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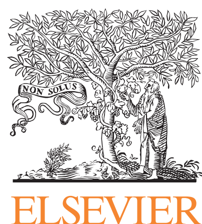

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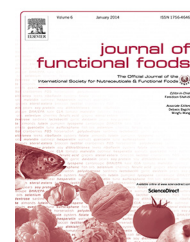

Perspectives on the biotechnological production and potential applications of lactosucrose: A review

Sara C. Silvério [a,*], Eugénia A. Macedo [b], José A. Teixeira [a], Lígia R. Rodrigues [a]

[a] CEB – Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] LSRE – Laboratory of Separation and Reaction Engineering-Associate Laboratory LSRE/LCM, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

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A B S T R A C T

Lactosucrose is a synthetic trisaccharide composed of galactose, glucose and fructose. This compound is obtained through enzymatic synthesis using lactose and sucrose as substrates. The enzymes involved in the process are able to catalyse both hydrolysis and transfer reactions. The yield and productivity of the process are usually affected by the occurrence of parallel hydrolysis of the newly formed product (lactosucrose). Therefore, it is important to find efficient strategies to avoid or minimize product degradation. Furthermore, in the last decades the demand for lactosucrose has significantly increased. This compound is considered a potential prebiotic and several beneficial effects associated to its consumption have been described. As a result, it has been included in the formulations of functional foods. This review covers the most relevant information about lactosucrose, including its synthesis and purification, beneficial effects at physiological level, and also its potential applications.

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* *Corresponding author.* CEB – Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Tel.: +351 601 972; fax: +351 253 604 429.
E-mail address: sarasilverio@deb.uminho.pt (S.C. Silvério).
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1. Introduction

The concept of prebiotic was first introduced in 1995 by Gibson and Roberfroid (1995). However, the original concept was updated and now prebiotics are defined as: "*a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora that confers benefits upon host well-being and health*" (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). In general, prebiotics are carbohydrate compounds; however, not all carbohydrates can be considered prebiotics. There are clear criteria that must be fulfilled to denote a food ingredient as prebiotic, namely (1) non-digestible in the upper gastrointestinal tract, thus reaching the colon intact. This condition requires resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (2) susceptibility to fermentation by the intestinal microflora; and (3) ability to selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being (Gibson et al., 2004).

Several physiological benefits associated with the intake of prebiotics have been recognized and almost all of them are a consequence of the prebiotic ability to maintain or restore a healthy gut microbiota. Generally, any dietary compound that is non-digestible and is able to reach the large intestine can potentially be classified as a prebiotic. Nevertheless, the currently accepted prebiotics are limited to a few non-digestible oligosaccharides including fructans, galactans and lactulose (Gibson et al., 2010). Lactosucrose is not yet included in this group since some researchers considered it only as a potential candidate (Cho & Finocchiaro, 2010; Gibson et al., 2010). Additional studies are still needed to unequivocally prove that lactosucrose fulfils all the criteria to be considered a prebiotic ingredient. However, it is already known that lactosucrose is barely digested in the upper gastrointestinal tract and it can reach the colon almost intact (Oku, Tanabe, Ogawa, Sadamori, & Nakamura, 2011). Moreover, some lines of evidence of the potential prebiotic effect of lactosucrose were also reported for humans and animals. One interesting characteristic of lactosucrose is its laxative threshold. Lactosucrose presents a higher laxative threshold when compared to other lactose-based prebiotics (Oku & Okazaki, 1999), such as lactulose and galacto-oligosaccharides (GOS). This fact represents an important advantage because diarrhoea is frequently described as a side effect of prebiotic intake (Vrese & Marteau, 2007). Additionally, lactosucrose has a significant water-holding capacity, which is considered an essential parameter both at physiological and industrial levels. High water-holding capacity increases bowel peristalsis and improves faecal formation and output (Jie et al., 2000). In the food processing industry, the increase of water-holding capacity can reduce syneresis or serum separation during product storage (Krasaekoopt, Bhandari, & Deeth, 2003). For consumer satisfaction, this is an important aspect when considering fermented milk products, such as yogurts or cheese, which are susceptible to serum or whey accumulation on the surface. The ability of lactosucrose to retain water may suggest its use as a fat replacer in this kind of products, contributing to a decrease in syneresis and improving some particular characteristics such as consistence and texture, as it was already reported for inulin (Arango, Trujillo, & Castillo, 2013).

Lactosucrose has been widely used in the preparation of functional foods, namely in Japan, where it was also included in the list of foods for specific health uses (FOSHU), in 2005. The demand for lactosucrose has increased since then, and in 2007 the market size of lactosucrose was estimated in approximately 3000 tons per year with an annual growth rate of 10% (Alfertsholt-Allen, 2007). Lactosucrose production is achieved by enzymatic processes involving enzymes able to catalyse both hydrolysis and transfer reactions. Different enzymes can be involved in lactosucrose synthesis, but all of them use the same substrates: lactose and sucrose. However, the reaction yields are not particularly high due to the occurrence of product degradation. To efficiently respond to the increasing demand of lactosucrose, it is important to improve the process of synthesis, which can be achieved by finding new and promising enzymes with enhanced catalytic activity, or by developing efficient strategies to minimize product degradation.

2. Lactosucrose properties and stability

Lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) is a synthetic trisaccharide composed of galactose, glucose and fructose. It can also be known as 4^{G}-β-D-galactosylsucrose or lactosylfructoside and presents a molecular weight of 504.44. Raffinose (O-α-D-galactopyranosyl-(1,6)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside), naturally found in some vegetables, is an isomer of lactosucrose and it can also present some potential prebiotic effect (Fernando et al., 2010).

Lactosucrose is obtained as a white solid with bland taste. It can form small monoclinic platelets which are described as very hygroscopic. The melting point of lactosucrose is around 181 °C and its specific rotation $[\alpha]_D^{20}$ was determined as +59°

(3.8 g/100 mL, H_2O), which gives a molecular rotation $[M]_D$ of 29,730 (Avigad, 1957).

Fujita et al. (1991b) studied some physical properties of a sugar mixture with 98% of lactosucrose (LS-98), obtained using β-fructofuranosidase from *Arthrobacter* sp. The solubility of LS-98 in water was found to be 3.67 g/L at 25 °C. Furthermore, its sweetness was evaluated and it was found that LS-98 presents 30% of sucrose sweetness. The powder mixture presented high hygroscopy, and when in solution (70% w/w), its water-holding capacity was higher than that found for sucrose. The water activity of LS-98 (70% w/w) was determined as 0.87 at 25 °C. The stability tests revealed that LS-98 was stable at pH 7.0 and 80 °C for 2 hours, and moderately stable under acidic pH conditions (pH 3.0, 80 °C) where it showed some decomposition (less than 20%) after 2 hours. It was also stable for 1 hour at pH 4.5 for temperatures up to 120 °C.

The use of lactosucrose as a sugar substitute in food was evaluated by adding 10% (w/w) of a sugar mixture composed of lactosucrose (35%, w/w, LS-35) and granulate sugar to agar gels (0.5 and 1%, w/w). For 0.5% (w/w) agar gels, the addition of LS-35 promoted a slight increase of syneresis after 24 and 48 hours of agar gel preservation at 6 °C. On the other hand, for 1% (w/w) agar gels, LS-35 contributed to a slight decrease in syneresis. This decrease was higher when the sweetener used was composed of 100% LS-35 then when a mixture of LS-35 and granulate sugar (1:1) was added to agar (Hirota, Furuuchi, & Mita, 1993).

When in contact with the reagents commonly used for sugar analysis, lactosucrose proved to be non-reducing to the alkaline copper reagent of Somogyi (1945) and it formed a green colour upon reaction with diazouracil, a substance that is used to specifically identify sucrose or sucrose-containing oligosaccharides (Avigad, 1957).

Some tests to evaluate the enzymatic cleavage of lactosucrose showed that this trisaccharide can be decomposed into equimolar amounts of fructose and lactose by an invertase from yeast and into equimolar amounts of galactose and sucrose by β-galactosidase. A combined action of invertase and β-galactosidase allowed the total cleavage of lactosucrose into glucose, galactose and fructose. When lactosucrose was incubated with levansucrase, in the absence of added aldose, three products were formed, namely levan, fructose and lactose. On the other hand, when glucose was combined with levansucrase, levan production decreased and a new non-reducing product (not identified) was detected. Enzymes like glucosucrase, dextran-sucrase, amylosucrase or yeast glucosucrase were not able to decompose lactosucrose when used separately. Nevertheless, mixed systems of β-galactosidase and one of those enzymes had the ability to cleave lactosucrose (Avigad, 1957).

Lactosucrose hydrolysis in supercritical water was investigated by Khajavi, Ota, Nakazawa, Kimura, and Adachi (2006) using a continuous flow-type reactor and operating at 10 MPa with different temperatures (200, 210 and 230 °C). For short residence times, decomposition of lactosucrose occurred with the formation of approximately equimolar amounts of fructose and lactose. Besides, no glucose or galactose were detected in those residence times, which indicates that the bond α-Glc-(1→2)-β-Fru is the most sensitive and susceptible for hydrolysis under the studied conditions. Lactose hydrolysis took place for longer residence times and it was confirmed by the detection of both galactose and glucose. The increase of temperature positively affected lactosucrose hydrolysis, being faster at higher temperatures (Khajavi et al., 2006).

3. Lactosucrose production

Avigad (1957) was probably the first to report the lactosucrose production. In his original work, the author describes the enzymatic synthesis and characterization of a new non-reducing trisaccharide (α-lactosyl-β-fructofuranoside) which was denominated "lactsucrose", later called lactosucrose. Levansucrase from *Aerobacter levanicum* was the enzyme involved in the synthesis, while sucrose and lactose were described as fructosyl donor and acceptor, respectively. The author also added *Torulopsis glabrata* cells to the reaction mixture to ensure continuous removal of hexoses from the system and to prevent both inhibition and shift of levansucrase action.

Later, Fujita, Hara, Hashimoto, and Kitahara (1990a) described the production and purification of an extracellular β-fructofuranosidase from *Arthrobacter* K-1 with wide acceptor specificity. The transfructosylation ability of this enzyme using lactose as acceptor was demonstrated (Fujita, Hara, Hashimoto, & Kitahara, 1990b) and the process of lactosucrose production was patented by Ensuiko Sugar Refining Co., Lda (Fujita, Hara, Hashimoto, & Kitahara, 1992).

At an industrial level, lactosucrose can be obtained in a batch reaction process through a transfructosylation reaction using sucrose and lactose as substrates (Arakawa et al., 2002). The enzyme involved is β-fructofuranosidase from *Arthrobacter* sp. and lactosucrose is produced by incubating the enzyme with both substrates and invertase-deficient yeast at 30–35 °C. The yeast has an important role in removing the resulting by-product glucose that can inhibit lactosucrose production (Arakawa et al., 2002). This methodology offers a saccharide mixture with about 65% of lactosucrose. However, higher lactosucrose content (between 70 and 75%) and low amount of by-products (less than 3%) can be achieved using other patented methodology for industrial application (Okabe, Aga, Kubota, & Miyabe, 2008). This methodology combines the use of β-fructofuranosidase from *Bacillus* sp. V230 and a sucrose-unassimilable yeast, such as *Saccharomyces cerevisiae*. The yeast is able to assimilate monosaccharides but it cannot consume or hydrolyse oligosaccharides, including disaccharides (Okabe et al., 2008).

The reaction involved in lactosucrose production can be described as follows:

$$\text{Sucrose} + \text{Lactose} \leftrightarrow \text{Lactosucrose} + \text{Glucose} \quad (1)$$

where β-fructofuranosidase (such as levansucrase) catalyses the transfer of the fructosyl residue from sucrose to lactose. However, other compounds rather than lactose can act as acceptors, such as water or sucrose. When water is the acceptor, a hydrolysis reaction occurs:

$$\text{Sucrose} \xrightarrow{H_2O} \text{Fructose} + \text{Glucose} \quad (2)$$

If sucrose is the acceptor, the synthesis of fructooligosaccharide (FOS) takes place. Besides the transfer reactions, the enzymes can also catalyse the parallel hydrolysis of the newly formed product, which may compromise the lactosucrose yields and productivity.

Another enzyme involved in the lactosucrose synthesis is β-galactosidase, which catalyses the transfer of the galactosyl residue from lactose (donor) to sucrose (acceptor). Again, the formed lactosucrose can also act as a galactosyl donor, thus decreasing the yield.

Product degradation during the synthesis is a problem that affects both enzymatic routes and strategies to avoid or minimize this limitation ought to be developed. These strategies may include the thermal deactivation of the enzyme to discontinue the catalytic action (Pilgrim, Kawase, Matsuda, & Miura, 2006) or the use of immobilized enzymes which can be easily separated from the reaction products (Mikuni et al., 2000). Alternatively, product degradation can be controlled through process integration combining synthesis and product separation. This strategy reduces the contact time between enzyme and product and generally favours the product formation and/or the elimination of potential inhibitors (Kawase, Pilgrim, Araki, & Hashimoto, 2001; Pilgrim et al., 2006).

Protein engineering can also give an important contribution to reduce product degradation by improving the catalytic performance of the enzymes involved in the synthesis. The proportion of transglycosylation to hydrolysis is dependent not only on the reaction conditions, namely the ratio of donor to acceptor, but also on the intrinsic properties of enzymes. Different sources of enzymes can provide different transglycosylation proportions, depending on the ability of the enzyme to bind the acceptor and to exclude water. The understanding of the structures and catalytic mechanisms involved in the enzymatic synthesis led to the development of mutated enzymes with increased transglycosylation activity and/or reduced ability to hydrolysis (Ballesteros et al., 2007). For example, Schroeven, Lammens, van Laere, and van den Enden (2008) reported a drastic shift from water to sucrose as preferred acceptor molecules, for mutant enzymes from *Triticum aestivum* expressed in *Pichia pastoris*. A mutagenesis approach applied to β-galactosidase from *Geobacillus stearothermophilus* resulted in a simultaneous improvement of the transglycosylation activity and reduction of hydrolysis to potential transglycosylation products (Placier, Watzlawick, Rabiller, & Mattes, 2009). Mutagenesis can also be useful to provide enzymes with enhanced thermostability, an important characteristic for the industrial use of enzymes (Ohta et al., 2014; Yuan et al., 2008), or to minimize the effect of some inhibitors, such as galactose (Hu, Robin, O'Connell, Walsh, & Wall, 2010) or glucose (Trollope, Nieuwoudt, Görgens, & Volschenk, 2014). Additionally, the heterologous expression of enzymes has been reported as a successful approach to achieve high levels of extracellular enzyme (Gimeno-Pérez, Linde, Fernández-Arrojo, Plou, & Fernandéz-Lobado, 2015; Oliveira, Guimarães, & Domingues, 2011) which clearly facilitate its recovery and further application.

Commercial lactosucrose production started in 1993 and the Japanese-based companies Ensuiko Sugar Refining Co., Lda. and Hayashibara Company are the main producers of this trisaccharide (Fujita et al., 1992; McSweeney & Fox, 2009; Okabe et al., 2008).

According to the literature, three different enzymes have potential to be used as biocatalysts in the enzymatic synthesis (Tables 1 and 2). Levansucrase and β -fructofuranosidase are the most widely studied. β-Fructofuranosidase is generally obtained from *Arthrobacter* sp., but for levansucrase, different microbial sources have been evaluated. Recently, β-galactosidase has also been reported for the production of lactosucrose (Li et al., 2009). The different reaction conditions and catalysts used in lactosucrose production will be discussed in the following sections, where the use of commercial or industrial preparations of enzymes is differentiated from the use of integrated processes of enzyme and lactosucrose production.

3.1. *Integrated process of enzyme and lactosucrose production*

The integrated process involves both the enzyme production and the lactosucrose synthesis. However, the biocatalysts used in the process can present different forms such as isolated enzymes or the whole cells. In the first case, two steps are included: the production of a suitable enzyme and its further application in lactosucrose production. Enzyme purification is frequently performed prior to its use in the synthesis. The most commonly techniques for enzyme purification are precipitation with salts, centrifugation and chromatography. Purification provides more active extracts and/or eliminates detrimental compounds but it also represents an additional step to the process. On the other hand, the use of whole cells as biocatalysts does not require the isolation of the enzyme and allows the simultaneous production of the enzyme and lactosucrose. Alternatively, cultivated cells can be harvested from the fermentation media by centrifugation and easily added to the reaction mixture containing the substrates for the enzymatic synthesis. Both cases represent a reduction in the time and/or costs associated to the process. Furthermore, enzymes in cells tend to be more stable since they are protected from external environment. However, it is important to highlight that when using whole cells, many other enzymes, besides the desired enzyme, are present in the reaction media, and consequently, additional unwanted by-products may be formed (Ishige, Honda, & Shimizu, 2005).

The reaction conditions and yields reported in the literature for lactosucrose production using free enzyme or whole cells are summarized in Table 1. In almost all cases, levansucrase was the enzyme involved in the synthesis; however, different microbial sources have been described.

3.1.1. *Free enzyme*

Avigad (1957) obtained crude levansucrase from *Aerobacter levanicum* (after cell disruption) and used it, for the first time, to produce lactosucrose at pH = 5.4, with lactose in excess (292 g/L lactose, 100 g/L sucrose) and in the presence of *T. glabrata* cells. The reaction mixture was incubated at 30 °C with toluene and was sporadically agitated. After 9 hours, fresh sucrose was added to the reaction mixture and 13 hours later the addition of ethanol (96% v/v) promoted the precipitation of the enzyme and also some lactose. Chromatographic analysis revealed that besides lactosucrose, a small amount of a

Table 1 – Integrated process of enzyme and lactosucrose production.

Enzyme	Microbial source	Conditions of use	pH	Temp. (°C)	Initial substrate concentration (g/L)	Sugar conversion[a] (%)	Maximum lactosucrose production (g/L)	Productivity (g/L h)	Reference
Levansucrase	*Aerobacter levanicum*	Crude free enzyme	5.4	30	292(lactose) 50(sucrose) at the beginning +50(sucrose) after 9 hours	NI[b]	NI[b]	NI[b]	Avigad, 1957
Levansucrase	*Bacillus natto*	Free purified enzyme	6.2	35	85.5 (lactose) 85.5 (sucrose)	42 (lactose) 54 (sucrose)	53[c]	26.5	Takahama et al., 1991
β-Fructofuranosidase	*Bacillus* sp. 417	Crude free enzyme	5.6	45	100 (lactose) 100 (sucrose)	NI[b]	54[c]	6.8	Ikegaki and Park, 1997
Levansucrase	*Paenibacillus polymyxa*	Free whole cells	6.0	55	225 (lactose) 225 (sucrose)	42 (lactose) NI[b] (fructose)	170	28	Choi, Kim, Kim, Jung, & Oh, 2004
		Crude free enzyme	6.0	40	225 (lactose) 225 (sucrose)	NI[b]	140[c]	210[c]	
Levansucrase	*Bacillus amyloliquefaciens* IFO15535	Free whole cells	6.0	55	24 (lactose) 34 (sucrose)	NI[b]	4.8	4.8	Park, Choi, & Oh, 2005
	Bacillus subtilis KCCM32835		6.0	55	225 (lactose) 225 (sucrose)	44 (lactose) 64 (sucrose)[c]	183	18.3	
	Geobacillus stearothermophilus ATCC12980		6.0	55	24 (lactose) 34 (sucrose)	NI[b]	10.4	10.4	
	Paenibacillus polymyxa KCCM35411		6.0	55	24 (lactose) 34 (sucrose)	NI[b]	14.1	14.1	
	Pseudomonas syringae IFO14086		6.0	40	24 (lactose) 34 (sucrose)	NI[b]	3.9	3.9	
	Rahnella aquatilis KCTC2858		6.0	40	24 (lactose) 34 (sucrose)	NI[b]	5.6	5.6	
	Sterigmatomyces elviae ATCC18894		6.0	50	24 (lactose) 34 (sucrose)	NI[b]	7.5	7.5	
Levansucrase	*Bacillus subtilis* NCIMB 11871 expressed in *Escherichia coli*	Crude recombinant enzyme	6.0	NI[b]	410 (lactose) 205 (sucrose)	NI[b] (lactose) 77 (sucrose)[c]	131[c]	21.9	Seibel et al., 2006
Levansucrase	*Pseudomonas aurantiaca*	NI[b]	4.0	45	360 (lactose) 510 (sucrose)	NI[b]	285	142.5	Han et al., 2007

(*continued on next page*)

Table 1 – *(continued)*

Enzyme	Microbial source	Conditions of use	pH	Temp. (°C)	Initial substrate concentration (g/L)	Sugar conversion[a] (%)	Maximum lactosucrose production (g/L)	Productivity (g/L h)	Reference
NI[b]	*Arthrobacter mysorens* ATCC 33408	Free whole cells	6.0	50	300 (lactose) 300 (sucrose)	NI[b]	15	1.0	Lee, Lim, Park, et al., 2007
	Klebsiella pneumoniae ATCC 25306	Free whole cells	6.0	50	300 (lactose) 300 (sucrose)	NI[b]	16	1.1	
	Rahnella aquatilis ATCC 55046	Free whole cells	6.0	50	300 (lactose) 300 (sucrose)	NI[b]	79	5.3	
	Sterigmatomyces elviae ATCC 18894	Free whole cells	6.0	50	300 (lactose) 300 (sucrose)	NI[b]	91	6.1	
		or mutant cells immobilized on calcium alginate beads	6.0	50	250 (lactose) 250 (sucrose)	NI[b] (batch) NI[b] (continuous)	192 (batch) 180 for 48 days (continuous)	9.6 (batch) –	
NI[b]	*Sterigmatomyces elviae* ATCC 18894	Mutant free whole cells	6.0	50	250 (lactose) 250 (sucrose)	NI[b]	141 (basal medium) 184 (optimal medium)	9.4 (basal medium) 12.3 (optimal medium)	Lee, Lim, Song, et al., 2007
Levansucrase	*Zymomonas mobilis* expressed in *Escherichia coli*	Crude recombinant enzyme	7.0	23	180 (lactose) 180 (sucrose)	28 (lactose)[c] 66 (sucrose)[c]	103	25.8	Han et al., 2009
Levansucrase + glucose oxidase			6.0	30	180 (lactose) 180 (sucrose)	33 (lactose)[c] 66 (sucrose)[c]	156	34.7	
Levansucrase	*Bacillus methylotrophicus*	Crude free enzyme	6.5	37	200 (lactose) 200 (sucrose)	40 (lactose)[c] 48 (sucrose)[c]	143	7.2	Wu et al., 2015

[a] When maximum lactosucrose production was obtained.
[b] NI, not indicated.
[c] Obtained from graphical extrapolation or text information and converted to appropriate units.

fructose-containing non-reducing tetrasaccharide and a trace of a fructose-containing reducing trisaccharide were also produced. The product was purified using a chromatographic column containing Darco G-60 and Celite 535 (1:1) and it was subsequently characterized. Nevertheless, no information about the sugar conversion or lactosucrose yield was reported.

Takahama et al. (1991) used free levansucrase from *Bacillus natto* to obtain lactosucrose. In this case, the enzyme was previously purified by precipitation with ammonium sulfate and through chromatographic methods. The enzymatic reaction occurred at the optimal pH and temperature found for levansucrase, i.e. 6.2 and 35 °C, respectively. Maximum lactosucrose production (approximately 53 g/L) was obtained after 2 hours of reaction using an equal substrate concentration. The production of lactosucrose by immobilized levansucrase was also evaluated using different supports, such as DEAE-Toyopearl 650M, hydroxyapatite and molecular sieves of 5 Å. However, in all cases, lactosucrose production was lower as compared to the results obtained for the free enzyme.

Ikegaki and Park (1997) selected *Bacillus* sp. No. 417 as a promising β-fructofuranosidase producer from a large number of microorganism strains isolated from soil, flower and fruit samples. Furthermore, this strain also presented high transfer activity and proved to be able to produce lactosucrose, using lactose and sucrose as substrates. The optimal conditions for lactosucrose synthesis were established at 45 °C, pH 5.6, and 20% (w/w) of lactose and sucrose (ratio 1:1). Under these conditions a concentration of 54 g/L of lactosucrose was obtained after 8 hours of reaction, which is very similar to that reported by Takahama et al. (1991).

Choi, Kim, Kim, Jung, and Oh (2004) studied the lactosucrose production using levansucrase from *Paenibacillus polymyxa*. Different biocatalyst conditions were compared, namely crude free enzyme and whole cells. Crude levansucrase was obtained after cell disruption and subsequent precipitation of the supernatant with sodium sulfate. For free enzyme conditions, approximately 140 g/L of lactosucrose were produced in 40 minutes. However, this lactosucrose concentration was lower than that obtained when whole cells were used (see Whole cells section for further details).

The use of recombinant levansucrase to obtain lactosucrose is also reported in the literature. Seibel et al. (2006) expressed a recombinant levansucrase from *Bacillus subtilis* in *Escherichia coli* and studied the acceptor specificity and catalytic mechanism involved in the production of new sucrose analogues and oligosaccharides. Productivity around 21.9 g/L h was achieved for lactosucrose using lactose as the acceptor. In a different study, recombinant levansucrase from *Zymomonas mobilis* was expressed in *E. coli* and the enzymatic extract obtained after cell disruption was used in lactosucrose synthesis, without further purification (Han et al., 2009). Lactosucrose production was investigated at the optimal conditions found (23 °C, pH = 7.0, 18% (w/v) lactose and 18% (w/v) sucrose). Under these conditions, a lactosucrose conversion efficiency of 28.5% was obtained. With the aim to increase lactose and sucrose conversion to lactosucrose, a mixed-enzyme system composed of levansucrase and glucose oxidase was also tested. Glucose oxidase contributed to reduce glucose content in the reaction mixture, by converting glucose to gluconic acid. In addition, it was demonstrated that the mixed-enzyme system increased the efficiency of conversion to 43.2%.

In a brief abstract report, Han et al. (2007) described the optimal conditions (45 °C, pH = 4.0, 36% (w/v) lactose and 51% (w/v) sucrose) to produce lactosucrose using levansucrase from *Pseudomonas aurantiaca*. After 2 hours of reaction, the lactosucrose concentration reached 285 g/L, representing the best yield found in the literature so far (Table 1). However, no details on the conditions of use of the levansucrase were reported.

The extracellular β-fructofuranosidase from *Bacillus* sp. V230, previously described as catalyst for lactosucrose production in the Japanese Patent Kokai No. 224665/97, was employed in a new methodology patented by Okabe et al. (2008). The enzyme was successfully used in combination with *Saccharomyces cerevisiae,* which is able to consume the monosaccharides present in the reaction medium and consequently enhance lactosucrose yield. In this methodology, crude β-fructofuranosidase was concentrated and purified through ion-exchange and hydrophobic chromatography prior to be added to the reaction mixture containing 20% (w/w) of sucrose, 20% (w/w) of lactose and yeast cells. Lactosucrose synthesis occurred at 30 °C, under pH conditions controlled to 4.0–5.5, and provided a saccharide mixture with more than 70% of lactosucrose after 24 hours (Okabe et al., 2008).

Recently, an intracellular levansucrase from *Bacillus methylotrophicus* was described for lactosucrose production (Wu, Zhang, Mu, Miao, & Jiang, 2015). The enzyme was obtained after cell disruption and it was used without further purification in the enzymatic synthesis under pH 6.5, 37 °C and 400 g/L substrate concentration (lactose to sucrose 1:1). Maximal lactosucrose concentration (143 g/L) was obtained after 20 hours of reaction. The study showed that high substrate concentration has a positive effect in the lactosucrose production, probably because at higher concentration, the substrate can be more effective when competing with water to act as acceptor. The study also revealed that concentration ratio of substrates has great influence in lactosucrose production. The enzyme performance was considerably better when a ratio of 1:1 sucrose to lactose was used instead of 1:2 or 2:1 (around 100 g/L in both cases).

3.1.2. *Whole cells*

Choi et al. (2004) reported for the first time the use of whole cells as biocatalysts in the lactosucrose production. These authors obtained a higher lactosucrose concentration with whole cells of *Paenibacillus polymyxa* when compared to free levansucrase from the same microorganism. Whole cells also presented a higher optimal temperature (55 °C). This fact can justify the higher lactosucrose concentration observed because increased temperature favours the substrate solubility and reaction rate. Furthermore, the cell wall seemed to shield levansucrase from denaturation caused by temperature. High substrate concentrations (225 g/L lactose, 225 g/L sucrose) also appeared to protect the enzyme in whole cells from denaturation. Maximum lactosucrose production (170 g/L) was detected after 6 hours of reaction.

Park, Choi, and Oh (2005) evaluated the production of lactosucrose by several bacteria containing levansucrase activity. A total of seven microorganisms (*Bacillus amyloliquefaciens*,

B. subtilis, *G. stearothermophilus*, *Paenibacillus polymyxa*, *Pseudomonas syringae*, *Rahnella aquatilis* and *Sterigmatomyces elviae*) were selected and their efficiency to produce lactosucrose when using whole cells was compared. The highest lactosucrose concentration after 1 hour of reaction at optimal pH and temperature was found for *B. subtilis* (15 g/L). Therefore, this microorganism was selected to be used in a further study in which the substrate concentration was increased to the values previously reported by Choi et al. (2004): 225 g/L lactose and 225 g/L sucrose. Under these conditions a maximal concentration of 183 g/L was reached after 10 hours of reaction, which represents an increase in lactosucrose production if compared with the production achieved by Choi et al. (2004). However, a higher reaction time was needed to obtain the maximum concentration.

In a different study, Lee et al. (2007) evaluated the lactosucrose-production activities of *Arthrobacter mysorens*, *Klebsiella pneumoniae*, *Rahnella aquatilis* and *Sterigmatomyces elviae* in order to select the most promising microorganism to be used in a continuous process of lactosucrose production involving cell immobilization. *Sterigmatomyces elviae* presents a higher cell size than bacteria, being more adequate for continuous production in large scale. Furthermore, it also showed a higher activity, providing a lactosucrose concentration of 91 g/L after 15 hours. However, its activity was considered reduced by the authors and the microorganism was subsequently mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in order to improve the lactosucrose-production activity. Continuous production of lactosucrose in a packed-bed reactor under optimized conditions (pH = 6.0, 50 °C, 25% lactose and 25% sucrose) were evaluated using the mutant whole cells immobilized in alginate beads. It was shown that the feed flow rate can influence lactosucrose production by accelerating the degradation of sucrose and inhibiting the fructosyltrasfer reaction. A lactosucrose production of 180 g/L was maintained for 48 days. However, after that period of time the beads were swollen and cell leakage occurred, leading to a decrease in the production. In order to increase the cell mass and lactosucrose production by *Sterigmatomyces elviae*, the same research group optimized the culture media using analysis of variance (ANOVA) and response surface methodology (RSM) (Lee et al., 2007). An increase in lactosucrose concentration was obtained comparing the results for the basal medium (140.91 g/L) and the optimized medium (183.78 g/L). Furthermore, the experimental value obtained for lactosucrose was not distant from the predicted maximum value determined (194.12 g/L).

3.2. *Lactosucrose production using commercial/industrial enzyme mixtures*

Table 2 summarizes the results obtained for lactosucrose production using commercial or industrial enzyme mixtures. β-Fructofuranosidase is the most used enzyme and it was tested both in free and immobilized conditions. β-Galactosidase is introduced as an alternative to the conventional route of lactosucrose synthesis using transfructosylation.

3.2.1. *Free enzyme*

Pilgrim et al. (2001) studied the lactosucrose production using β-fructofuranosidase obtained from a sugar refining company. They reported the transfructosylation mechanism as an ordered bi-bi type where sucrose is first bound to β-fructofuranosidase and lactosucrose is released afterwards. Furthermore, it was shown that side reactions of sucrose and lactosucrose hydrolysis were inhibited by glucose and lactose, respectively, but no inhibition effect was observed from fructose. In order to improve the lactosucrose production and reduce side reactions, Kawase et al. (2001) used a simulated moving bed reactor (SMBR) and β-fructofuranosidase obtained from the same sugar refining company. In this process, lactosucrose and glucose were separated inside the reactor, migrating to the raffinate and extract ports, respectively. Thus, enzymatic reaction and separation occurred simultaneously, improving the lactosucrose yield. Nevertheless, the expected lactosucrose yield was not reached because strong product hydrolysis was detected around the raffinate port. The maximum overall yield was 53% when sucrose conversion reached 70%. To overcome this problem, some changes were suggested, namely the enzyme cannot be eluted into the raffinate port; the enzyme has to be inactivated when it is eluted into the raffinate port; or the use of SMBR with columns of immobilized enzyme. This process of lactosucrose production using SMBR was later optimized (Pilgrim et al., 2006). An appropriate model was developed taking into account the effect of several process parameters, including enzyme deactivation (to reduce produce hydrolysis). It was shown that temperature influences both the sugar reaction rates and the deactivation rate of the enzyme. The maximum predicted lactosucrose yield obtained by simulation was 69% at 65 °C.

The potential of β-galactosidase from *B. circulans* to produce lactosucrose and other galactosylated derivatives using lactose and sucrose as substrates was already reported (Farkas et al., 2003; Li et al., 2008). Nevertheless, Li et al. (2009) showed for the first time, in a complete study, the effectiveness of a commercial β-galactosidase from *B. circulans* to catalyse lactosucrose synthesis, being the chemical structure of the trisaccharide confirmed by NMR analysis. Besides lactosucrose, other transfer products (tri- and tetrasaccharides) were detected and identified. Furthermore, it was shown that the amount and composition of all transfer products changed considerably during the enzymatic reaction. For that reason, the reaction conditions (temperature, reaction time, substrate concentration, molar ratio donor/acceptor and enzyme concentration) were optimized to increase the production of lactosucrose. A maximum of 56 g/L of lactosucrose was obtained after 4 hours of reaction under the optimal conditions.

3.2.2. *Immobilized enzyme*

As far as we know, the work from Mikuni et al. (2000) is the only reference available in the literature regarding the use of an immobilized enzyme for lactosucrose production. In this work, β-fructofuranosidase from *Arthrobacter* sp. was obtained from a sugar refining company and immobilized onto a carrier resin (FE4611). The continuous production of lactosucrose on a pilot plant scale was studied using a column reactor containing the immobilized enzyme. The reactor was successfully operated for 35 days, providing concentrations of lactosucrose around 120 g/L. These results suggested the possibility of lactosucrose production at an industrial scale.

Table 2 – Lactosucrose production using commercial/industrial enzyme mixtures.

Enzyme	Microbial source	Supplier	pH	Temp. (°C)	Initial substrate concentration (g/L)	Sugar conversion[a] (%)	Maximum lactosucrose production (g/L)	Productivity (g/L h)	Reference
β-Fructofuranosidase immobilized on carrier resin (FE4611)	*Arthrobacter* sp.	Ensuiko sugar refining company, Japan	6.5	55	200 (lactose) 200 (sucrose)	NI[b]	120[c] for 35 days (continuous)	–	Mikuni et al., 2000
β-Fructofuranosidase	*Arthrobacter* sp.	Ensuiko sugar refining company, Japan	NI[b]	50	137 (lactose)[c] 137 (sucrose)[c]	47 (lactose)[c] 56 (sucrose)[c]	93[c]	63.4[c]	Pilgrim et al., 2001
			NI[b]	43	137 (lactose)[c] 222 (sucrose)[c]	33 (lactose)[c] 25 (sucrose)[c]	202[c]	60.6[c]	
β-Fructofuranosidase	*Arthrobacter* sp.	Ensuiko sugar refining company, Japan	NI[b]	50	181 (lactose) 171 (sucrose)	NI[b] (lactose) 70(sucrose)	133[c] (overall yield 53%) 9[c] (in the raffinate after 1320 min) using a simulated moving bed reactor	NI[b]	Kawase, Pilgrim, Araki, & Hashimoto, 2001
β-Fructofuranosidase	*Arthrobacter* sp.	Ensuiko sugar refining company, Japan	NI[b]	50	342 (lactose) 342 (sucrose)	NI[b]	348[c] (overall yield in the simulation 69%) 40[c] (in the raffinate after 3005 min)using a simulated moving bed reactor	NI[b]	Pilgrim, Kawase, Matsuda, & Miura, 2006
β-Galactosidase	*Bacillus circulans*	Daiwa Kasei Co. Ltd., Japan	6	40	300 (lactose) 300 (sucrose)	70 (lactose) 47 (sucrose)	56	14	Li et al., 2009

[a] When maximum lactosucrose production was obtained.
[b] NI, not indicated.
[c] Obtained from graphical extrapolation or text information and converted to appropriate units.

4. Lactosucrose quantification and purification

Lactosucrose detection and quantification is essentially based on chromatographic methods. In the first work describing lactosucrose production, Avigad (1957) detected the presence of lactosucrose using paper chromatography and specific reagents to identify fructose-containing saccharides. Additionally, clear confirmation of the trisaccharide structure was obtained from the information collected from differential identification of glycosidic radicals using enzymatic reagents, from the products of lactosucrose oxidation with periodate, and also from the value of optical rotation.

All other references about lactosucrose production describe high performance liquid chromatography (HPLC), with refractive index or electrochemical detector, as the technique used for carbohydrate determination and quantification. Nevertheless, different types of HPLC columns have been reported for sugar separation, such as

- silica-based columns with: aminopropyl groups (Agilent Zorbax NH_2), secondary/tertiary amine (COSMOSIL Sugar-D) or carbamoyl groups (TSKgel Amide-80), eluted with mixtures of acetonitrile/water (Kawase et al., 2001; Lee et al., 2007; Li et al., 2009);
- polymer-based column with amino groups (Shodex Asahipak NH_2P-50 4E), eluted with mixtures of acetonitrile/water (Wu et al., 2015);
- C18-amine column (Kromasil KR100-10NH_2) eluted with mixtures of acetonitrile/water (Han et al., 2009; Park et al., 2005);
- cation-exchange columns packed with sulfonated copolymer in the sodium form (Na^+) (Shodex Sugar KS-801) or in the calcium form (Ca^{2+}) (Rezex RCM-monossacharide Ca^{+2}) eluted with ultrapure water (Li et al., 2009; Seibel et al., 2006);
- anion-exchange columns packed with quaternary ammonium functionalized stationary phase (Dionex, CarboPac PA1), using gradient elution with NaOH and sodium acetate (Choi et al., 2004).

To unquestionably confirm the presence of lactosucrose, its structure can be determined by ^{1}H and ^{13}C NMR analyses after separation and purification from the reaction mixture (Han et al., 2009; Li et al., 2009; Seibel et al., 2006). Other alternative *technique* to assess carbohydrate structures could be mass spectroscopy. The use of a separation technique, such as chromatography, coupled to mass spectroscopy, with electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), has been described for oligosaccharide separation and analysis (Kailemia, Ruhaak, Lebrilla, & Amster, 2014). These coupled techniques could also be suitable for lactosucrose separation and detection. However, to the best of our knowledge, there are still no reports on their use for this compound.

The composition of the sugar mixtures obtained at the end of enzymatic synthesis of lactosucrose is dependent on the reaction conditions and type of biocatalyst used. These mixtures may contain variable amounts of unreacted substrates (lactose and sucrose), glucose, fructose and galactose (formed from enzymatic hydrolysis of sucrose or lactose) and unknown saccharides resulting from the transfer action of the enzymes. The removal of monosaccharides, such as glucose and galactose, can be easily achieved using microbial fermentation. Suitable yeasts, like *Saccharomyces cerevisiae*, can successfully reduce the amount of monosaccharides in sugar mixtures, by fermenting them into ethanol and CO_2, without any negative effect on the oligosaccharide content (Hernandéz, Ruiz-Matute, Olano, Moreno, & Sanz, 2009). Some patented methodologies for industrial production of lactosucrose include this kind of fermentation also for reducing enzyme inhibition by monosaccharides during the synthesis (Fujita et al., 1992; Okabe et al., 2008).

Some lactosucrose purification can also be obtained using SMB technology, as described in Section 3.2. This technique can be applied to different kinds of chromatography and has been used in the sugar industry for large scale separations (Geisser, Hendrich, Boehm, & Stahl, 2005). In lactosucrose production, the use of a SMB reactor composed of several columns filled with ion-exchange resin (Amberlite) allowed the trisaccharide separation from glucose during the enzymatic synthesis (Kawase et al., 2001).

Lactosucrose has been isolated and purified from the reaction mixture using conventional techniques, such as decolonization and removal of unreacted substrates using activated charcoal and Celite columns (Li et al., 2009); deionization and separation of oligosaccharides using ion-exchange resins (Seibel et al., 2006); or removal of the unreacted substrates by crystallization (Okabe et al., 2008) or precipitation with ethanol (Avigad, 1957). These techniques are described both for lab and industrial purification of lactosucrose.

Lactosucrose crystallization from its supersaturated solution can be achieved using temperature control and addition of organic solvents, such as methanol, ethanol and acetone to control the degree of supersaturation and viscosity (Okabe et al., 2008).

There are also some techniques described in the literature for the separation and purification of oligosaccharide mixtures which could be adequate for mixtures containing lactosucrose. For example, nanofiltration using suitable ultrafiltration membranes (Goulas, Kapasakalidis, Sinclair, Rastall & Grandison, 2002) and supercritical extraction based on carbon dioxide (Montañés, Olano, Reglero, Ibáñez & Fornari, 2009) were successfully used to fractionate and concentrate mixtures of oligosaccharides. Nevertheless, as far as we know there are no reports on their use for lactosucrose.

5. Benefits associated to lactosucrose ingestion

Several physiological benefits associated with the consumption of lactosucrose have been reported. These benefits result essentially from its resistance to digestion and ability to be fermented by intestinal microbiota. Lactosucrose, as a potential prebiotic, has an important role in the modulation of microbiota composition. Furthermore, microbial fermentation of lactosucrose generates interesting end products such as short-chain fatty acids (SCFAs). These compounds have recognized beneficial effect to the host by reducing the pH and enhancing mineral bioavailability (Roberfroid et al., 2010). Intestinal

Table 3 – Physiological action of lactosucrose and its practical effects in animals and humans.

		Animal	Human
Physiological action	Enhancement of beneficial bacteria and/or inhibition of pathogenic bacteria	Rats (Honda et al., 1999; Yoneyama et al., 1992b) Dogs (Terada et al., 1992) Cats (Terada et al., 1993) Chickens (Terada et al., 1994)	Fujita et al., 1991a; Iwagaki et al., 1991; Kumemura et al., 1992; Iwagaki et al., 1993; Takumi et al., 2001; García-Cayuela et al., 2014; Li et al., 2015
	Decrease of faecal pH, production of SCFAs[a] and gases, reduction of putrefactive products	Rats (Yoneyama et al., 1992b) Dogs (Terada et al., 1992) Cats (Terada et al., 1993) Chickens (Terada et al., 1994) Fishes (Kihara et al., 1995; Kihara & Sakata, 2001; 2002)	Kumemura et al., 1992
	Enhancement of intestinal absorption of minerals	Rats (Fujita et al., 1999; Kishino et al., 2006) Fishes (Kihara et al., 2007)	Teramoto et al., 2006
Practical effects	Prevention of obesity	Rats (Mizote et al., 2009; Okuda & Han, 2001)	–
	Improvement of the immune response	Rats (Hino et al., 2007) Fishes (Chu et al., 2013)	–
	Prevention of allergic diseases	Rats (Taniguchi et al., 2007)	–
	Reduction of faecal odour	Dogs (Terada et al., 1992) Cats (Terada et al., 1993) Chickens (Terada et al., 1994)	Kumemura et al., 1992
	Treatment of chronic inflammatory bowel diseases	Rats (Honda et al., 1999; Zhou et al., 2014, 2015a, 2015b)	Teramoto et al., 1996
	Contribution to calcium accumulation in bones	Rats (Fujita et al., 1999; Kishino et al., 2006)	Teramoto et al., 2006
	Normalization of intestinal microflora	Rats (Honda et al., 1999)	Iwagaki et al., 1991; Iwagaki et al., 1993
	Prevention of abdominal symptoms of lactose intolerance	–	Oku, Kasagi, Sawatani, Fukuda & Kurimoto, 2002

[a] SCFAs – short chain fatty acids.

fermentation of lactosucrose, as well as its positive action in health, has been demonstrated using both *in vitro* and *in vivo* studies with humans and animals. The physiological action and practical effects of lactosucrose consumption are summarized in Table 3.

5.1. *In vitro studies*

The *in vitro* studies performed with lactosucrose were essentially focused on its resistance to digestion and its ability to be selectively fermented by intestinal microbiota.

Lactosucrose resistance to digestion in the upper gastrointestinal tract was confirmed in a comparative *in vitro* study using human and rat small intestinal homogenates. The results obtained indicated that lactosucrose was barely hydrolysed and the hydrolysing activity was similar for human and rat intestinal enzymes (Oku et al., 2011).

In vitro fermentation of lactosucrose was evaluated using potential probiotic stains such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* and it was observed that lactosucrose promoted the growth of four bacterial strains (*Streptococcus salivarius*, *Lactobacillus casei*, *Lactobacillus reuteri* and *Lactobacillus acidophilus*) out of seven tested (García-Cayuela et al., 2014).

In fishes, *in vitro* fermentation of lactosucrose was demonstrated using intestinal microbiota collected from the carnivorous teleost *Pagrus major* (Kihara, Ohba, & Sakata, 1995), the carp *Cyprinus carpio* L. (Kihara & Sakata, 2002) and the rainbow trout *Oncorhynchus mykiss* (Kihara & Sakata, 2001). The degree of fermentation was evaluated by the release of gases and the production of SCFAs. The results obtained demonstrated that *in vitro* fermentation of lactosucrose can occur in herbivorous, omnivorous and carnivorous fishes (Kihara et al., 1995).

The increase of bifidobacteria and lactobacilli populations and the reduction of the numbers of bacteroides and clostridia confirmed the occurrence of *in vitro* digestion of lactosucrose by the human intestinal microbiota (Li et al., 2015). The production of SCFAs was also observed.

5.2. *In vivo studies with animals*

The effects of lactosucrose consumption were evaluated in several animals and positive results were obtained (Table 3). The enhancement of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* spp., and the inhibition of pathogenic bacteria, like *Clostridium perfringens* or Bacteroidaceae, was observed after lactosucrose consumption by rats (Yoneyama et al., 1992b), broiler chickens (Terada et al., 1994), shepherd dogs (Terada et al., 1992) or cats (Terada et al., 1993). In some cases, all these parameters returned to normal when lactosucrose was removed from diet (Yoneyama et al., 1992b).

Production of SCFAs, like acetic and butyric acids, and consequent reduction in the pH of faecal contents were also noticed, confirming the lactosucrose fermentation by the microbiota

(Terada et al., 1992, 1993, 1994; Yoneyama et al., 1992b). Furthermore, a decrease in putrefactive substances, such as ammonia, phenol or skatole, was observed, which contributed to a reduction of faeces odour (Terada et al., 1992, 1993, 1994).

The production of SCFAs by lactosucrose fermentation lowers the intestinal pH value, which has a practical effect in the enhancement of calcium solubility and consequently its absorption from the intestine. Calcium distribution and accumulation in bones can be followed through ^{45}Ca administration into the stomach or analysed by X-ray densitometry. Rats fed with lactosucrose presented a significant increase of calcium accumulation in femur and tibia, suggesting that lactosucrose consumption can stimulate osteogenesis and help in the maintenance of bone strength (Fujita et al., 1999; Kishino et al., 2006). The ingestion of lactosucrose increased calcium retention not only in mammals but also in marine teleosts. Studies carried out with red sea bream *Pagrus major* showed that dietary lactosucrose affected the calcium metabolism, leading to a greater increase of calcium content in scales (Kihara, Kiryu, & Sakata, 2007).

Furthermore, lactosucrose can have a trophic effect by stimulating the growth of intestinal tunica muscularis, which resulted in increased thickness and toughness of this muscular layer in *Pagrus major* (Kihara et al., 1995).

The maintenance of intestinal microflora in the small intestine of rats also resulted in a protective effect on intracolonic indomethacin-induced ulcers (Honda et al., 1999).

Other practical effect of lactosucrose intake was the inhibition of the 2-mono-oleoyl-glycerol absorption by the brush border membrane vesicles and also the reduction of plasma triacylglycerol levels in female mice (Han et al., 1999). Moreover, lactosucrose caused a reduction in the parametrial adipose tissue weight in rats (Okuda & Han, 2001) due to the suppression of body fat accumulation. For that reason, lactosucrose was suggested as a promising dietary supplement for obesity prevention (Mizote et al., 2009).

Lactosucrose supplementation can positively affect the serum metabolome in trinitrobenzene sulfonic acid-induced colitic rats, resulting in a beneficial protection to the host against ammonia toxicity and oxidative injury (Ruan et al., 2013). The potential nutraceutical action of lactosucrose on the combat of colitis in rats was demonstrated by its positive effect on the prevention or inhibition of trinitrobenzene sulfonic acid-induced chronic inflammation (Zhou et al., 2014, 2015b). Therefore, lactosucrose can be used as a functional food for patients with inflammatory bowel disease (Zhou et al., 2015a).

The changes that occurred in the intestinal environment of microflora of rats after lactosucrose ingestion can indirectly enhance the levels of immunoglobulin A, an antibody which has an important role in mucosal immunity (Hino et al., 2007). As a result, lactosucrose can indirectly regulate mucosal immune response. Furthermore, lactosucrose supplementation to mice had a suppressive effect on antigen-specific immunoglobulin E (IgE) production, which suggested the potential use of lactosucrose in the prevention of IgE-mediated allergic diseases (Taniguchi et al., 2007).

The addition of lactosucrose to the conventional diet of grass carp juveniles (*Ctenopharyngodon idella*) showed an improvement in the growth performance, surviving rate and immunity of the specie. Moreover, the results obtained suggested the potential use of lactosucrose as an alternative dietary antibiotic in fish aquaculture (Chu et al., 2013).

5.3. In vivo *studies with humans*

There are also some studies reporting the effect of lactosucrose on humans (Table 3). Lactosucrose consumption by healthy adults (Fujita et al., 1991a; Hara et al., 1994; Ogata et al., 1993; Takumi et al., 2001; Yoneyama et al., 1992a) and elderly patients with constipation (Kumemura et al., 1992) had a positive impact in the number of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* spp., and a detrimental effect on the number of pathogenic bacteria, like *Clostridia* sp. Furthermore, the levels of blood glucose or serum insulin were not significantly changed after lactosucrose ingestion (Fujita et al., 1991a) by healthy adults. The most significant changes occurred on faecal pH, which suffered a considerable reduction due to the production of SCFAs, namely acetic and *n*-butyric acids (Kumemura et al., 1992). The concentration of harmful putrefactive products in faeces, such as *p*-cresol, indole, skatole and ammonia, was also reduced (Kumemura et al., 1992).

Lactosucrose intake can have a practical effect in the increase of stool frequency, faecal weight and moisture. Furthermore, the subject sensation of defection can be improved after lactosucrose ingestion and inconvenient symptoms such as abdominal pain, distension, stomach rumble, or nausea were not reported (Kumemura et al., 1992).

The administration of lactosucrose had a positive impact in the recovery of colonic environment in patients with resected colon (Iwagaki et al., 1991) and also in normalization of the intestinal microflora in a female patient suffering from diarrhoea after total colectomy (Iwagaki et al., 1993).

Lactosucrose can also have a beneficial effect in the treatment of patients with chronic inflammatory bowel diseases, like Crohn's disease and ulcerative colitis (Teramoto et al., 1996). The prevention of abdominal symptoms in lactose-intolerant patients can be achieved by the administration of lactosucrose (Oku, Kasagi, Sawatani, Fukuda, & Kurimoto, 2002). Additionally, long-term consumption of lactosucrose enhanced the intestinal absorption of calcium, magnesium and phosphorus and reduced bone resorption in healthy young women (Teramoto et al., 2006).

The effective dosage of lactosucrose with physiological effect on faecal flora and faecal metabolites for healthy humans was determined as 1–3 g/day (Hara et al., 1994; Ogata et al., 1993; Yoneyama et al., 1992a). Furthermore, the ingestion of a given daily amount of lactosucrose divided into two or more doses reduced the occurrence of diarrhoea comparatively to the ingestion of the same amount in a single dose (Oku & Okazaki, 1999). The laxative threshold of lactosucrose was determined as 0.802 g/kg body weight, which is significantly higher than those reported for other non-digestible carbohydrates such as the disaccharide lactulose (0.26 g/kg body (Oku & Okazaki, 1998)).

6. Potential applications of lactosucrose

In the past two decades, a large number of functional foods containing lactosucrose have been introduced into the market. In Japan, lactosucrose was classified as FOSHU ingredient and

it is widely used in diverse healthy foods and drinks to modify gastrointestinal conditions and improve well-being.

Lactosucrose, as a low-digestive and low-cariogenic sweetener, can be included in foods and beverages such as bakery products (Ueda et al., 2000), yogurts (Takumi et al., 2001), ice creams, infant formula, snacks, cookies, desserts or candies (Naito, Aoyama, Fujita, & Takahashi, 1994). Its incorporation in chocolates, chewing gum, instant juice, instant soup and mineral water was also claimed in several patents (Fujii, Mizoguchi, Okimura, & Shinoda, 2006; Okabe et al., 2008).

Furthermore, lactosucrose was added to pet food to simultaneously regulate intestinal microflora and reduce the unpleasant odour of faeces and urine (Fujimori, 1994), and also to fish feed to improve nutrient absorption and decrease self-contamination by excretion (Kihara et al., 2001).

The application of lactosucrose in pharmaceutical and cosmetic products was described in some patents, where it acts as excipient (Bassarab et al., 2005), as nutritional support and microflora regulator (Garssen, van Tol, Jben, & Verlaan, 2009), or as active ingredient in the prevention of some skin diseases (Nobuaki, 1998).

Additional applications of lactosucrose include its use to mimic transglycosylation products in enzymatic tests of hydrolysis by mutated β-galactosidase (Placier et al., 2009), to induce the production of α-galactosidase (Rezessy-Szabó et al., 2003), and to stabilize proteins (Schüle, Schulz-Fademrecht, Garidel, Bechtold-Peters & Frieβ, 2008) or polyplexes (Kasper, Schaffert, Ogris, Wagner, & Friess, 2011).

The diversity of lactosucrose applications proposed since its commercial production reflects its great potential. Hence, the use of lactosucrose is not restricted to the examples described herein and it is expected that new and attractive applications will emerge in the near future.

7. General conclusions and future perspectives

Lactosucrose presents interesting physical and chemical properties which make it an attractive and advantageous substitute to the conventional sugar. Furthermore, its hydrolysis by intestinal enzymes is apparently very low, while its fermentation by intestinal microflora seems to be high. This important feature is associated to numerous beneficial effects to health and has been extensively explored during the past decades. New and promising applications of lactosucrose have been proposed, and consequently, the demand for lactosucrose has significantly increased. Besides its importance in the medical area for treatment of some specific diseases, the consumption of lactosucrose in the form of functional foods has been widely associated to good health practices and improvement of well-being. For that reason, lactosucrose has been incorporated in food and beverages, in dietary supplements and also in animal feed. Lactosucrose market is expected to increase, and as a result, the development of efficient strategies for its synthesis is of utmost importance.

The enzymatic production of lactosucrose was essentially based on the ability of levansucrase or β-fructofuranosidase to catalyse the hydrolysis of sucrose and subsequent transfer of fructosyl moiety to the acceptor lactose. Alternatively, β-galactosidase was successfully proposed as biocatalyst for the production of lactosucrose. The optimal reaction conditions for lactosucrose synthesis depend both on the type of enzyme and on the microbial source. In the same way, the product composition obtained at the end of the reaction is strongly dependent on the biocatalyst used and its ability to catalyse transfer reactions.

Protein engineering can provide a crucial contribution to the enhancement of lactosucrose yield. New mutants can be obtained with interesting specificity such as improved transglycosylation activity or reduced capacity to lactosucrose hydrolysis. This allows the possibility to guide the enzymatic reaction towards the synthesis of the desired compounds.

For lactosucrose, besides the challenge of enhancing the reaction yield, it is also necessary to validate it as an unquestionable prebiotic to be accepted by the scientific community. Lactosucrose already showed promising features as prebiotic; however, more clinical studies are needed to obtain robust data about the lactosucrose effect in humans. Additional *in vitro* and *in vivo* tests involving a large number of individuals have to be performed to definitely confirm its resistance to digestion in the upper gastrointestinal tract, its susceptibility to fermentation by the intestinal microflora and also its capability to selectively stimulate the growth and/or activity of intestinal bacteria, namely *Bifidobacterium* and *Lactobacillus* genera. Furthermore, a detailed study about lactosucrose fermentation and decomposition by gut microflora can provide valuable information and contribute to the design of novel compounds with improved properties and enhanced prebiotic effect. There is an increasing interest in finding prebiotics which could offer a higher activity at lower dosages, higher persistence in the colon without side effects, good storage and processing stability, and also additional biological activities.

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