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Review

Systematic Review on Application of Whey Towards Production of Galacto-oligosaccharide Using β-Galactosidase Enzyme from *Pichia pastoris*

Ramachandran C*, Deog-Hwan Oh*
Department of Food Science and Biotechnology, Kangwon National University, Chuncheon, Korea

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ABSTRACT - Galacto-oligosaccharides (GOS) are prebiotics that have a beneficial effect on human health by promoting the growth of probiotic bacteria in the gut, in addition to having various applications in the food industry. GOS are generally produced from lactose in a reaction catalyzed by ß-galactosidase. Synthesis of GOS from whey permeate (WP) (ultrafiltration of whey, concentrated then spray dried) using surface engineered β-galactosidase in *Pichia pastoris* (*P. pastoris*) is a novel method to convert waste into a valuable product. Cell-surface display is the expression of peptides and proteins on the surface of living cells by fusing them to functional components of cells. Surface engineered cells have many potential uses. The Flo1p flocculation functional domain, thought to be located near the N terminus, recognizes and adheres non-covalently to cell-wall components such as α-mannan carbohydrates, causing reversible aggregation of cells into flocs.

Key words : Galactooligosaccharides, ß-Galactosidase, Whey, Mannan, Flocculation

Galacto-oligosaccharides (GOS), also known as oligo-galactosyllactose, oligo-galactose, oligo-lactose or trans-galacto-oligosaccharide (TOS), belong, because of their indigestible nature, to the group of prebiotics. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and activity of beneficial bacteria in the colon. GOS are mainly used in infant milk formula, and infant foods[1]. Because of their stability, in addition to infant foods, GOS can also be incorporated into other foods like beverages (fruit juices and other acid drinks), meal replacers, fermented milks, flavored milks, and confectionery products[2]. GOS have a pleasant taste and can increase the texture and mouth feel of foods providing bulk properties similar to sucrose. GOS are resistant to salivary degradation and are not used by the oral micro biota and can therefore be used as low-cariogenic sugar substitutes[3].

β-D-Galactosidases (EC 3.2.1.23) are also referred as lactases, hydrolyze The β (1→4) linkage of lactose to glucose and galactose. It also transfer the galactose formed from lactose cleavage onto the galactose moiety of other lactose to yield GOS[4]. Different strategies have been published on the chemical synthesis[5] of oligosaccharides where chemical glycosylation seems truly nonrealistic for industrial purposes, and hence enzymatic synthesis using galactosidase is the mostly adopted method for the production of GOS from lactose[6]. Selection of whey protein as feed for the production of GOS can change the role of whey from waste to valuable products and also aiming to judge the extent of meeting the antipollution regulation demands that required for the dairy industry. Yadav[7] indicated that recovery of lactose from whey and its utilization for valuable nutraceuticals preparations solves both the problems encompassing the improved economics of whey utilization and pollution reduction, as lactose recovery itself can reduce the biological oxygen demand (BOD) value of whey by more than 80%[8].

Importance of whey and other nutritional benefit

Whey is a by-product of cheese-making and casein manufacture in the dairy industry. After the casein curd separates from the milk, the remaining watery and thin liquid is called whey. Whey can be obtained from any type of milk, with cows′ milk being the most popular[9]. There are two basic types of whey: sweet whey, originating from manufacture of cheese and casein production by the rennet coagulation of milk, and acid whey, resulting from processes

*Correspondence to: Deog-Hwan, Oh, Department of Food Science and Biotechnology, Kangwon National University, Chuncheon, Gangwon 24341, Korea
*Co-corresponding author: Ramachandran, C, Department of Food Science and Biotechnology, Kangwon National University, Chuncheon, Gangwon 24341, Korea
Email: deoghwa@kangwon.ac.kr, ramachandran865@gmail.com

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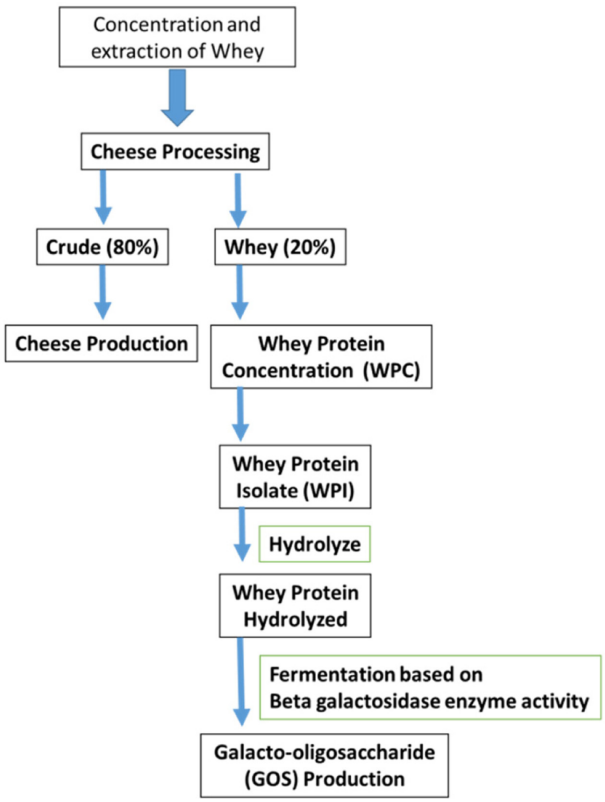

Fig. 1. Flow chart of whey protein towards biosynthesis of oligosaccharide (Prebiotic) compounds.

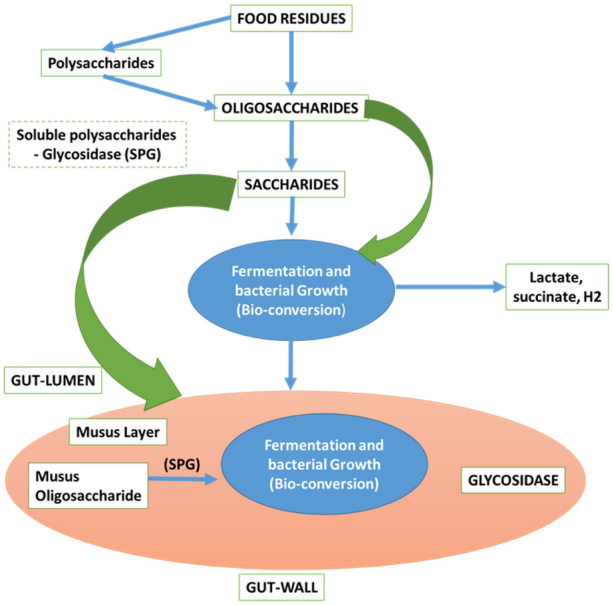

Fig. 2. Schematic representation of oligosaccharide digestion in intestine.

based on destabilization of the milk casein colloid by acidification to bellow pH 5.0[10]. Sweet whey and acid whey are generally distinguished by their pH values, which are pH>6.4 and pH 4.6-6.4, respectively[11].It comprises of 20% of total milk protein 6 g of whey protein is present in 1 L whey. It contains the full spectrum of AAs including essential AA (EAAs) and branched-chain AAs (BCAAs) which are important in tissue growth and repair. It is easily digestible so helpful for all types of target groups. Bioavailability and Protein Digestibility Corrected Amino Acid Score (PDCAA) value of whey protein is 1. Annual global milk production in 2007 is estimated of over 534 thousands of metric tons, whose transformation to cheese gave up to two thousands of metric tons of whey[12]. Whey is defined as the greenish-yellow colored liquid obtained after the coagulation of casein[13] contains approximately 20% of original milk protein. It is produced from the process that leads to curds formation during the cheese making process[14]. Whey contains nearly half of all the solids found in whole milk[15]. It has about 6.5% solids, of which 4.8% is lactose, 0.6% protein, 0.15% lactic acid, 0.25% non-protein nitrogen compounds and 0.1% fat[16]. Whey Permeate also known as De-proteinized Whey (DPW) is a modified dairy product obtained by the removal of protein from whey. Removal of the whey protein is accomplished by physical separation techniques such as precipitation, filtration or dialysis. When producing Whey Protein Concentrate (WPC), typically by ultra-filtration, a lactose rich fraction, the cheese whey permeate, is obtained. This can be used directly as feed, to produce lactose, or as substrate for fermentation such as alcoholic fermentation[17]. The lactose, the largest component in whey, is the most problematic to dispose of economically[18] (Fig. 1).

Significance of oligosaccharides

Oligosaccharides are largely indigestible in the upper intestine so they can be considered as low-molecular weight, non-viscous, water-soluble dietary fiber and useful as prebiotics. In the gastrointestinal tract they serve as substrates for probiotic or “beneficial” bacteria. The most common NDOs used as food ingredients are fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS). FOS and GOS are generally produced by enzymatic trans-glycosylation by fructano-transferase and ß-galactosidase respectively. The end products of fermentation of oligosaccharides by colonic bacteria are short chain fatty acids (SCFA), which are efficiently absorbed and utilized by human colonic epithelial cells. They are able to inhibit the growth of undesirable bacteria such as *Escherichia coli* (*E. coli*) and *Clostridium perfingens (C. perfingens)* [19] (Fig. 2).

Table 1. Physiological importance and health benefits claimed for non-digestible oligosaccharides

S.No	Physiological effects	Health factors	References
1	Stimulated carbohydrate metabolism in colonic bacteria; increased bacterial cell mass, short chain fatty acids, and fermentation gases	Through short chain fatty acids, they provide energy sources for the colonic epithelium and control of differentiation. Flatulence may be a problem. Laxative effects	22
2	Selection of *Bifidobacterium* and lactic acid bacterial growth in large bowel	Enhanced resistance to invading pathogens	23, 24
3	Not hydrolyzed by oral micro-organisms	Protection against caries	25, 26
4	Not glycemic	Potentially useful for diabetics	27, 28
5	Non-specific stimulation of immune function	Resistance to infection	29, 30
6	Modulation of carcinogen metabolism	Anticancer properties	31, 32, 33
7	Reduced hepatic synthesis of very low density lipoprotein cholesterol and serum triglycerides	Coronary heart disease	34, 35, 36
8	Increased absorption of Mg and Ca	Osteoporosis	37

Nature of galacto-oligosaccharides (GOS)

GOS, have D-Glucose-[ß-D-Galactose]^{n} where n ranges between three and ten sugar moieties. GOS is the non-digestible oligosaccharides or soluble dietary fibers because they are not digestible by the enzymes of the small intestine, but they are fermentable by bacteria in the large intestine[20]. This is due to the substrate specificity of human gastrointestinal digestive enzymes, which are mostly specific for a glyosidic bonds whereas GOS glyosidic bonds have a ß-configuration. Some ß-galactosidases, localized in the small intestine, are able to digest GOS but their activity is usually weak or often deficient[21] (Table 1).

Need and health benefits of galacto-oligosaccharides (GOS)

Oligosaccharides serve as growth factors for indigenous *Bifidobacterium* in the colon. The increased population of *Bifidobacteria* antagonistically suppresses the activity of putrefactive bacteria such as *Clostridia sp.* and thereby reduces the formation of toxic fermentation products[38]. GOS promote intestinal health by keeping unfriendly bacteria such as *E. coli* from sticking to the walls of the intestine. They do this by mimicking the appearance of the intestinal walls, so that the *E.coli* attaches themselves to the GOS instead and are then flushed through the intestinal tract. GOS also make the body better able to absorb certain minerals, such as calcium. Therefore, GOS supplementation may prevent developing osteoporosis. GOS and beneficial bacteria reduce the severity and number of allergic reactions in the body and help to maintain the integrity of the intestinal tract and prevent vaginal and urinary infections. GOS also help to regulate bowel movements relieving from constipation. GOS and beneficial bacteria also help to alleviate dermatitis and other skin conditions. Studies show that GOS significantly reduce colorectal cancer (CRC) associated risk factors.

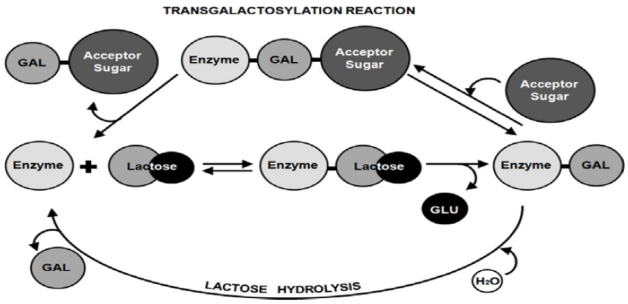

Fig. 3. Reaction pathway for trans-glycosylation and hydrolysis β-galactosidase.

β-Galactosidase enzyme and mechanism on lactose

β-galactosidase (β-gal) (E.C 3.2.1.23) is a galactosyl hydrolase which cleaves lactose, releasing glucose and galactose. This enzyme was one of the first enzymes isolated and purified from various natural sources, such as plants, animal organs and microorganisms[39]. The reaction mechanism for β-galactosidase has been elucidated and proceeds by two steps: - Step (a): enzyme–galactosyl complex formation and simultaneous glucose liberation. - Step (b): the enzyme–galactosyl complex is transferred to nucleophilic acceptor containing a hydroxyl group. Transfer to water produces galactose (hydrolysis reaction). Transfer to another sugar produces di-, tri- and higher galactosyl-saccharides, collectively termed galacto-oligosaccharides (Fig. 3).

Galacto-oligosaccharide production

Glycoside hydrolases with β-galactosidase activity occur in a variety of microorganisms from Archaea, Bacteria, and Eukaryota. Some of these enzymes have been expressed in host organisms, and purified by a combination of several conventional techniques, such as salting-out fractionation, ion exchange, gel filtration, hydroxyapatite, and hydrophobic interaction chromatography's[40]. Generally, the yield of GOS synthesis from lactose using glycoside hydrolases can be increased by: using highly concentrated starting lactose solution; decreasing water thermodynamic activity (for example, using a micro-aqueous environment); removing the final product and/or inhibitors from the reaction medium; and modifying the enzyme[41]. Lactose hydrolysis and trans-galactosylation reaction are both catalyzed by β-galactosidase, depending on the sugar concentration in solution[42]. GOS mixtures produced by trans-galactosylation always contain considerable amounts of non-reacted lactose and monosaccharides. The efficient removal of these non-GOS impurities allows the commercialization of added-value GOS products[43]. Large-scale separation of monosaccharides is usually conducted by a chromatographic process with ion-exchange resins or activated charcoal. Recently, a comparison of fractionation techniques to obtain high-content GOS mixtures, at preparative scale, concluded that size-exclusion chromatography was the most appropriate method to obtain fractions with high purity, enabling the purification of GOS with different degrees of polymerization (DP). Although not in a mature stage, membrane techniques, particularly nano-filtration, also show potential for large-scale fractionation of oligosaccharides from complex mixture. Supercritical fluid extraction technology also has shown satisfactory performances in the isolation of monosaccharides, disaccharides, and higher saccharides from complex mixtures[43]. The synthesis of GOS with a high yield of 55 % from 275 g/L lactose at 50°C for 12h was performed using trans-glycosylating β-galactosidase producing *Enterobacter cloacae* (*E. cloacae*)[44]. The figure shows two possible types of GOS products: Gal-β-1 → 4-Gal-β-1 → 4-Glc (4β-galactosyllactose) and Gal-β-1 → 6-Gal-β-1 → 4-Glc (6β-galactosyllactose), where Gal: Galactose, Glc: Glucose[45] (Fig. 4).

Surface displayed enzyme production in *Pichia pastoris*

The β-galactosidase most studied for GOS production is from *E.coli* and is encoded by the lacZ gene. It is not considered suitable for use in foods owing to toxicity problems associated with the host coli form. Hence, the β-galactosidase from *E. coli* is generally not preferred for use

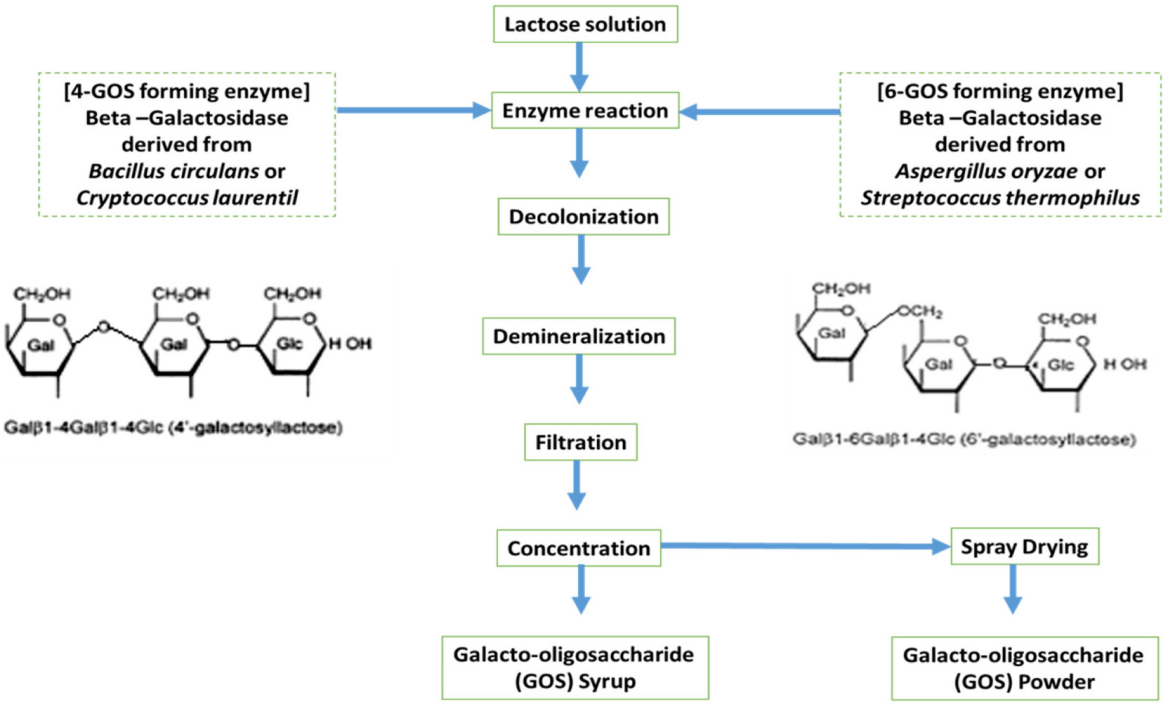

Fig. 4. Schematic representation of Industrial (Biosynthesis) production process for GOS. (The figure shows two possible types of GOS products: Gal-β-1→4-Gal-β-1→4-Glc (4β-galactosyllactose) and Gal-β-1→6 Gal-β-1→4-Glc (6β-galactosyllactose), where Gal: Galactose, Glc:Glucose[45].

in food industry[46] have cloned a novel gene encoding trans-glycosylating β-galactosidase (BGase) from *Penicillium expansum (P. Expansum)* F3 and subsequently expressed on the cell surface of *Saccharomyces cerevisiae* (*S. cerevisiae*) EBY-100 by galactose induction. The BGase-anchored yeast could directly utilize lactose to produce GOS, as well as the by-products glucose and a small quantity of galactose. The glucose was consumed by the yeast, and the galactose was used for enzyme expression, thus to a great extent facilitating GOS synthesis. The GOS yield reached 43.64%, when the recombinant yeast was cultivated in yeast nitrogen base-casamino Acids medium containing 100 g/L initial lactose at 25°C for 5 days. The separation of lactose from a disaccharide fraction has proven to be difficult by all the reported processes, and usually results in large losses of GOS products, mainly non-lactose disaccharides. To overcome this difficulty, *S. cerevisiae* was used to improve the purity of a commercial mixture of GOS obtained with β-galactosidase from *Bacillus circulans* (*B. circulans*) by completely removing the monosaccharides. A combination of *S. cerevisae* and *Kluyveromyces lactis* (*K. lactis*) was used to improve the purity of a GOS mixture produced by β-galactosidase from *Penicillium expansum* (*P. expansum*) from 29% to 98% by selective fermentation of monosaccharides and lactose[47]. *S. cerevisiae* most widely studied & advanced host strain for cell surface display system due to safety, simplicity in genetic modification and cell wall structure rigidity. But the large scale production and induction is a major problem. But *Pichia pastoris can be* strongly induced by methanol using AOX1 promotor (pAOX1)[48]. The disadvantage of the pAOX1 is that the need for methanol as inducer. Methanol is a hazardous substance due to its high flammability and toxicity. Also, cells growing on methanol have a very high oxygen consumption, which usually requires the addition of pure oxygen to the culture, increasing the cost of the process and limiting the cultivation capacity at high scale. An alternative strategy to overcome the need for methanol based on, the constitutive glyceraldehyde dehydrogenase promoter (pGAP), which can yield high expression levels without the use of methanol. The GAP promoter has shown to be a strong constitutive promoter and several examples of high level expression of heterologous proteins have been published[49].

The pGAPZ series constitutive vectors including the intracellular expression vectors pGAPZ A, pGAPZ B, GAPZ C and secreted expression vectors-pGAPZα A, pGAPZα B and pGAPZα C are widely. These vectors are only suitable for using the method of electroporation transformation but not of spheroplasts transformation. Because these vectors only contain zeocin resistance gene as selection marker and spheroplasting involves removal of the cell wall to allow DNA to enter the cell and cells must

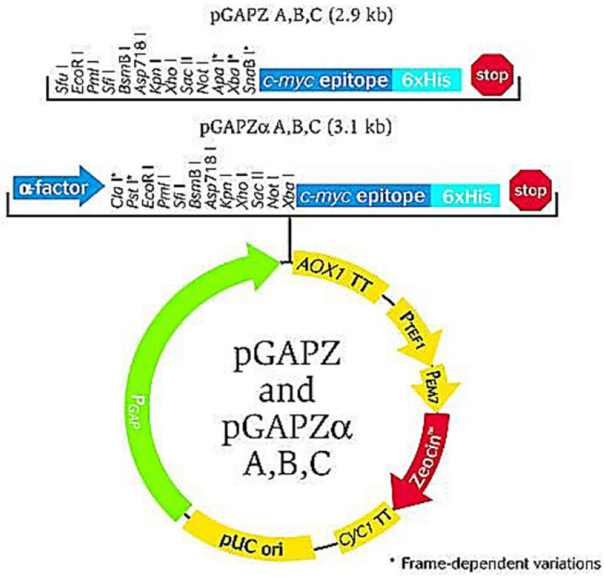

Fig. 5. The constitutive glyceraldehyde dehydrogenase promoter (pGAP-vector), which results in the yield of high expression levels (The GAP promoter reported to be a strong constitutive promoter which leads in high level of heterologous protein expression, which has been previously reported by Waterham et al.)[52].

first regenerate the cell wall before they are able to express the Zeocin resistance gene. For this reason, plating spheroplasts directly onto selective medium containing Zeocidoes not yield any transformants[50]. Compared to pAOX1, pGAP has shown to allow for higher expression levels in some cases (Fig. 5). For instance, the expression of the mammalian peptide transporters hPEPT1 and rPEPT2 using the pGAP yielded up to five times more expression levels than using the classical pAOX1[51].

Additional benefits of oligosaccharide towards anti- flocculent (negative –interaction)

Biological systems involve a wide spectrum of regulated cell adhesion. This involves fungal, viral and bacterial infections in microbiology or the development of ordered cell structures such as biofilms. Cell adhesion is generally characterized as clumping (attaching) a cell to a substrate that can be another cell, a surface, or an organic matrix in general[53]. The *S. cerevisiae* strain S288C family of adhesion proteins that is subdivided into two classes. Protein first community is encoded by genes including FLO1, FLO5, FLO9, and FLO10[54]. The morphogenetic events (flocculation, filamentation and invasive growth) are tightly regulated[55]. Flocculation often occurs upon depletion of sugar during late-exponential or stationary phases of growth[55]. The

mannan residues form an outer and inner core of the cell wall, which is attached to an asparagine residue of the protein component, which is linked through B-(1→4) glycosidic bonds[56].

Conclusion and future perspectives

Numerous microbial glycoside hydrolases were indicated for GOS production on lactose. Those proteins, in this case. Their capacity to accelerate the trans-galactosylation initial response comparative to hydrolysis and their affinity to hydrolysis significantly differ. The GOS was shaped as opposed with the lactose linkage. The end product range acquired during processing of lactose and the glycosidic relation between both the monomers is dependent on the source of the enzyme and the catalyzed physicochemical conditions.

The commercial GOS based product, where prepared based on the combinations of galactose-based oligosaccharides with various extents of polymerisation and glucose, galactose and binding structure Lactose. GOS mixtures are well known ingredients of the prebiotics. But GOS varies on their composition of oligosaccharides, in which the products differed in terms of their bifidogenic and other defensive properties.

The major goal of current review focuses on the future developments in GOS production, mainly to produce pure and more effective formulations, the goal ranges are narrow and precise. A comprehensive picture of modulation of intestinal microbiota achieved with GOS, which are applied in food, feed and pharmaceutical applications.

국문요약

Galacto-oligosaccharides(GOS)는 프로바이오틱스 미생물의 성장을 증진시켜 인류 건강에 유익한 작용을 갖게 하는 프리바이오틱스이며 식품 산업에서 다양한 활용성을 갖는다. GOS는 보통 β-galactosidase에 의해 촉매 반응이 일어난 lactose로부터 생성된다. 한편, 세포 표면 발현은 살아있는 세포 표면의 펩타이드와 단백질을 세포의 기능성 성분에 융합시켜 발현시키는 것이다. 표층 발현 세포는 다양한 잠재적 이용가치를 갖는다. N 말단 부근에 위치하는 것으로 생각되는 Flo1p 응집 functional domain은 세포의 flocs로의 가역적인 응집을 유발하면서 α-mannan carbohydrates와 같은 세포벽 성분과 비공유결합을 한다. 한외여과한 유청을 농축, 분무건조한 유청막투과액(Whey Permeate, WP)을 이용하여 β-galactosidase 재조합 *Pichia pastoris* (*P. pastoris*) 로 표층 발현 처리 (surface engineering) 하는 GOS의 합성법은 폐기물을 활용하는 새로운 효율적인 방법이라 할 수 있다.

Conflict of interests

The authors declare no potential conflict of interest.

ORCID

Deog-Hwan Oh https://orcid.org/0000-0002-7472-0436
Ramachandran C https://orcid.org/0000-0003-2060-9297

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