (Padilla *et al.* 2012). All *Kluyveromyces* crude cell extracts produced GOS, such as 6-galactobiose and 3'-, 4'-, and 6'-galactosyl lactose and, when lactulose was the substrate, together with the above indicated disaccharide, 6'-galactosyl lactulose and 1-galactosyl lactulose were also formed. However, the most remarkable result is the fact that *K. marxianus* strain O3 produced a very high yield of OsLu with 450 g/kg of total carbohydrates after 4 h of reaction.

Guerrero *et al.* (2013) compared the formation of GOS and OsLu using β-galactosidases from *K. lactis*, *A. oryzae* and *B. circulans* and found higher yields for OsLu than for GOS production in the case of *K. lactis* and *A. oryzae*, whereas *B. circulans* enzyme gave rise to greater formation of GOS than OsLu. No identification of individual compounds was done. These results underline the importance of substrate and enzyme source for the production and yield of oligosaccharides.

9.4.3. Chemical isomerisation

As is well known, lactose is converted into lactulose by isomerisation of the glucose moiety to fructose with high yields (70-80%) using electrolytes such as aluminium hydroxide (Aider & Halleux 2007). Therefore, GOS can be also transformed into the corresponding isomers under basic media. Moreover, GOS mixture reactions obtained during the hydrolysis and transgalactosylation of lactose present noticeable amounts of mono- and disaccharides without prebiotic functionality and with high caloric value and glycaemic index. Thus, the development of synthesis procedures focused on the production of reaction mixtures with high yields of oligosaccharides and low concentration of mono- and disaccharides are of interest. In this sense, a new strategy to obtain isomeric oligosaccharides with different potential prebiotic properties was proposed by Cardelle-Cobas *et al.* (2008c). In this study, at first, GOS were synthesised using the β-galactosidase from *K. lactis* under the optimal conditions reported by Martínez-Villaluenga *et al.* (2008a). Then the reaction mixture was isomerised by the action of sodium aluminate at a certain concentration and under controlled temperature and time conditions. During the reaction, lactose, glucose, and galactose were isomerised to lactulose, fructose, and tagatose, respectively; in addition, allolactose, 6-galactobiose, and 6'-galactosyl lactose were converted into the corresponding keto-sugars (**Figure 9.6**). The optimal reaction conditions for obtaining the highest conversion of lactose and 6'-galactosyl lactose to lactulose and 6'-galactosyl lactulose, respectively, were 40 ºC for 9 h and an aluminate/lactose ratio of 3:1. Under these conditions, the isomerisation yield was > 60%, and the amount of final carbohydrates was close to 90% of the initial product. This procedure could be considered as adequate for people with lactose intolerance or diabetes since the concentration of lactose and glucose is considerably decreased. In addition, another

aspect that should be taken into account is the fact that, the synthesis of OsLu by isomerisation of GOS proposed in the paper by Cardelle-Cobas *et al.* (2008c) is more economically profitable than the utilisation of lactulose as substrate. However, the main drawback of this procedure is the utilisation of aluminium as catalyst of isomerisation, which must be removed from the reaction mixtures (Dendene *et al.* 1994; Zokaee *et al.* 2002).

As indicated in Section 9.4.1., GOS can be produced either in buffered solutions of lactose or in WP, the latter being more profitable from an economic point of view. Very recently, Corzo-Martínez *et al.* (2013) have developed a new approach to obtain OsLu together with GOS, based on the isomerisation of lactose of WP and a subsequent transgalactosylation reaction of lactulose with the β-galactosidase from *B. circulans*. The main advantage of this procedure is the utilisation of egg shell which is a food-grade catalyst (as compared to aluminium and boron) and, therefore, any intermediate purification step is not necessary. The maximum formation of oligosaccharides with DP 2-4 was achieved after 5 h of reaction at pH 6.5 and 50 ºC with 300 g/kg carbohydrates and 3 U/mL of enzyme. Under these conditions the mixture was composed by glucose, galactose, lactulose, lactose, allolactose, β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc, β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Fru. This application represents an opportunity for WP upgrading.

These findings indicate that the chemical isomerisation performed before or after the transgalactosylation reaction constitutes a good and economic option for obtaining mixtures of GOS and OsLu with potential prebiotic properties.

9.4.4. Assessment of beneficial effects of oligosaccharides derived from lactose and lactulose

The beneficial effects of GOS on human health have been largely reviewed in the literature according to its well-established prebiotic status (Gibson *et al.* 2004; Macfarlane *et al.* 2006; Roberfroid 2007; Macfarlane *et al.* 2008; Playne & Crittenden 2009; Tzortzis & Vulecic 2009; Torres *et al.* 2010). Moreover, the reader can find detailed information on the nutritional benefits of GOS for infants and young children, as well as on the physico-chemical and physiological properties of GOS in chapter 26 of this book. In this context, moreover, GOS are well known as effective prebiotic ingredients presenting a major effect on intestinal microbiota, barrier functions, and other health benefits in animal and human adults (Lamsal 2012; Walton *et al.* 2012; Culpepper *et al.* 2012). Thus, GOS has been shown to exert beneficial physiological effects such as improvement of stool consistency and frequency, increased mineral (calcium and magnesium) absorption, immunomodulation properties for the prevention of allergies and gut inflammatory conditions, anti-pathogenic properties, trophic effects of SCFAs on the colonic epithelium, reduced toxigenic microbial metabolism that might reduce risk factors for colon cancer and immunomodulation properties for the prevention of allergies and gut inflammatory conditions.

Gopalakrishnan *et al.* (2012) suggested that GOS could be a novel strategy for inflammatory bowel disease since these oligosaccharides significantly reduced the severity of colitis in mice treated with the pathogen *Helicobacter hepaticus* and this was accompanied by an increase in the percentage of NK cells.

Other effects of GOS related with lactose intolerance have also recently been studied. Thus, Savaiano *et al.* (2012) investigated in a multi-centre randomised, double-blinded, placebo-controlled, parallel group trial the symptoms of lactose intolerance in

individuals who had intake novel short-chain GOS (RP-G28) and, although more research is required, a significant reduction in negative symptoms was observed.

In the case of the new generations of oligosaccharides synthesised in the last few years, it is also necessary to carry out *in vitro* and *in vivo* studies to demonstrate the potentiality as functional ingredients, particularly as prebiotics. Concerning OsLu, very recent papers have been addressed with this aim. The fermentative properties of *Bifidobacterium*, *Lactobacillus* and other important human gut species toward the OsLu synthesised during the hydrolysis and transgalactosylation of lactulose have also been tested by our research group. Thus, Cardelle-Cobas *et al.* (2011b), in a study with purified trisaccharides derived from lactose or lactulose, showed that, in general, pure cultures of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* have preference towards β-galactosyl residues having β-(1→6) and β-(1→1) linkages over those of β-(1→4). Similar or higher cell densities and growth rate were achieved on 6'-galactosyl lactulose than on 6'-galactosyl lactose. In addition to this study, the bifidogenic properties of mixtures of OsLu with DP ≥ 3 in human faecal slurries were investigated by quantitative PCR by Cardelle-Cobas *et al.* (2009). Very recently, in an *in vitro* fermentation study with mixed faecal microbiota, Cardelle-Cobas *et al.* (2012) assessed the prebiotic properties of these oligosaccharides. Among the different populations studied (*Bifidobacterium, Lactobacillus, Enterococcus, Eubacterium, Bacteroides, Clostridium* and *Atopobium*), bifidobacteria and lactobacilli selectively fermented the OsLu, producing a higher concentration of SCFA than other bacteria tested. Comparing the different oligosaccharides tested (**Figure 9.7**), the OsLu produced by means of the β-galactosidase from *K. lactis* presented higher selective index scores, particularly after 24 h of fermentation, than lactulose, the commercial preparation Vivinal-GOS, GOS derived from *K. lactis* and *A. aculeatus* and OsLu formed with the enzyme of *A.*

aculeatus. The selective index gives a comparative relationship between the growth of beneficial faecal bacteria (bifidobacteria, *Lactobacillus/Enterococcus* group, and *Eubacterium rectale* group) and less desirable ones (for example, clostridia and bacteroides), related to the changes of the total number of bacteria (Sanz *et al.* 2005; Ruiz-Matute *et al.* 2011).

These investigations have recently been completed by evaluating *in vivo* their fermentation properties. Thus, Hernández-Hernández *et al.* (2012) compared the *in vivo* ileal digestibility and modulatory effects in faecal microbiota of commercial GOS and laboratory synthesised OsLu. Taking into account the ileal digestibility rates of samples, the trisaccharide fraction of OsLu was significantly more resistant to gut digestion than GOS (12.5 vs. 52.9%), underlining the specific role of the monomer and linkage involved in the formation of these molecules. Both GOS and OsLu reached the large intestine and were fermented since no presence of these oligosaccharides was detected in the faecal samples.

All of these findings highlight the importance of the chemical structure of oligosaccharides (DP, sugar monomeric composition, linkages, etc.) on their fermentation properties by gut microbiota. However, the most striking feature is the fact that the OsLu, obtained as previously mentioned in the different papers of our research group, may constitute an alternative as prebiotics to the original disaccharide lactulose and also to GOS. Further *in vivo* studies carried out with humans are currently underway in order to definitively establish the usefulness of oligosaccharides derived from lactulose as new functional ingredients with important applications in a number of products.

9.4.5. Uses of galactooligosaccharides

Given the similarity of GOS and OsLu structures, it is easy to expect that the applications and uses of both might be fairly similar. GOS food applications are well known, as has been extensively reported by Wang (2009), Torres *et al.* (2010), Sangwan *et al.* (2011) and Lamsal (2012) (please, see also chapter 26). Given that the transgalactosylation reaction produces an array of oligosaccharides with different DP, the commercial preparations available are formed by mixtures with variable compositions depending on aforementioned factors (source, initial concentration of substrate and enzyme, temperature, time). Consequently, this affects their physiological effects and physicochemical properties, which determine their applications. In general, solubility, osmolality, crystal formation ability, sweetness and reactivity decrease as the molecular size increases, contrary to viscosity.

GOS are versatile ingredients to be incorporated in a wide range of food products because of their stability to pH and temperature, good taste quality, and relatively low sweetness and caloric value (See Chapter 26). The main applications of GOS (and supposedly OsLu) can be fermented milks and yogurts, health drinks, nutrition bars, breakfast cereals, beverages (fruit juices and other acid drinks), bakery products, meat products, soups and sauces, mineral supplements, weight loss products, green foods, infant food and pet food. These prebiotic ingredients can be used for their nutritional and also for their functional (bioactivity and technofunctional) properties. In some examples they are used for fat replacement, texture modification and also moisture retention (Figueroa-González *et al.* 2011; Sangwan *et al.* 2011).

Particularly interesting is the application of GOS in infant formula and growing-up milk due to their resemblance to human milk oligosaccharides. It has been found that a low level of GOS in infant formula (2.4 g/L) could stimulate the growth of intestinal bifidobacteria and lactobacilli. Another potential application is in specialised foods for the elderly and hospitalised people (Lamsal 2012).

In addition to the food area, other sectors such as pharmaceutical and cosmetic companies can also use the excellent properties of these oligosaccharides. In fact, fact, prebiotic oligosaccharides can selectively stimulate beneficial bacteria of skin and and some formulation for that purpose has already been studied (Krutmann 2009).

In spite of the beneficial effects of GOS and OsLu, more research is needed to investigate their stability, not only during processing but also during storage within the shelf-life period of the processed food in which these ingredients could be incorporated. In the case of OsLu, clinical assays are mandatory to evaluate the possibility of introducing this new generation of prebiotics as functional ingredients in food and food-stuffs.

9.5. Other oligosaccharides

Currently, as indicated above, the development of simple and convenient methods for the enzymatic synthesis of novel oligosaccharides with biological activities is attracting high interest. In this context, oligosaccharides derived from lactose by transglucosylation using dextransucrases (E.C. 2.4.1.5) can be obtained. These enzymes are glucansucrases produced by various species of *Leuconostoc, Lactobacillus,* and *Streptococcus,* which polymerises the glucosyl moiety of sucrose to yield the α-(1→6) linked polysaccharide dextran (see Chapter 10 for more details on these types of enzymes). When carbohydrates other than sucrose are used, the D-glucosyl unit could

be transferred to the carbohydrate called the acceptor; if this acceptor is a disaccharide such as lactose, the resulting oligosaccharide increases only by the molecular weight of the D-glucose group added (Robyt & Eklund 1983).

Seo *et al.* (2007) studied oligosaccharide synthesis during a novel symbiotic fermentation process of cow's milk using as starters a coculture consisting of *Leuconostoc citreum* with *Lactobacillus casei*, *Lb. delbrueckiisb sp*. *bulgaricus* and *Streptococcus thermophilus*. The trisaccharide β-D-Glc-(1→6)-β-D-Gal-(1→4)-α-D-Glc was formed during fermentation of milk by dextransucrase activity of *L. citreum* after 1-2 days of fermentation at 15 or 25 ºC and when 4% sucrose was added in milk. These authors proposed the possibility of using leuconostoc as a starter along with addition of sucrose in milk to produce oligosaccharides-containing fermented types of milk.

Another oligosaccharide formed by means of dextransucrase activity has been described by Díez-Municio *et al.* (2012a). These authors synthesised and characterised D-Gal-(1→4)-α-D-Glc-(2→1)-α-D-Glc using different lactose/sucrose ratios or WP and sucrose, and *Leuconostoc mesenteroides* B 512F dextransucrase. They proposed this trisaccharide as an excellent candidate for a new prebiotic ingredient due to the reported high resistance of α-(1→2) linkages to the digestive enzymes in humans. Moreover, these results would extend the use of agricultural raw materials such as sucrose or WP as renewable substrates for enzymatic synthesis of oligosaccharides of nutritional interest.

Through a transglucosylation reaction using dextransucrases, other potential prebiotic oligosaccharides can be formed when lactulose is used as acceptor of glucose released from sucrose. Lactulosucrose (β-D-Gal-(1→4)-β-D-Fru-(2→1)-α-D-Glc) formation was described by Suzuki & Hehre (1964) using cultures of *Leuconostoc*

mesenteroides strain NRRL B-1299 and by the transfer of the glucosyl moiety of sucrose to lactulose formed during sterilisation of culture media containing lactose. Subsequently, these same authors (Hehre & Suzuki 1966) confirmed, using dried cells of leuconostoc and lactulose, that dextransucrase was the enzyme responsible for lactulosucrose formation. Recently, Díez-Municio *et al.* (2012b) have performed an exhaustive study in which they described an efficient enzymatic synthesis of lactulosucrose using dextransucrase from *L. mesenteroides* B-512F. These authors obtained higher yields of lactulosucrose than Suzuki & Hehre (1964). They attributed this result to the fact that purified dextransucrase efficiently catalysed the formation of lactulosucrose.

In addition, oligosaccharides with other functional groups, such as *N*-acetyl, sialyl or fucosyl, are also of interest due to their similarity to those present in human milk. These carbohydrates also form part of a large number of membrane glycoproteins and glycolipids and play a central role in cellular recognition phenomena in biological processes, such as inflammation and cancer development (Kunz & Rudloff 2006; Bode 2009; Dall'Olio *et al.* 2012).

One of these carbohydrates is the *N*-acetyl-lactosamine that can be synthesised by chemical and enzymatic methods. Two kinds of enzymes can be used in its synthesis, galactosyltransferases and β-galactosidases, the former having higher selectivity and producing higher yields than the latter, although they are more expensive and need a complex substrate (uridine diphosphogalactose) as donor (Fang *et al.* 1998).

The enzymatic synthesis of *N*-acetyl-lactosamine using β-galactosidases is performed via the hydrolysis of lactose in the presence of *N*-acetyl-glucosamine giving rise to β–(1→4) and β–(1→6) linkages, different from those β–(1→3) present in human milk (Urashima *et al.* 2012). The enzymes used in this synthesis are β-galactosidases

from *Escherichia coli* (Ajisaka*et al.* 1987), *K. lactis* (Sakai *et al.* 1992), *B. circulans* (Sakai *et al.* 1992; Li *et al.* 2010; Bridiau & Maugard 2011), *Bullerasingularis* (Nilsson *et al.* 1997), *Bifidobacterium bifidum* (Yoon & Rhee 2000), *Lactobacillus bulgaricus* and *Lb. plantarum* (Black *et al.* 2012). β-Galactosidase from *B. circulans* has been the most used biocatalyst for the synthesis of this type of oligosaccharides. The compounds isolated from the reaction mixture were derived from *N*-acetyl-glucosamine with one or two galactose moieties and the optimal synthetic conditions were different for the production of oligosaccharides with β–(1→4) and β–(1→6) linkages (Li *et al.* 2010). To improve yield, this β-galactosidase has been tested in water-miscible ionic liquids as 1,3-di-methyl-imidazolmethyl sulphate which suppresses the secondary hydrolysis of the product formed, resulting in doubling the yield to almost 60% with respect to the initial *N*-acetyl-glucosamine amount (Kaftzik *et al.* 2002).

Synthesised *N*-acetyl-lactosamine can be used for the enrichment of infant formula, since cow milk is poor in oligosaccharides (Gopal & Gill 2000). Moreover, other properties of these disaccharides are related to their availability to bind toxins, particularly in the case of the isomer with β-(1→3) linkage (El-Hawiet *et al.* 2011).

Finally, the galactose moiety of lactose can be sialylated or fucosylated to form sialyllactoses and fucosyllactoses, respectively (Bode 2009). They can be obtained by chemical synthesis, although these methods are tedious and toxic reagents are used (Pereira *et al.* 2012). Therefore, other procedures can be used such as recovery from whey and biological methods (Seki & Saito 2012).

For both trisaccharides there are four types of biological methods: (i) using a special precursor such as cytidine monophosphate (CMP)-sialic acid or guanosine diphosphate-L-fucose and sialyltransferases or fucosyltransferases (Carlson *et al.* 1993; Albermann *et al.* 2001). (ii) Coupling two enzymes to produce the precursor and the

final product as a CMP-sialic acid synthetase and a sialyltransferases respectively (Endo *et al.* 2000; Albermann *et al.* 2001). (iii) Constructing a fusion protein having the two activities, CMP-sialic acid synthetase and sialyltransferase, over-expressed in *E. coli*. Starting with lactose, sialic acid, phosphoenolpyruvate, ATP and CMP the obtainment of α-2,3-sialyllactose at the 100 g scale was possible (Gilbert *et al.* 1998) (iv) when using whole cell biosynthesis methods by metabolically engineered *E. coli* expressing a multifunctional glycosyltransferase. In the case of syalyltransferases, glycerol as carbon substrate, IPTG as inducer and lactose, 3' or 6-sialyllactose were obtained, depending on the enzyme cloned (Drouillard *et al.* 2010). With a fucosyltransferase from *Helicobacter pylori*, a maximum of 1.23 g/L 2-fucosyllactose was obtained from a batch fermentation with 14.5 g/L lactose (Lee *et al.* 2012). However, all the above methods are very expensive and the product obtained is used only for research or pharmaceutical purposes since it has been observed that fucosylated oligosaccharides inhibit *Campylobacter* binding to human intestinal mucosa *ex vivo* (Bode 2009), and sialyllactose modified the *Helicobacter pylori* and host interactions (Simon *et al.* 1997).

It is known that sialylated and fucosylated oligosaccharides of milk are absorbed from digestive tracts of babies and used for the biosynthesis of glycoproteins and glycolipids in their brains (Kobata 2010). Since cow's milk contains less oligosaccharides than human milk, fractions enriched in oligosaccharides obtained from an ultrafiltration permeate could be used. In this sense, Shimatani *et al.* (1992) patented a process for obtaining a concentrate of sialic acid-bound oligosaccharides, peptides and lipids, using a cation exchanger column. Furthermore, Holst *et al.* (2007) developed a patent to obtain a concentrate enriched in oligosaccharides using UF and diafiltration. This method can be more useful applied to goat milk permeate, richer in

oligosaccharides than cow's milk permeate (Martínez-Férez *et al.* 2006); moreover, the former contains fucoyl-oligosaccharides (Nakamura & Urashima 2004).

9.6. Purification of carbohydrates derived from lactose

Purification of carbohydrates derived from lactose represents the most expensive operation in the production of this group of compounds. In base-catalysed aldose-ketose isomerisation, aluminate and borate are removed from the final product with great difficulty. Methods to remove these chemical catalysts, including chromatographic purification systems and nanofiltration, have been successfully applied. Kozempel *et al.* (1995) developed a commercially feasible pilot plant to produce lactulose from lactose using boric acid to boost the conversion from about 75% and the final removal of boric acid was by liquid chromatographic purification system to produce lactulose containing less than 1-5 ppm of boric acid. Recently, Zhang *et al.* (2011) studied the retention behaviour of the lactose, lactulose and boric acid mixture in a pilot scale test of nanofiltration by using a Sepro-NF2A membrane and they found that more than 96.5% of boric acid was removed from lactulose syrup with only a small loss (11%) of disaccharides.

As can be inferred from what is mentioned above, the final products derived from the transglycosylation reaction comprise a mixture of oligosaccharides with a high amount of mono- and disaccharides without prebiotic properties and with high caloric value. Since the removal of sugar components other than prebiotic carbohydrates is a positive factor for improving the quality of a commercial product, several purification procedures have been developed based on supercritical fluid extraction, nanofiltration, selective fermentation and enzymatic oxidation. (See Chapter 13 for more detailed information on fractionation of bioactive oligosaccharides.)

Two loose nanofiltration membranes and one dense ultrafiltration membrane were used by Goulas *et al.* (2003) to fractionate commercial oligosaccharide mixtures and the results obtained suggest that nanofiltration could be a practical tool for the large-scale fractionation of oligosaccharides from complex mixtures.

A process for obtaining non-monosaccharide and high-purity GOS was proposed by Li *et al.* (2008) in which the GOS mixture produced by β-galactosidase was subjected to fermentation by *S. cerevisiae* L1 or *K. lactis* L3, resulting in an increase of GOS purity from 28.7% to 39.4% and 97.5%, respectively. This process was effective in producing high-purity GOS at a low cost on an industrial scale.

To date, fractionation of GOS has been mainly carried out by size exclusion chromatography (SEC) (Tzortzis *et al.* 2005; Shoaf *et al.* 2006; Huebner *et al.* 2007). Later, Hernández *et al.* (2009) compared four different fractionation techniques to obtain prebiotic GOS free of mono- and disaccharides. Diafiltration did not show any selectivity among mono- di- and oligosaccharides, whereas yeast treatment allowed the removal of monosaccharides with high recovery of di- and oligosaccharides. Treatment with activated charcoal showed a different selectivity in GOS recovery depending on the ethanol percentage (1-15%) in the aqueous solution used. Ethanolic solutions of 8% led to a high GOS recovery (90%), but 20% of disaccharides were also recovered, whilst 10% ethanolic solutions gave almost complete removal of disaccharides and only approximately 53% of trisaccharides were recovered. The purest GOS fractions (DP up to 8) were obtained using SEC, a fact of particular interest in the case of a subsequent characterisation of new compounds.

Splechtna *et al.* (2001) assayed the selective enzyme oxidation for GOS purification using fungal cellobiose dehydrogenase, which displays an approximately 100-fold preference for reaction with lactose compared to reaction with GOS. Oxidation

of lactose was coupled to reduction of 2,6-dichloro-indophenol, which was added in catalytic concentrations. The oxidised redox mediator was regenerated continuously by a fungal laccase-catalysed reduction of molecular oxygen into water. Ion exchange chromatography were employed to remove lactobionic acid, other ions and monosaccharides

Supercritical carbon dioxide, with different ethanol/water mixtures as co-solvents, has been tested for the selective fractionation of mixtures of commercial GOS. Under appropriate conditions, the almost complete removal of monosaccharides and disaccharides from the mixture is possible, which leads to a residue mainly composed of GOS with 75% purity and 94% recovery (Montañés *et al.* 2009).

9.7. Conclusions

Lactose, the major component of cheese whey, is an abundant waste material with limited industrial uses that can be transformed into a wide variety of carbohydrates via chemical or enzymatic reactions. Some of these carbohydrates, such as lactulose, of proven utility in medicine and human nutrition, and GOS, used as prebiotics, are commercially available. In mixture with other carbohydrates, lactose can produce a wide variety of oligosaccharides that are of particular interest for biological, medical, and food applications. Although clinical studies on the effects of GOS in humans prove their effectiveness as prebiotics, more research is needed not only on the prebiotic properties of the new generation of oligosaccharides synthesised from lactose but also on other potential new uses on the protection of human and animal health.

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Table1. Formation of lactulose through lactose isomerisation.

FORMATION OF LACTULOSE			
Lactose isomerisation			
Mechanism	**Type of catalyst**		**References**
Heat treatment of milk	Buffer system of milk		Adachi (1958); Montilla *et al.* (2005b)
Chemical reaction	Hydroxyl groups	Sodium, calcium or potassium hydroxide	Montgomery & Hudson (1930); Zokaee *et al.*(2002); Abdolreza & Zokaee (2010)
	Sulphites and Phosphates		Panesar & Kumari (2011)
	Tertiary amines		Hicks & Parrish (1980)
	Complexing reagents	Aluminates; borates	Mendicino (1960); Aider & Halleux (2007); Zang *et al.* (2011); Panesar & Kumari (2011)
	Ion Exchange resins		Aider & Halleux (2007); Panesar & Kumari (2011)
	Heterogeneous:	Alkaline sepiolites	De la Fuente *et al.* (1999); Troyano *et al.* (1996); Villamiel *et al.* (2002)
		Zeolites	Shukla *et al.* (1985)
		Calcium carbonate: Egg shell Oyster shell	Montilla *et al.* (2005a); Paseephol *et al.* (2008)
	Electro-isomerisation	Sodium chloride and DC-electric field	Aider & Gimenez-Vidal (2012)
Enzymatic reaction	Redox isomerisation: Selective oxidases and reductases	Pyranose oxidase and aldose reductase	Schuster-Wolff-Bühring *et al.* (2010)
	Thermostable cellobiose 2 epimerase	(*Caldicellulosiruptor saccharolyticus*)	Kim & Oh (2012); Kim *et al.* (2012b)

Table 2. Formation of lactulose by transgalactosylation of lactose using different types of enzymes.

FORMATION OF LACTULOSE BY TRANSGALACTOSYLATION			
BIOCATALYSTS	**SUBSTRATES**	**MICROORGANISMS**	**REFERENCES**
Permeabilised cells (β-galactosidase activity)	Lactose (donor) and fructose (acceptor)	*Kluyveromyces lactis*	Lee *et al.* (2004)
β-Galactosidases	Lactose (donor) and fructose (acceptor)	*Aspergillus oryzae*	Mayer *et al.* (2004); Lee *et al.* (2004); Guerrero *et al.* (2011); Vera *et al.* (2011)
	Lactose and fructose	*Escherichia coli* *Saccharomyces fragilis*	Lee *et al.* (2004)
	Lactose and fructose	*Kluyveromyces lactis*	Lee *et al.* (2004); Fattahi *et al.* (2010); Guerrero *et al.*(2011); Shen *et al.* (2012); Hua *et al.* (2013); Song *et al.* (2013)
	Lactose and fructose	*Sulfolobus solfataricus*	Kim *et al.* (2006)
	Lactose and fructose	*Bacillus circulans*	Guerrero *et al.* (2011)
	Lactose and fructose	*Arthrobacter* sp.	Tang *et al.* (2011)
	Ultrafiltered Whey-permeate	*Kluyveromyces lactis* *Kluyveromyces fragilis* *Aspergillus oryzae*	Adamczak *et al.* (2009)
	Whey powder and glucose, galactose, fructose	*Kluyveromyces lactis*	Song *et al.* (2013)
β-Glycosidases	Lactose and fructose	*Pyrococcus furiosus* (CelB)	Mayer *et al.*, (2004; 2010)
Dual enzymatic system: β-galactosidase + glucose isomerase	Lactose and fructose	β-galactosidase from *Kluyveromyces lactis*	Hua *et al.*, (2010)

Figure 1. Simplified model of the alkaline isomerisation of lactose (Taken from Montilla *et al.* 2005).

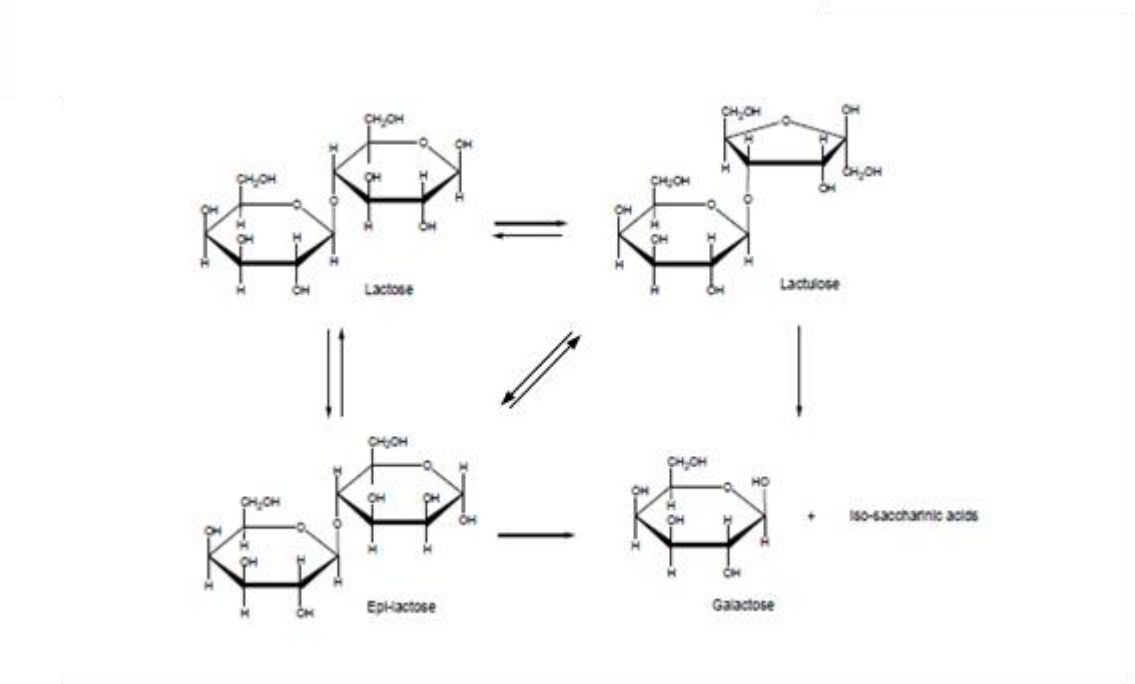


Figure 2. Schematic transglycosylation pathway catalysed by β-galactosidase using lactose and fructose as substrates (Taken from Hua *et al.* 2013).

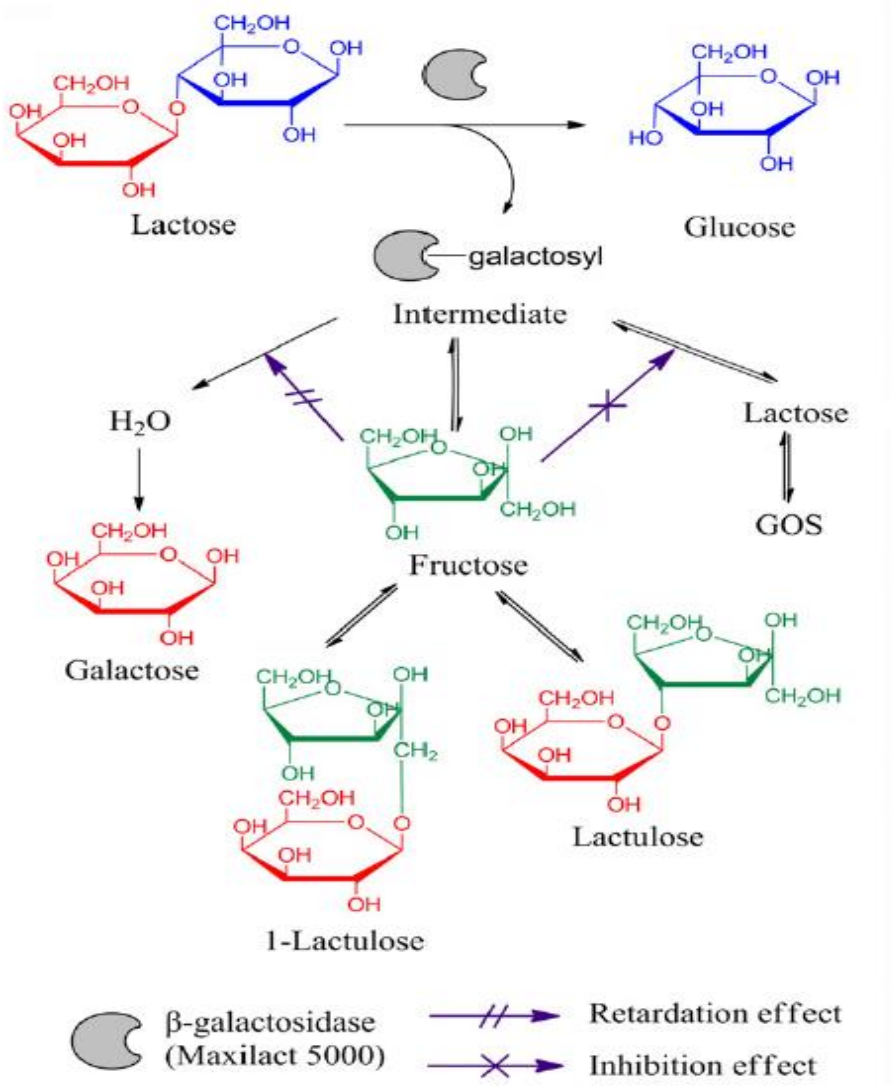


Figure 3. General model of lactose hydrolysis and GOS synthesis. (a, b, and c: glycosidic linkage position; X: donor; Y: acceptor).(Taken from Torres *et al.* 2010).

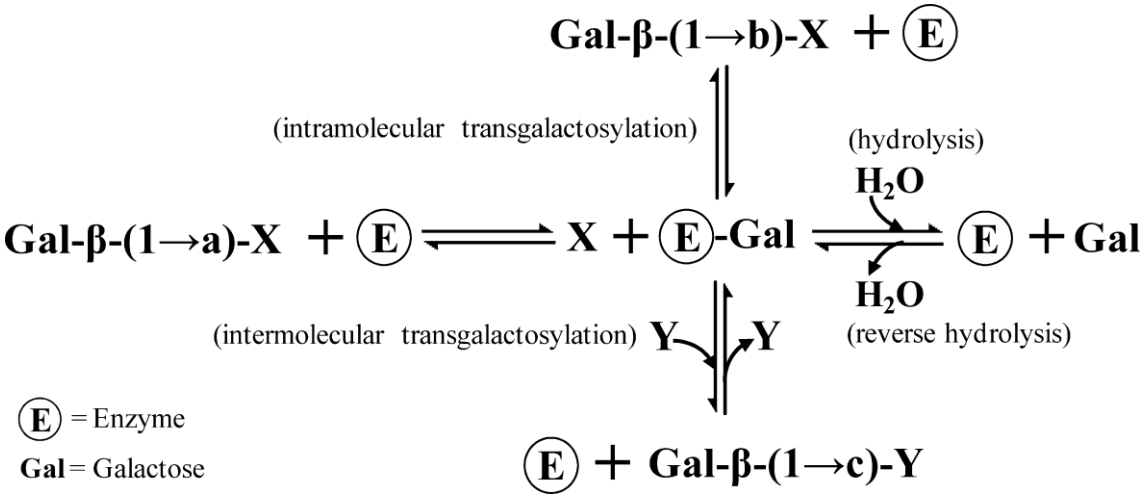


Figure 4. Structure of 6’-galactosyl lactulose.

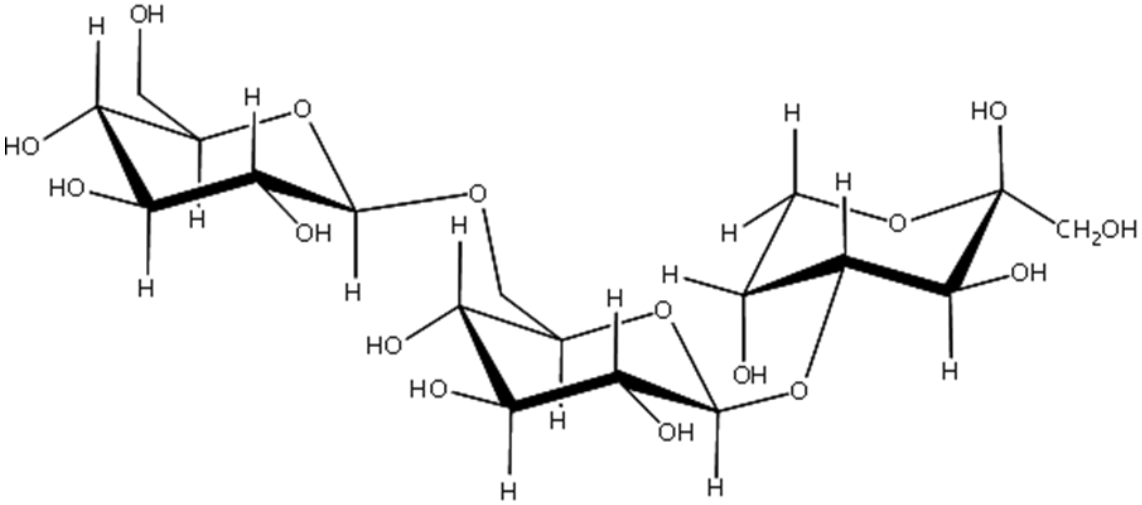


Figure 5. Struture of 1-galactosyl lactulose.

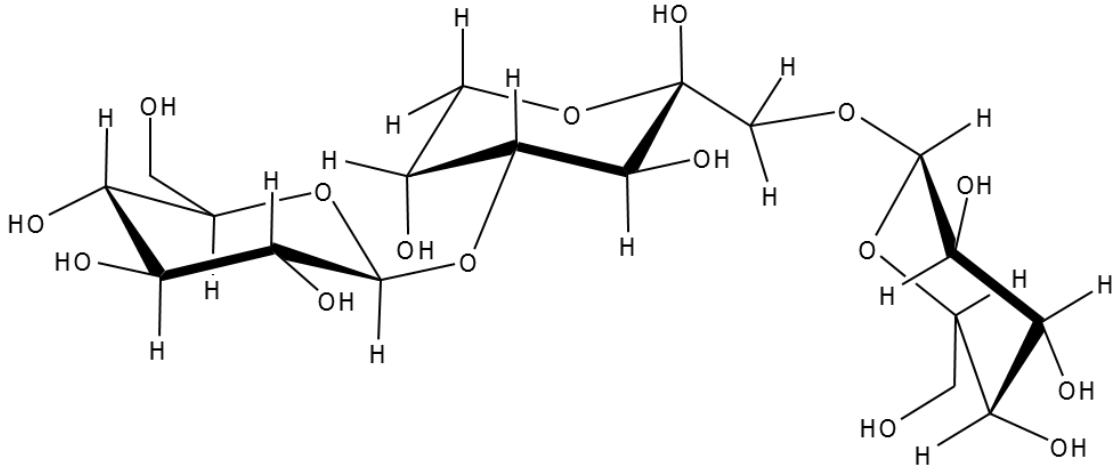
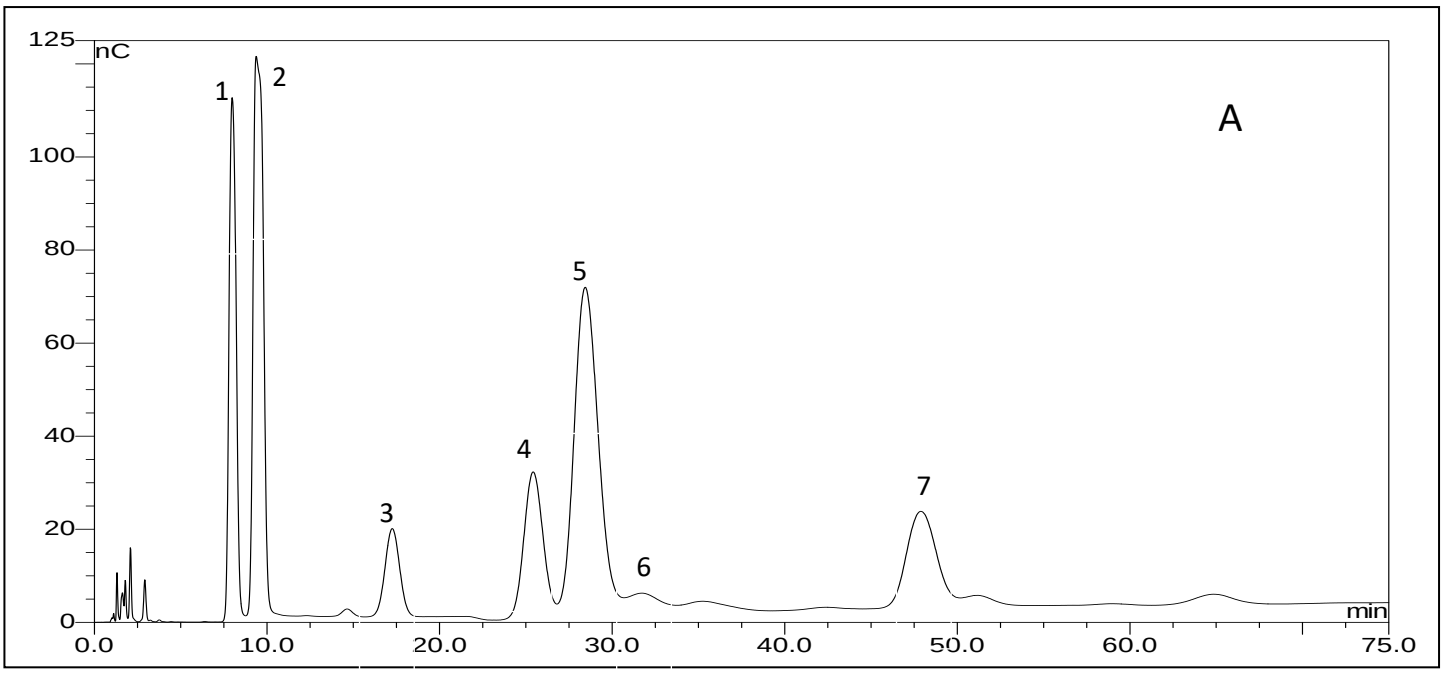


Figure 6. HPAEC-PAD profiles of carbohydrate mixture obtained by enzymatic hydrolysis of lactose and β-galactosidase from *Kluyveromyces lactis* (Lactozym 3000 L HP G) before (A) and after nine hours of isomerisation at 40 ºC using sodium aluminate: lactose molar relation 3:1 (B). **1**: Galactose, **2**: Glucose, **3**: 6-Galactobiose (β-D-Gal-(1→6)-D-Gal), **4**: Allolactose (β-D-Gal-(1→6)-D-Glc), **5**: Lactose, **6**: 4-Galactobiose (β-D-Gal-(1→4)-D-Gal), **7**: 6'-galactosyl lactose (β-D-Gal-(1→6)- β-D-Gal-(1→4)-D-Glc), **1'**: Tagatose, **2'**: Fructose, **5'**: Lactulose and **7'**: 6'-galactosyl lactulose (β-D-Gal-(1→6)- β-D-Gal-(1→4)-D-Fru). (Taken from Cardelle-Cobas *et al.* 2008c).



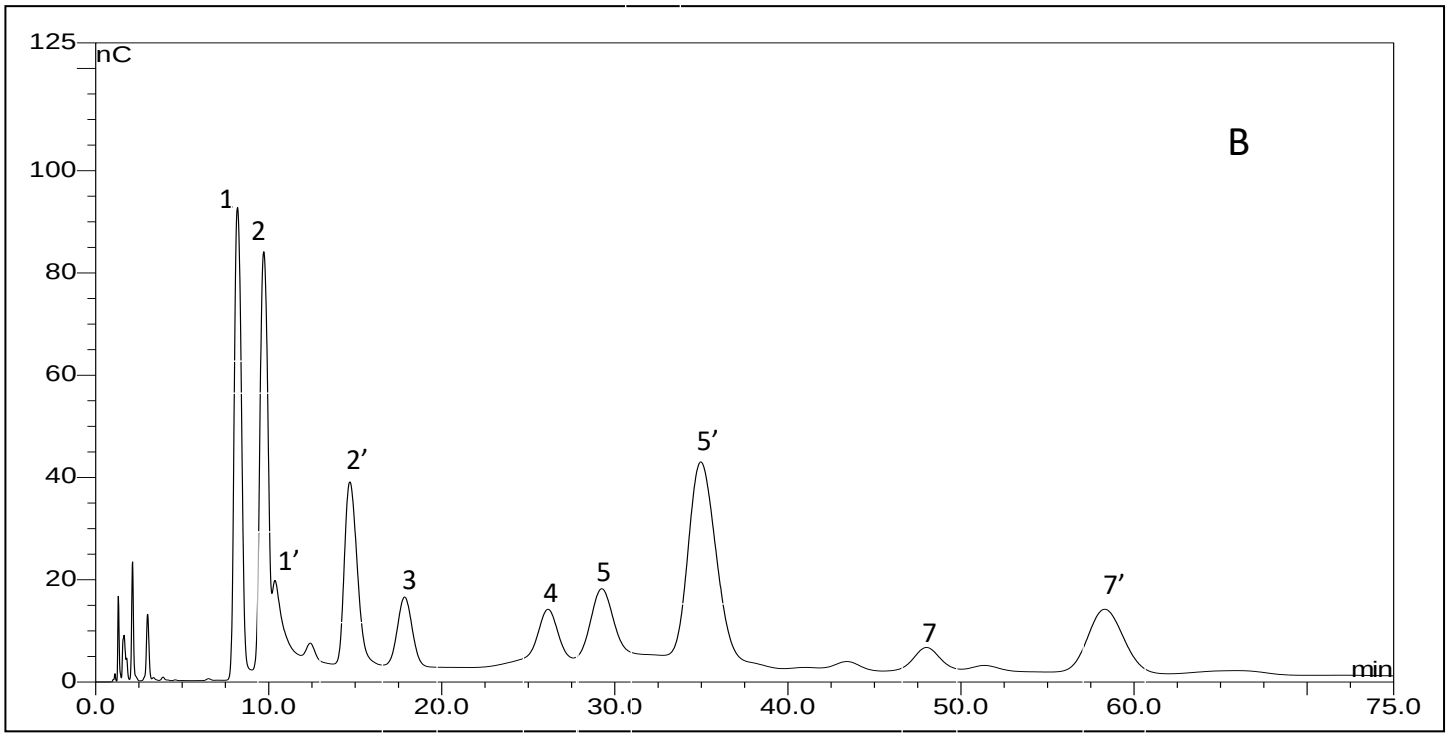


Figure 7. Selective Index (SI) scores from pH-controlled batch culture fermentations of lactulose (Lu), enriched fraction of oligosaccharides derived from lactose (GOS-1 and GOS-2), lactulose (OsLu-1 and OsLu-2) and commercial Vivinal-GOS. 1, oligosaccharides obtained with Lactozym 3000 L HP G; 2, oligosaccharides obtained with Pectinex Ultra SP-L. (Taken from Cardelle *et al.* 2012).