

ABSTRACT: Fructooligosaccharides (FOSs) are short-chain sugar molecules, which are useful for a variety of purposes. They possess a number of desirable characteristics, such as low calories, no carcinogenicity, and usefulness as prebiotics. There has been increasing interest in neo-FOSs recently due to their superior *Bifido*-stimulating effect and chemical and thermal stability compared to their counterpart FOSs. This article reviews their enzymatic preparation and the purification of the end products.

KEYWORDS: fructooligosaccharides, neo-fructooligosaccharides, fructosyltransferase, oligosaccharide

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Introduction

Beyond basic nutritional considerations, functional foods have been demonstrated to have physiological benefits or to reduce the risk of chronic disease. Consumer awareness and acceptance of functional foods and natural health products are increasing and consequently their global market continues to expand.¹ The international market for functional foods is estimated to be approximately US\$30–US\$60 billion, which represents 3% of the total food market.² Probiotic bacteria have attracted much commercial and research interest in recent years due to their role in promoting human intestinal health. These bacteria, usually species of *Bifidobacterium* and *Lactobacillus*, confer various beneficial effects on the host, especially by reducing the incidence of intestinal diseases.^{3–5} It has also been suggested that the colonic population of autochthonous or exogenous probiotic bacteria is significantly influenced by the nondigestible oligosaccharides that reach the colon.⁶ These so-called prebiotic carbohydrates are selectively metabolized by a limited number of microorganisms residing in the colon, including *Bifidobacterium* and *Lactobacillus*. In particular, independent studies^{7–10} have shown that the growth and activity of these bacteria are stimulated by fructooligosaccharides (FOSs), prebiotic fructans that are either derived from inulin and other edible plant materials or enzymatically synthesized from sucrose via transfructosylation.^{11–13}

Chemically, FOSs are polymers (degree of polymerization, 3–9) consisting of a sucrose molecule that is elongated by a chain of fructosyl units, having the generic structure GF_n (where G refers to glucose molecule, F to fructose molecule, and n to the number of fructose units). Depending on the linkage type between the monosaccharide residues, different types of FOS series can be distinguished (Fig. 1).

(i) inulin-FOSs (¹F-FOSs), which consist of linear non-reducing chains with β -(2→1) linkages, such as 1-kestose (¹F- β -D-fructofuranosylsucrose) (GF2), 1-nystose [¹F (¹F- β -D-fructofuranosyl)₂ sucrose] (GF3), and ¹F- β -fructofuranosylnystose (GF4); (ii) levan-FOSs (⁶F-FOSs), which have a β -(2→6) linkage formed between fructose units, such as 6-kestose (⁶F- β -D-fructofuranosylsucrose); and (iii) a neo-FOS (⁶G-FOS) series in which the D-glucose moiety of sucrose is linked directly to a fructose unit through a β -(2→6) linkage, such as in the case of neo-kestose (⁶G- β -D-fructofuranosylsucrose), giving the possibility that chain elongation occurs on D-fructose residues by both β -(2→1) or β -(2→6) bonds.¹⁴ All FOS preparations actually commercialized for their prebiotic properties belong exclusively to the inulin type and exhibit low degrees of polymerization (three to nine).¹⁵ Levan, a polysaccharide from *Zymomonas mobilis*, was hydrolyzed in a microwave oven to obtain oligofructans that beneficially affect the host by selective stimulation of probiotic bacteria in the colon.¹⁶ The neo-FOS structure is very close to inulin type because both are fructans in which fructosyl units are bound by a β -(2→1) linkage. The difference is in the nature of linkage between the first fructosyl unit and the D-glucose residue, which are β -(2→6) and α -(1→2) for neo-FOS and inulin type, respectively. There has been increasing interest in neo-FOS recently due to its superior *Bifido*-stimulating effect and chemical and thermal stability compared to ¹F-FOS.^{17,18} Most particularly, neokestose was shown to improve the population of *Bifidobacteria* and *Lactobacilli* to a greater extent than currently available FOSs and to inhibit the growth of clostridia.¹⁷

Neo-FOSs such as neokestose can be extracted from vegetables (eg, onions, garlic, and asparagus) or cereals (eg, oats, barley, and rye). Because neo-FOSs are present in very low

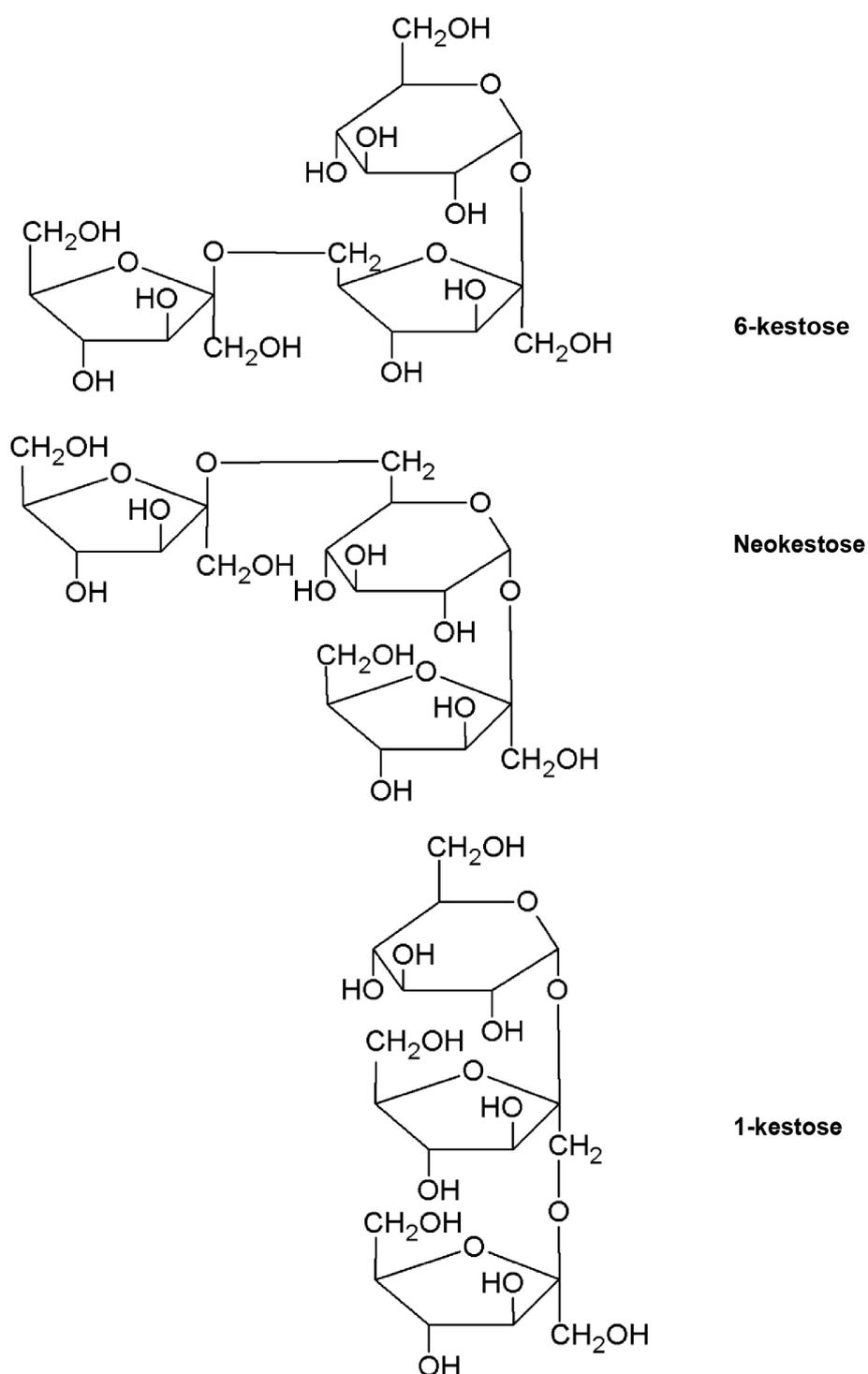


Figure 1. Chemical structures of the fructooligosaccharides 6-kestose, neokestose, and 1-kestose.

amounts in plants, their direct industrial extraction could not be performed. The enzymatic production of neo-FOSs with fungal enzymes has been reported.¹⁹

Considering the structural diversity of oligosaccharides (three different amino acids would allow the synthesis of only six different peptides, while three different hexopyranose moieties would yield up to 720 trisaccharides), the stereo- and regioselectivity of enzymes are considered a valuable

alternative to chemical synthesis, which needs complex protection and deprotection steps for the preparation of structurally well-defined oligosaccharides. Actually, enzymatic processes are preferred in the food industry for the production of most important oligosaccharides.

Several novel microbial enzymes producing specific oligosaccharides have been discovered since 1970. Using these new enzymes, it is now possible to produce, on an industrial

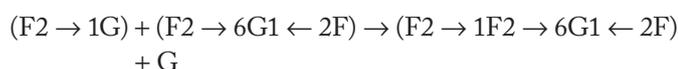
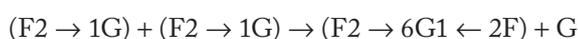
scale, various oligosaccharides such as glycosylsucrose, fructooligosaccharides, maltooligosaccharides, isomaltooligosaccharides (branched oligosaccharides), galactooligosaccharides, xylooligosaccharides, palatinose (isomaltulose), and lactosucrose.²⁰ Mass production of neo-FOSs has been carried out using a free-whole-cell biotransformation or culture of *Xanthophyllomyces dendrorhous*.^{20–22}

Although many research articles and review articles on FOS synthesis were published,^{23–25} review articles on neo-FOS synthesis scarcely appeared in the literature until now. The aim of this review is to summarize the latest findings on neo-FOS research and to explore their enzymatic reaction mechanisms, production techniques, and future directions.

Enzymatic Reaction Mechanisms

Although the normal function of transglycosidases is the transfer of a glycosyl residue to another sugar acceptor (transfer reaction), they can also use water as the acceptor of the glycosyl–enzyme intermediate (hydrolysis reaction). Only an enzyme that possesses a significant transglycosylation-to-hydrolysis ratio should be considered a transglycosidase.²⁶ Fructosyltransferases are transglycosidases that transfer the fructosyl moiety of sucrose, yielding fructose oligomers (fructooligosaccharides, FOSs) and/or polymers (fructans such as inulin or levan).²⁷ Enzymes with transfructosylating activity are present in many higher plants (asparagus, Jerusalem artichoke, chicory, onion, and so on) and microorganisms.^{28–31} FOS-producing enzymes can be divided into two classes: one is β -D-fructofuranosidase, one of the hydrolases, numbered as EC.3.2.1.26; the other is fructosyltransferase, concentrating on the nature of transfructosylation of the enzyme, classified as EC.2.4.1.9. The amount of enzyme in plants such as asparagus, sugar beet, onion, Jerusalem artichoke, etc, is low and quite limited by seasonal conditions; therefore, industrial production depends chiefly on microbial enzymes.³¹ Generally, neo-FOS is one of the products of enzymatic reaction. Neokestose occurs as a minor product of reactions in whole cells from various plants, yeast (eg, *Saccharomyces cerevisiae*), and some filamentous fungi. Culture of the astaxanthin-producing yeast *Xanthophyllomyces dendrorhous* accumulated neokestose as a major product when it was grown on sucrose.

The enzyme mechanism depends on the source of the enzyme. In plants and some microorganisms, a series of enzymes act together, whereas a single enzyme works in most other microorganisms. The reaction mechanism of *Claviceps purpurea* enzyme produces mainly neokestose-based oligosaccharides, which can be summarized as follows:



where the number indicates the position of the carbonyl carbon atoms and arrows represent the direction of glycosidic linkage. In addition to the above two synthetic reactions, hydrolyzing reactions also occur. A hydrolysate such as $F2 \rightarrow 6G$ acts again as fructose donor and acceptor for the synthesis of neokestose and its tetraoligomer.³² Lee and Shinohara³³ proposed a model (Fig. 2) for enzymatic production of neo-FOS from sucrose using *Penicillium citrinum* cells. This reaction route showed that neo-FOS was only formed from inulin-type sugars, such as sucrose and 1-kestose; however, when neo-FOS, such as neokestose, was used as the substrate, no reaction product was obtained.³³

Production Methods

Conventionally, FOSs are industrially produced by microbial processes including a two-stage process that uses the extracted enzymes, produced by microbial fermentation, in free or immobilized conditions, and a single-step process using whole cells of a given microorganism, either suspended or immobilized. The enzymes involved in FOS production can be either intra- or extracellular. The different FOS production processes have been widely reviewed by several authors; however, articles on neo-FOS production have not been reviewed. Table 1 shows microbial sources, enzymes properties, and yield of neo-FOS-producing methods appearing in the literature.

Gross et al³⁴ were the first to report the enzymatic synthesis of neokestose using the action of yeast invertase preparations on sucrose. During the hydrolysis of sucrose by invertase, which is also responsible for fructosyl transfer to the primary hydroxyl groups of sucrose, the production of ~10% neokestose was identified. Itoh and Shimura used the fructose-transferring enzyme (EC.3.2.1.26) isolated from *Penicillium oxalicum* to produce FOS.³⁵ It was found that the enzyme synthesized mainly neokestose and 1-kestose from sucrose. Their data showed the reaction products were 15.6%

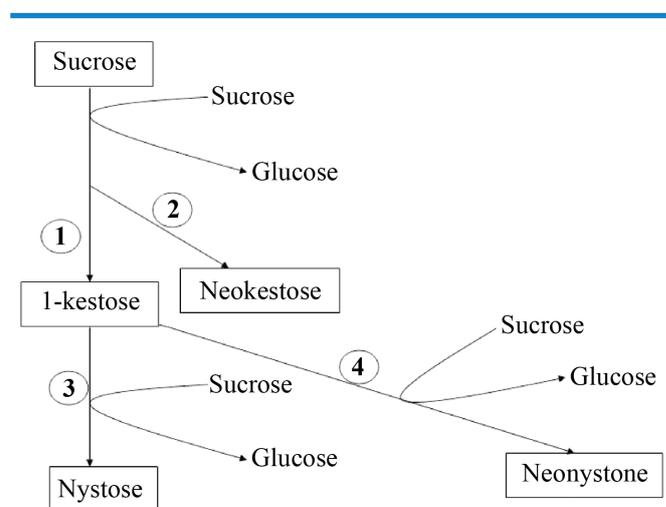


Figure 2. Schematic reaction route for production of the fructooligosaccharides (redrawn from Ref. 33). Routes 1–4 indicate the real reactions that occurred in the experiments.

Table 1. Microbial sources, enzyme properties, and yield of neo-FOS-producing methods.

ENZYME	SOURCE	REACTION CONDITIONS	YIELD (% W/W Neo-FOS/ TOTAL CARBOHYDRATE)	REFERENCE
Invertase	<i>Saccharomyces cerevisiae</i>	Enzyme reaction: 50% sucrose pH = 4–6	~10%	Gross et al, 1954 ³⁴
Cytolase PCL5	<i>Penicillium oxalicum</i>	Enzyme reaction: 50% sucrose 50–55°C, pH = 5	15.6%	Itoh and Shimura, 1987 ³⁵
	<i>Aspergillus awamori</i>	Enzyme reaction: 55°C, pH = 5.5	49.5%	Grizard and Barthomeuf, 1999 ³⁶
	<i>Penicillium citrinum</i>	Fermentation: 70% sucrose, 50°C, pH = 6, 72 hours	18.5%	Hayashi et al, 2000 ³⁷
	<i>Xanthophyllomyces dendrorhous</i> UOFS Y-0175	Fermentation: 11% sucrose, 25°C, pH = 7	75.11 g/L	Kritzinger et al, 2003 ³⁸
	<i>Penicillium citrinum</i> KCCM 11663	Immobilization of whole cells: 60% sucrose, 50°C, pH = 6	49 g/L	Park et al, 2005 ³⁹
	<i>Penicillium citrinum</i>	Fermentation: 12.65% sucrose and 2.9% yeast extract 50°C, pH = native	43.44 g/L	Lim et al, 2005 ⁴⁰
Neo-fructosyltransferase	<i>Penicillium citrinum</i> KCTC 18080P	Coimmobilized whole cells and Neo-fructosyltransferase: 60% sucrose, 50°C, pH = native	108.4 g/L	Lim et al, 2007 ¹⁸
β-fructofuranosidase	<i>Rhodotorula darirenensis</i>	Enzyme reaction: 75% sucrose, 55–60°C, pH = 5	10.6 g/L	Gutiérrez-Alonso et al, 2009 ⁴¹
	<i>Xanthophyllomyces dendrorhous</i> ATCC MYA-131	Enzyme reaction: 41% sucrose, 50°C, pH = 5.6	65.9 g/L	Linde et al, 2009 ⁴²
	<i>Xanthophyllomyces dendrorhous</i> 269	Fermentation: 40% sucrose, 30°C, pH = native	227.72 g/L	Ning et al, 2010 ²⁰
	<i>Xanthophyllomyces dendrorhous</i> ATCC MYA-131	Enzyme reaction: 60% sucrose, 60°C, pH = 5.5	168 g/L	Linde et al, 2012 ⁴³
α-G-fructofuranosidase	<i>Xanthophyllomyces dendrorhous</i> BCRC 21346	Fermentation: 25% sucrose, 20°C, pH = 6.9±0.1, 72 hours	49.6%	Sheu et al, 2013 ⁴⁴

neokestose and 14.7% 1-kestose when they used 11.2 U/mL enzyme and incubated for 24 hours with 50% sucrose at pH 5.0, 30°C. The ratio of synthesized neokestose to 1-kestose increased with lowering pH and with increasing concentration of sucrose. Cytolase PCL5 from *Aspergillus awamori*³⁶ was found to convert sucrose into high neo-FOS syrup containing 30% neokestose, 17% neonystose, and 2.5% neofructofuranosylneystose. This enzymatic synthesis were carried out at temperatures ranging between 20 and 70°C, pH between 3.0 and 7.0, and enzyme/substrate ratios (U/g sucrose) from 0.59 to 37.7. Maximum yield of conversion was obtained at 55°C and optimum pH was found to be 5.5. Hayashi et al³⁷ studied the production of neo-FOS by the cells of *P. citrinum*. Sucrose (70%, w/v) and wet cells (99:1, w/w) were incubated at 50°C for 40 hours with shaking at 100 rpm. The main components of oligosaccharides in the syrup produced were found to be 1-kestose (21.8%), nystose (14.1%), and neokestose (10.5%). Yields of neo-FOS/total saccharides and FOS/total saccharides were 18.5% and 56.7% after 40 hours of reaction, respectively. Among the media, sucrose and yeast extract were thought to have more effect on neo-FOS production.

Kritzinger et al³⁸ investigated the effects of important process parameters on the production of the novel prebiotic

trisaccharide neokestose from sucrose by *X. dendrorhous* cells suspended in buffer. Their statistical analysis of the data showed that sucrose concentration had a large positive effect on the maximum concentration of neokestose produced, the maximum specific rate of neokestose production, and the maximum yield coefficient of neokestose.

Using whole-cell immobilization of *P. citrinum* KCCM 11663, Park et al³⁹ found that under optimum reaction conditions, the maximum neo-FOS production was 49 g/L. In a packed-bed reactor, continuous production of neo-FOS was possible for 50 days. Lim et al⁴⁰ investigated the optimization of culture media (sucrose as carbon source and yeast extract as nitrogen source) and culture conditions of *P. citrinum* to increase the neo-FOS production by free cells of *P. citrinum*. They found that the optimal concentrations of sucrose and yeast extract were 12.65% and 2.90%, respectively, and for culture conditions, optimal points of inoculum age (78.5 hours), initial pH (7.34), and inoculum size (11.36%, v/v) were determined. Under these conditions, neo-FOS production was approximately 41 g/L. Neo-FOS production using a coimmobilized procedure, a combination of *P. citrinum* mycelia and neo-fructosyl-transferase from *P. citrinum*, was also studied.¹⁸ They found CaCl₂ concentration was



an important factor in alginate entrapment because it could affect the support matrix structure and hence the stability of beads. Maximum neo-FOS production was achieved at 0.25 M CaCl_2 . The decrease of neo-FOS production at higher CaCl_2 concentrations implied the formation of excessive alginate gel structure, which led to a substrate diffusion limit and thus inhibited the reactions of cells and enzymes in the combined reaction. In batch production, coimmobilization of whole cells together with neo-fructosyl-transferase produced more neo-FOS (108.4 g/L) than whole immobilized cell (49.4 g/L).¹⁸

An extracellular β -fructofuranosidase from the yeast *Rhodotorula dairenensis* was characterized biochemically by Gutierrez-Alonso et al.⁴¹ The enzyme produced a varied type of FOS. The maximum concentration of FOS was reached at 75% sucrose conversion and it was 87.9 g/L, which contained 6-kestose, neokestose, 1-kestose and tetrasaccharides, respectively.⁴¹ So far, β -fructofuranosidase from the yeast *X. dendrorhous* is the only enzyme that mainly synthesizes neo-FOSs (neokestose and neonystose). For the production of high-purity neo-FOSs, several studies on this enzyme were performed. Linde et al.⁴² had characterized an extracellular β -fructofuranosidase from the yeast *X. dendrorhous*. They found that the enzyme from *X. dendrorhous*, unlike the other microbial β -fructofuranosidases, produced neokestose as the main transglycosylation product. Using a 41% sucrose solution, the maximum FOS concentration reached was 65.9 g/L. The reaction mixture contained 132 g of fructose, 179 g of glucose, 49 g of sucrose, 40 g of neokestose, 18 g of 1-kestose, and 8 g of tetrasaccharides (mainly nystose) per liter.

Two β -fructofuranosidases from this yeast have been characterized biochemically and kinetically.⁴³ The extracellular glycoprotein (Xd-INV) of 160 kDa (66 kDa for the non-glycosylated form) displays maximum hydrolase activity at 65°C–70°C, and the intracellular enzyme of 33 kDa has an optimal temperature at 45°C. Unlike the intracellular 33 kDa enzyme, which produced only neokestose, the extracellular 160 kDa enzyme produced neokestose and 1-kestose. Linde et al.⁴³ showed that the yield of neo-FOSs by the extracellular enzyme from *X. dendrorhous* reached 168 g/L and that of 1-kestose was only 20 g/L when using 60% sucrose at 60°C for 3 hours. Sheu et al.⁴⁴ compared the yields and compositions of neo-FOSs in fermentations involving *X. dendrorhous* BCRC 21346 with high enzyme activity and *X. dendrorhous* BCRC 22367 with low enzyme activity. Their results showed that the culture with high enzyme activity produced neo-FOSs much faster than that with low enzyme activity. However, neo-FOSs at a purity of up to 87.4% could be produced by culturing *X. dendrorhous* BCRC 22367, which was of much higher purity than that (60.7%) obtained by culturing *X. dendrorhous* BCRC 21346. That is because most of the monosaccharide by-products were consumed by the *X. dendrorhous* BCRC 22367 yeast cells.

So far, the largest neo-FOS levels have been reported by Ning et al.,²⁰ whereby they showed that the yeast

X. dendrorhous produced neo-FOSs at a maximum concentration of 227.72 g/L from 40% sucrose under optimal conditions.

Purification Methods

As mentioned above, FOS products are not pure but mixtures containing different amounts of FOSs that consist of 50–60% ¹F-FOS, 30–40% glucose, and 10–20% sucrose, mainly due to the low yields of their industrial production processes. Several downstream techniques currently available could be used for the purification of FOSs obtained in industrial fermentative processes.^{24,25} These commercial products contain about 5% of other sugars, although products with 55–99% purity can be found in the market typically.

High-purity ¹F-FOSs could be obtained from sucrose by a mixed-enzyme system or by a mixed-cell system, in which glucose is converted into gluconic acid by the catalytic action of glucose oxidase or intracellular glucose dehydrogenase, respectively, of *Gluconobacter oxydans*. Consequently, high-purity ¹F-FOS consisting mainly of 1-kestose, nystose, and ¹F-fructosyl nystose was obtained.^{45,46} Furthermore, a purity of ¹F-FOS up to 98.2% has been obtained from sucrose by a process incorporating immobilized *Aspergillus japonicus* and *Pichia heimi* cells.⁴⁷ Chromatographic separation has been performed using mobile and stationary chromatographic systems for the separation of FOSs. FOSs were separated from a mixture of sugars by activated charcoal fixed-bed column. Its purity was 80% and recovery was 97.8%.²⁴ Furthermore, high-purity ¹F-FOSs could be obtained alternatively by adding a yeast fermentation step following the catalytic reaction of ¹F-fructofuranosidase on sucrose.⁴³

For neo-FOSs, although there is no article focused on its purification technology, it should theoretically be the same in application. Until now, neo-FOS with a purity of up to 87.4% could be produced by culturing *X. dendrorhous* Biore-source Collection and Research Center (BCRC) 22367⁴³ and 89.4% was reached by using *X. dendrorhous* American Type Culture Collection (ATCC) MYA-131.⁴²

Conclusion

FOSs are noncariogenic, low-calorie, fibrous, and strong *Bifido*-stimulating factors, which have scientifically proven health benefits, including role in absorption of calcium and minerals, replacement of sugar and fat in food products, reduction of cholesterol, control of various diseases such as cancer, and so on. There has been increasing interest in neo-FOSs due to their superior *Bifido*-stimulating effect and chemical and thermal stability compared to ¹F-FOSs. Particularly, neokestose was shown to improve the population of *Bifido*-bacteria and *Lactobacilli* to a greater extent than commercial FOSs and to inhibit the growth of clostridia. So far, the β -fructofuranosidase from the yeast *X. dendrorhous* is the only reported enzyme that mainly synthesizes neo-FOSs (neokestose and neonystose). However, novel microorganisms producing potential enzymes are needed to be explored for



their application in neo-FOS production, and scale-up studies should be conducted for their industrial applications.

Author Contributions

Conceived the concepts: TH-W. Wrote the first draft of the manuscript: TH-W. Developed the structure and arguments for the paper: TH-W. Made critical revisions: TH-W. The author reviewed and approved of the final manuscript.

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