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Green Chemistry

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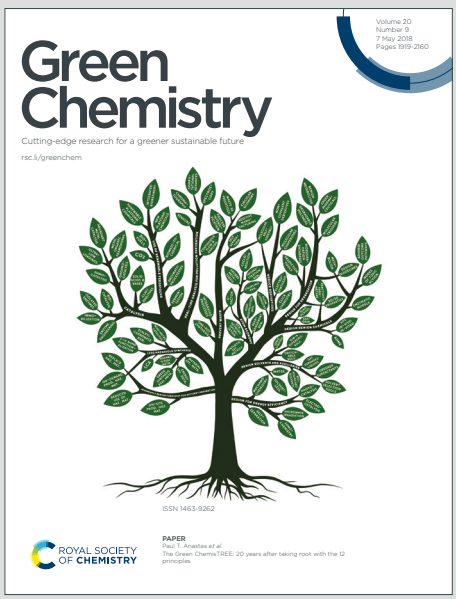

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GREEN FOUNDATION BOX

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Green foundation

1. We discuss greener methods to replace complex and tedious chemical processes in the synthesis of emerging oligosaccharides with prebiotic activities by catalytic conversion using enzymes. This allows for their synthesis in one-step in aqueous media and at low or moderate temperatures.
2. Prebiotics has critical effect as health promoting agent with a global market estimated in $13 billion at 2030. Their role is based on that beneficially affects the host stimulating the growth of “good” bacteria and inhibiting the “bad” bacteria. They have been evaluated as nondigestible food ingredient and more recently for skin problems.
3. While some promising catalysts have been developed for the synthesis, the use of enzymes as catalysts offers a significant advantage due to their selectivity and specificity, with more sustainable and efficient synthesis. This review will discuss methodologies to improve the final biocatalytic process.

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Developing and improving enzyme-driven technologies to synthesise emerging prebiotics

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Abstract

Emerging prebiotics, mainly oligosaccharides and phenolic compounds, are gaining increasing attention in the scientific community due to their health benefits and broad industrial potential. Prebiotics are widely used in foods, cosmetic formulations and dietary supplements. Emerging prebiotics offer additional advantages as they can be derived from low-cost, renewable materials and produced sustainably, in line with the principles of the circular economy. Green technological approaches, integrating expertise from different scientific disciplines, will be essential to develop efficient and environmentally friendly methods for the production of emerging prebiotic-enriched products.

This review provides a comprehensive overview of advances in the field, highlighting the advantages and optimisation of enzyme-based catalysis. It provides insight into how enzymes enhance control over oligosaccharide production by enabling selective synthesis of regioisomers with desired chain lengths and modification of phenolic prebiotics, different technologies to improve biocatalysts and contribute to novel bioprocess intensification strategies applicable to emerging prebiotic processing.

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1. Introduction

Prebiotics are stable chemical molecules, mainly carbohydrates, which has a critical effect as health promoting agents.[1] Their role is based on that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria in the colon, and thus improves host health, and, as ideal case, inhibiting the pathogenic bacteria.[2] Current targets for prebiotics have now expanded, beyond typical probiotic genera *Lactobacillus* and *Bifidobacterium*, to a wider range of microbial responders.[3] Similar to probiotics, these include candidate health-promoting genera such as *Roseburia spp., Eubacterium spp., Akkermansia spp., Christensensella spp., Propionibacterium spp., and Faecalibacterium spp*. A significant health-promoting benefit of these genera is their capacity to produce short-chain fatty acids (SCFAs), which have been demonstrated to regulate a variety of functions within the gastrointestinal system, including those of gut epithelial cells and mucus barrier function, immunity, inflammation, glucose and lipid metabolism, energy expenditure, and satiety.[4] Particularly they have been extensively evaluated as nondigestible food ingredient[2,3,5-6] but also more recently their role in treatment of illnesses such as psoriasis, atopic dermatitis, skin health in addition to others such as colon cancer, COVID, bone health and calcium absorption.[7-11] They have been highlighted in the area of prebiotics as an area of relevance.

The global prebiotics market, estimated at $6 billion in 2022, is forecast to reach a revised size of $13.8 billion by 2030, growing at a CAGR (Compound Annual Growth Rate) of 11% over the analysis period 2022-2030. Taking into account the ongoing post-pandemic recovery, the growth of the galacto-oligosaccharides (GOS) segment is adjusted to a revised CAGR of 12.1% over the next 8 years.[11]

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In recent times range of compounds classified as prebiotics expanded beyond "established prebiotics" (inulin, galacto- and fructo-oligosaccharides (GOS and FOS) on "emerging prebiotics" comprising new carbohydrate-based molecules[12-14] such as xylooligosaccharides (XOS),[15-20] isomaltooligosaccharides (IMO),[21-22] pectin oligosaccharides (POS),[23-25] mannooligosaccharides (MOS),[26] chitooligosaccharides (CHOS),[27] lactosucrose, raffinose, epilactose, and glucomannans.[12]

Furthermore, since latest definition of prebiotics included skin microbiota as target of their activity,[3] non-carbohydrate substances, such as polyphenols and other phenolic compounds have been identified as candidates to exert prebiotic effects on the host.[12,3,28] Since they are derived from plant-based materials or by-products generated during food and beverage processing, they align well with current market trends and the principles of a circular economy. Therefore, research on emerging prebiotics - comprising their synthesis and/or extraction and modification, *in vitro* models for confirmation of their prebiotic activity, green processing and incorporation in food products- is a propulsive multidisciplinary research topic. Due to the importance of this topic, the number of publications has increased over the last 20 years. In fact, there has been an exponential increase in the number of publications on this topic over the last 10 years (Fig. 1).

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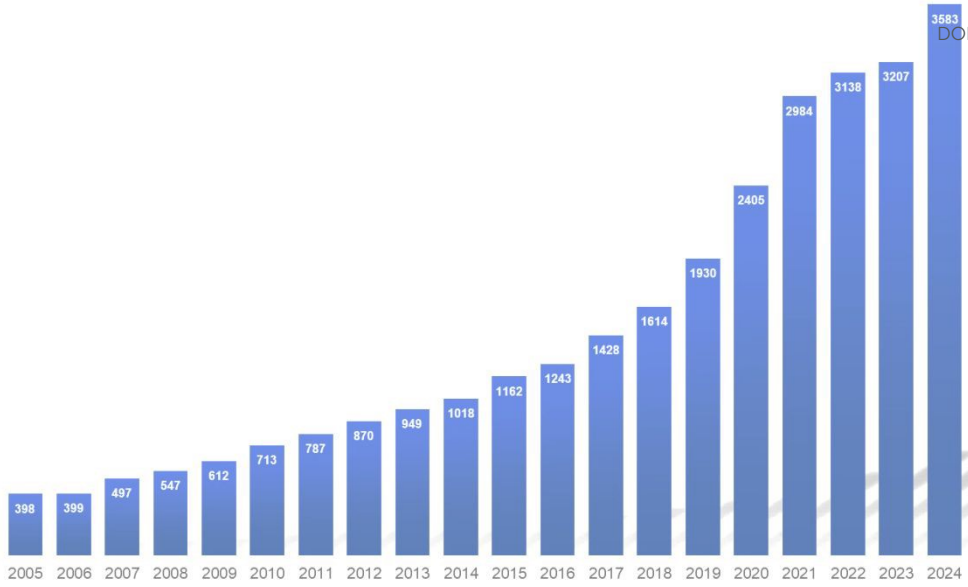

Fig. 1. Number of publications on prebiotics per year. Source: Scopus.

Emerging prebiotics are at an earlier development stage than established ones, facing challenges in discovery, testing, and application. Ensuring resistance to gastric digestion is key for prebiotics to reach the colon and be fermented by gut microbiota, which depends on their molecular structure. For instance, long-chained IMO is more resistant to degradation in the upper GI tract, making it more potent than short-chained IMO.[29]

Selectively stimulating beneficial bacteria, like *Bifidobacterium* and *Lactobacillus*, is also crucial, as different prebiotics can impact microbial composition differently. XOS, for example, was found to stimulate *Bifidobacteria* growth more effectively than FOS in rats.[30] Validating prebiotic efficacy is challenging due to variations in microbiomes and metabolic responses, though new evaluation methods like dynamic in vitro digestion and fecal fermentation experiments are helping. Despite these challenges, ongoing research is uncovering novel prebiotics that show resistance to digestion and promote health benefits, both *in vitro* and *in vivo*.[31]

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One of the most interesting aspects is the preparation of oligosaccharides, which can be produced either by a top-down approach (hydrolysis of parent polysaccharides to oligosaccharides) or a bottom-up approach (synthesis of oligosaccharides from simple sugars) (Fig 2). The first approach makes possible to produce these molecules from different sources by hydrolysis, especially from waste from the agri-food or marine industry, lignocellulosic wastes,[32] seaweeds,[33] or crustacean waste[34] (Fig.2). In these cases, the production of carbohydrates involves treatment with strong acids, whereas the use of enzyme allows to obtain them in aqueous media under mild conditions, and therefore higher degree of purity with respect to other by-products.

Enzymatic hydrolysis of polysaccharides serves a dual purpose in the production of emerging prebiotics. It not only facilitates oligosaccharide generation but also acts as a tool for breaking down plant cell walls, enhancing the extraction of phenolic compounds—another class of emerging prebiotics—through a green method commonly known as enzyme-aided or enzyme-assisted extraction.[35]

A second approach, a synthetic method, starts from single building blocks (Fig 2), and different chemical glycosylation reactions (using metal catalysts, base, etc.) have been described.[36] The versatility of the chemical processes allows the selective production of various molecules such as di-, tri- or tetra-saccharides, but makes the production of larger compounds more difficult.[35-36]

In this sense, the use of enzymes could overcome this drawback,[37-42] being able to produce different regioisomers of a given chain length with high selectivity facilitating the formation of larger oligomers more easily (Fig 2).

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To date, most of these compounds on the market are based on a mixture and their prebiotic efficacy has been evaluated in a general way, obviously considering a question of the price-performance ratio.

Therefore, several issues such as final overall yield of products, waste production, number of synthetic steps (based on protection and purification steps) could be improved using enzymes as catalysts (Fig 2). The extraordinary selectivity and specificity of the enzymes (particularly, glycosidic bond enzymes) allow to obtain these molecules in one or few synthesis steps in aqueous media and moderate temperature, also with high versatility, permitting to be used the same enzyme in several processes. One example is shown in Fig 2 about the synthesis of a hexamer-oligosaccharide.[43] The product was synthesized in 94% overall yield using glycosyltransferases (in four enzymatic steps with by using cofactor-recycling[43]), whereas the chemical approach required multiple glycosylation steps (with a high number of previous synthesized building blocks prepared) given a final overall yield of 2.6%.[45]

More recently, synthesis strategies have also been developed where enzymatic and chemical steps are combined to obtain tailor-made oligosaccharides.[44-45,42]

Thus, a large number of enzymatic glycosylation processes have been described in which, in addition to taking advantage of their excellent natural properties, strategies have been applied to improve them by increasing their stability under reaction conditions, or by increasing their versatility in recognizing other similar substrates, for example through protein engineering, or by improving their properties and recyclability, thus improving the economic sustainability of the process, which is of clear industrial interest.

This review discusses the major advances in the enzymatic synthesis, modification and extraction of various types of emerging prebiotics, as well as contributions in the area of

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property improvement processing and equipment through new intensified technologies for the food and cosmetics industries.

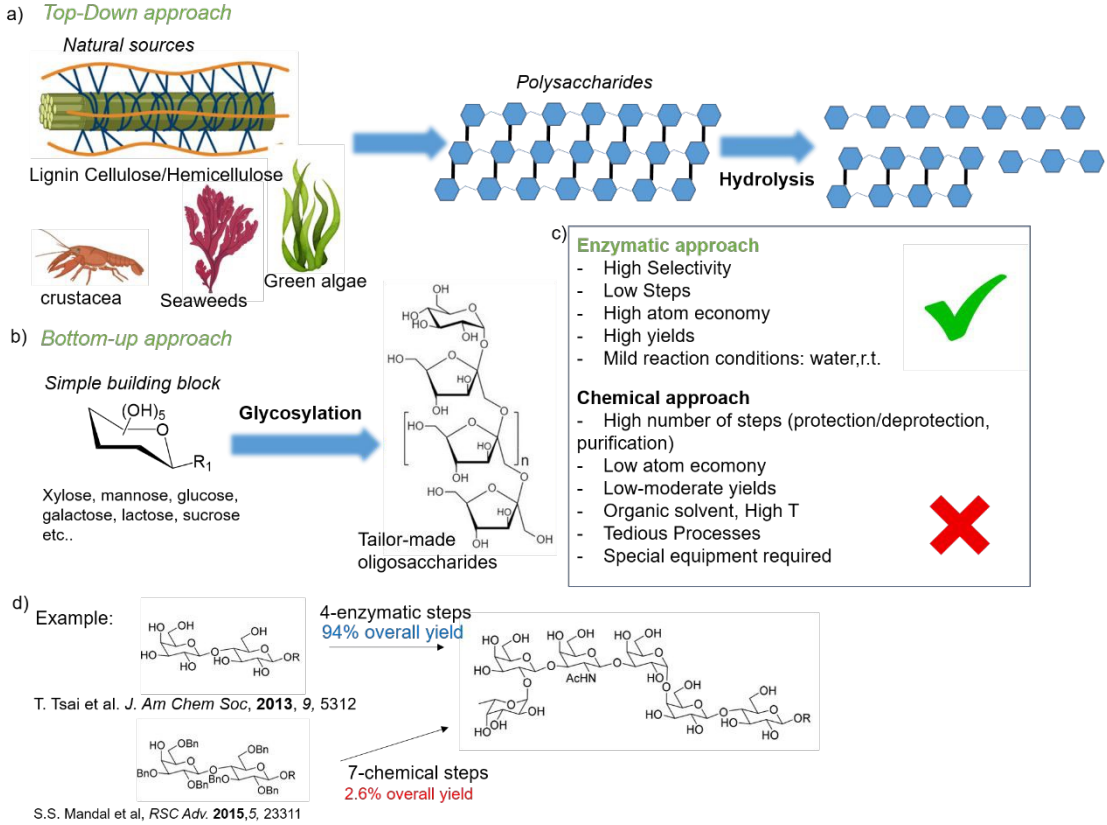

Fig. 2. Advantages of enzymatic strategy in the green synthesis of prebiotics oligosaccharides. a) Top-down approach synthesis, b) bottom-up approach synthesis, c) comparison between enzymatic and chemical approach, d) example of synthesis of tailor-made bioactive oligosaccharide by enzymatic versus chemical approach.

2. Enzyme-driven development of emerging prebiotics

2.1. Oligosaccharide emerging prebiotics

Currently, GOS, FOS and lactulose are the carbohydrates with confirmed prebiotic activity by clinical studies. However, with the expanded definition of prebiotics, there is a growing demand for identification, production and evaluation of new prebiotic carbohydrates that could affect the broader range of beneficial microorganisms and targeted hosts.[46] Those compounds, such as XOS, POS, MOS, IMO or CHOS (Fig 3) are known as "emerging" prebiotics, since evidence of their resistance to gastric digestion

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and selective growth stimulation of beneficial bacteria is still lacking. These compounds present great scientific and technological challenge, due to their structural diversity, arising from complex natural substrates. The latest achievements and trend towards sustainable transformation of different biomaterials in this field will be presented next.

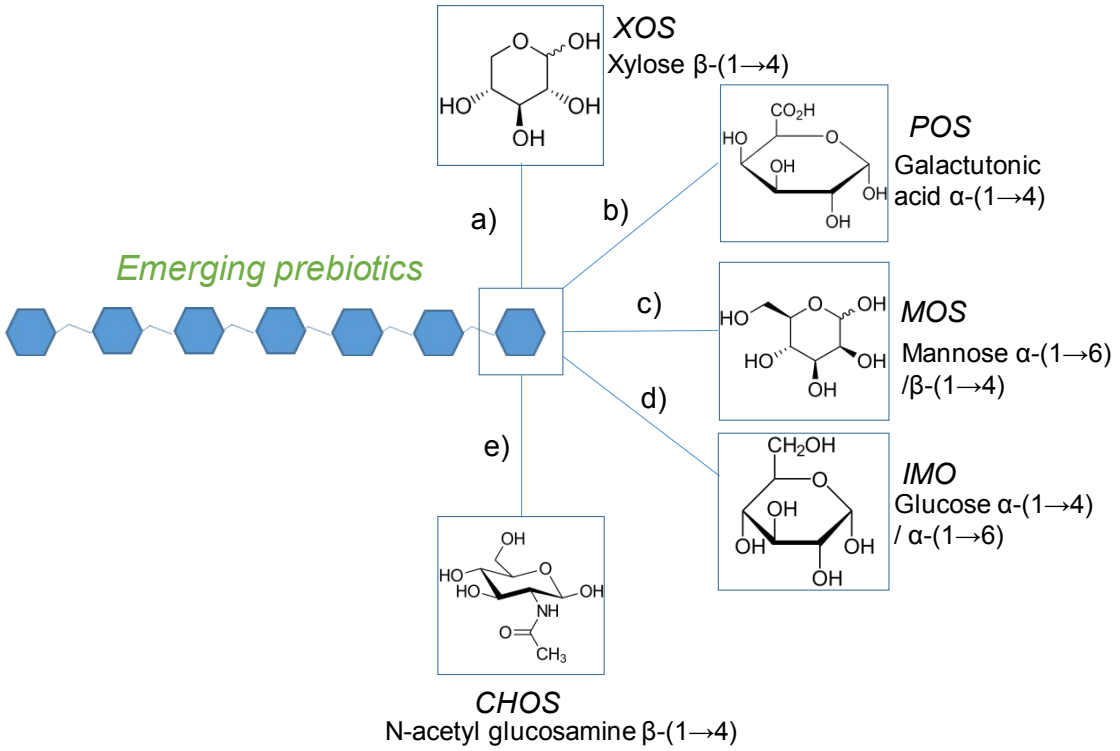

Fig 3. Typical structure of emerging prebiotics, emphasizing the monomeric unit of each one.

XOS are non-digestible oligomers consisting of 2 to10 linked xylose units by β-1,4 linkages (Fig 3a). Naturally XOS are present in small amounts in vegetables, fruits, honey and dairy products. XOS are considered to be competitive emerging prebiotics, ideal for incorporation into various products in the food and feed sector, especially considering their excellent application properties such as high stability in a wide range of

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temperatures (up to 100 ºC) and pH (2.5-8), good sweetening power and low calorie value.[12] Depending on the xylan sources used for XOS production, the structures of XOS are different in degree of polymerization (DP), monomeric units, and types of linkages. Generally, XOS are mixtures of oligosaccharides formed by xylose linked through β-(1→4)-linkages.[47] Several examples of the synthesis of XOS from sugar monomers have been described. The enzyme β-xylosidase is capable of synthesising various alkyl β-xylosides through transxylosylation processes.[48] The β-xylosidase from *A. niger* IFO 6662 has strong transxylosyl activity and has been reported to produce a novel non-reducing disaccharides. The synthesis of XOS from β-(1→4)-xylobiose in the presence of D-mannose by trans-xylosylation was reported, resulting in the production of two xylosylmannoses and non-reducing XOS[49] (Fig. 4a).

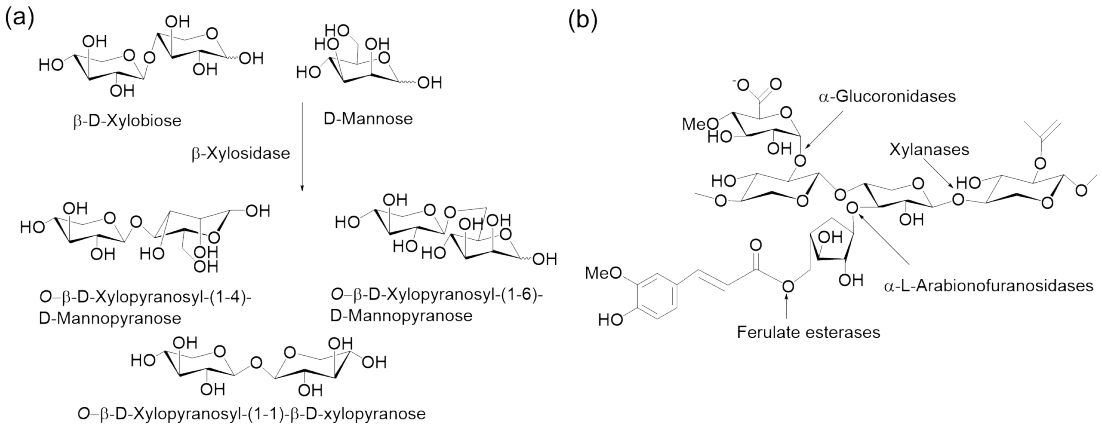

Fig. 4. Synthesis of XOS. A) synthesis using β-xylosidase; B) Enzyme hydrolysis of xylan.

However, in most cases, XOS are produced using commercially available xylan, but in order to enable their economically viable production, much emphasis has been placed nowadays on the use of lignocellulosic biomass, which is an abundant (up to 34% w/w) and, more importantly, cheap source of xylan.[50] Lignocellulosic materials can be hydrolyzed to XOS using combinations of thermal and chemical pre-treatments to

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degrade its complex structure, mainly composed of cellulose, hemicellulose and lignin, followed by enzymatic hydrolysis as shown in Fig.4b. To date, a large number of lignocellulosic materials (wheat and rice straw, sugarcane bagasse, corncob, beech and birch wood) have been studied for XOS production, with varying efficiencies depending on the amount and type of xylan present. Namely, xylan is a biopolymer with a β-1,4-linked xylose backbone with α-D-glucopyranuronic acids and/or L-arabinofuranose residues, and accordingly can be classified into one of four main groups (homoxylan, glucuronoxylan, arabinoxylan, arabinoglucuronoxylan and glucuronoarabinoxylan) based on the presence and distribution of substituents.[26] Therefore, different methods of XOS production and the great diversity of potential xylan substrates result in a wide spectrum of different XOS structures with different substituents on the xylose backbone and degrees of polymerization, which consequently have a great impact on their prebiotic and other functional properties.

For example, Vieira et al.[51] demonstrated that the lignocellulosic by-product of palm processing (*Bactris gasipaes* Kunth) can serve as an excellent substrate for XOS production. After mild alkali pretreatment of peach palm waste (inner sheath and peel), XOS were obtained by enzymatic hydrolysis using a commercial xylanase from *Aspergillus oryzae* with XOS yields from xylan inner sheath and xylan peel of 50.1% and 48.8%, respectively. The XOS obtained showed exceptional antioxidant capacity, significantly higher than that obtained from commercial xylan. In the last decades, several processes for XOS production have been evaluated, but it is still an evolving field where the best methods, catalysts and substrates are not completely clear. Therefore, special evaluation of xylan structure and content is of utmost importance for the development of a viable XOS synthesis process, especially in terms of enzyme selection. Endo-1,4-β-xylanases (EC 3.2.1.8) are the main enzymes responsible for the hydrolysis of xylan to

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XOS. Given their diversity, some of them work well on unsubstituted xylan, while others are more susceptible to substituted xylan. Nevertheless, the use of auxiliary enzymes to remove xylan substituents such as α-glucuronidases (EC 3.2.1.139), α-arabinofuranosidases (EC 3.2.1.55) and acetyl esterases (EC 3.1.1.72) has been investigated. An example of enzyme synergism is the degradation of oat spelt xylan using α-L-arabinofuranosidases from *Aspergillus hortai*, produced and purified from a medium containing citrus pulp and orange peel, and a previously described purified endoxylanase from the same microorganism. The experiments were carried out at 40ºC using 2% (w/v) oat spelt xylan solution in ammonium acetate buffer (pH 4.5), and the enzymes were used individually or in sequential reactions. The results showed that the prior action of the α-L-arabinofuranosidases, which removed the side arabinose substituents and made the main chain more accessible to xylanase action, resulted in a two-fold increase in the hydrolysis yields achieved.[52] It should be noted, however, that some xylanases show a preference for substituted xylans when auxiliary enzymes should be included in the process to generate unsubstituted XOS and allow for their better utilization by target microorganisms.[53] For example, Zhou et al. carried out the hydrolysis of hardwood xylan (glucoronoxylan) using *Thermotoga maritima* xylanase (XynB) and α-glucuronidase (AguA) co-expressed in *Escherichia coli via* dual-promoter and bicistronic constructs to reduce enzyme costs. The α-glucuronidase enabled the removal of 4-O-methyl-D-glucuronic acid residues from branched XOS, resulting in an increase in the antioxidant capacity of XOS mixtures produced with both XynB and AguA. In addition, these unsubstituted XOS are believed to significantly accelerate the growth of some bacteria from *Bifidobacterium* sp.[53] Xylanases are thus excellent tools to tailor prebiotic oligosaccharides with the intention of stimulating different types of bacteria from different niches, in congruence with the newly established definition of prebiotics.[54]

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Recently, in order to achieve better XOS production, tremendous efforts have been invested in the development of new enzymes with improved activity and stability by increasing the XOS/xylose ratio.

Also selected microorganisms are cultivated to produce extracellular xylanases that would thereafter hydrolyze xylan to XOS. A new strain, *Bacillus subtilis* KCX006, was found to constitutively produce endo-xylanase and xylan debranching enzymes without *β*-xylosidase activity in the presence of lignocellulosic biomass.[55] Therefore, this microorganism was used for simultaneous production of xylanase and XOS from lignocellulosic biomass (wheat bran, rice bran, rice husk and sugarcane bagasse SB) throughout the solid-state fermentation. In order to provide nitrogen sources for *Bacillus* growth organic and inorganic nitrogen sources together with different oil-cakes (ground nut oil-cake, sunflower oil-cake, castor oil-cake, and cotton seed cake) were used. Among various substrates wheat bran and groundnut oil-cake supported highest xylanase and XOS production, which under optimized conditions yielded 3102 IU/g and 48 mg/g, respectively.

POS (Fig 3b) represent the most diverse group of functionally active carbohydrates within the group of emerging prebiotics. Numerous studies have showed different physiological activities of POS mixtures, such as prebiotic, antibacterial, anticancer and antioxidant properties.[56] The main reason for this diversity is a complexity of structure of molecule of origin - pectin. This heteropolysaccharide, which is major constituent of the higher plants cell wall, consists of four main structural components: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II) and xylogalacturonan (XG).[57] The most abundant pectin substructure (more than 65%) is homogalacturonan (HG) that represents sequence of galacturonic acid residues with *α*-

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1,4-linkages, occasionally esterified with methyl (C6) or acetyl groups (O2 and O3). The rest of the molecule comprises of different monosaccharides (rhamnose, arabinose, galactose, and xylose) and various linkages.[56] POS can be produced from pectin by several methods such as enzymatic and acid hydrolysis, hydrothermal treatment method or combination of acid/enzymatic/hydrothermal treatment. The choice of employed methods is primarily dependent of pectin source; however, the application of enzymes showed the greatest potential (Fig.5).

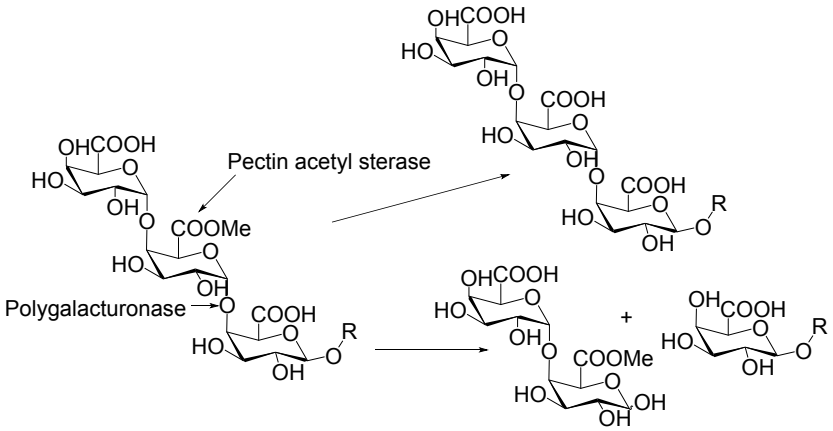

Fig. 5. Enzymatic hydrolysis of pectin.

Up to date pectin from various sources has been utilized for POS production, especially since various agricultural by-products (apple pomace, sugar beet pulp, citrus waste, berry pomace…) were recognized as cheap and abundant source.[56-57] Enzymatic hydrolysis procedure is highly complex, and requires the synergistic action of different groups of enzymes: hydrolases, esterases and lyases.[58] Enzymes responsible for pectin degradation can be majorly divided into HG degrading (polygalacturonase, EC 3.2.1.15, pectate lyase EC 4.2.2.2 and pectin lyases EC 4.2.2.10, pectin methyl esterase EC 3.1.1.11, and pectin acetyl esterase EC 3.1.1.6) and rhamnogalacturonan-I (RG-I) degrading enzymes (α-arabinofuranosidase EC 3.2.1.55, endoarabinase EC 3.2.1.99, *β*-galactosidase EC

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3.2.1.23, endogalactanase EC 3.2.1.89 and feruloyl EC 3.1.1.73 and *p*-coumaroyl esterases EC 3.1.1.B10).

Even though there are examples of newly isolated enzymes that are utilized for POS synthesis,[57] most of the publications present the results of commercial hydrolytic enzyme mixtures derived from *Aspergillus* sp.[56] For instance, Sabater and co-workers used a commercial enzyme preparation Viscozyme® L, multi-enzyme complex from *Aspergillus aculeatus* for hydrolysis of artichoke pectin into POS. Enzymatic process was optimized using an experimental design and further analyzed by application of artificial neural networks, yielding 65.9% of pectin conversion to POS at optimal conditions. The structural elucidation of obtained POS revealed presence of oligosaccharides DP2–DP6 (from dimer to oligomers with 6 units of monomers) that exhibited strong radical scavenging activities.[59] The same enzyme was utilized for conversion of pectin from industrial byproducts, such as lemon peels, sugar beet pulp [60] and onion skin[61] to form a functionally active POS. The crude pectic extract from onion skins, mostly made of homogalacturonan with very scarce rhamnogalacturonan regions, obtained by sodium hexametaphosphate (SHMP) extraction was processed by utilization of a continuous crossflow membrane bioreactor. The enzymatic hydrolysis and *in situ* membrane separation were combined to obtain high yields of tailor-made POS, because membrane allows the continuous removal of products of targeted DP simultaneously protecting the POS from further hydrolysis and monosaccharide formation, hence avoiding enzyme inhibition by reaction products. The reaction resulted in high POS yields (around 60%) provided that enzyme and substrate concentrations were 41.4 U/ml and 50 g/L, respectively. Under these conditions, the highest POS volumetric productivity (22.0 g/L/h), as well as the lowest POS/monosaccharide ratio (4.5 g/g) were achieved. Also it

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must be noted that a stable production was achieved for whole reaction period and short POS (DP2, DP3, and DP4) were observed as main reaction products.[61]

In addition to dominant processes which include utilization of hydrolases, lyases are often applied for POS synthesis by cleaving the α-1,4-glycosidic bond of substrate molecules by trans *β*-elimination reactions.[62] The recent example of cold-active pectate lyases drew particular attention due to their ability to retain high catalytic efficiency under lower temperatures, simultaneously enabling energy saving, cost reduction and potentially the preservation of physiochemical properties of treated products. A new cold-tolerant pectate lyase (ErPelPL1) gene from *Echinicola rosea* was cloned and thereafter heterologously expressed in bacteria *Escherichia coli*. The enzyme was purified by high-affinity Ni-charged resin FF (Ni–NTA Sepharose) and the molecular mass of 50 kDa was observed. The enzyme exhibited high catalytic activity at a low temperature (4°C) although it exhibited optimal activity at 35℃, pH 8.0 in the presence of 1 mM of Ca^{2+}. It was found that wide range of oligosaccharides (DP2-6) can be observed during the reaction course with predominance of DP2-3.[62]

MOS (Fig 3c) are group of non-digestible oligosaccharides, which comprise of 2-10 mannose units linked by two types of glycosidic bonds (α-1,6 and *β*-1,4). Accordingly, MOS can be further divided into two major groups: *α*- and *β*-MOS, mostly based on the substrate source. Small amounts of naturally occurring MOS can be extracted from structural and storage parts of plants, however the greatest deal of MOS is nowadays generated by the hydrolytic cleavage of different naturally occurring mannans (Fig 6). While *α*-MOS are products of yeast cell wall (*α*-1,6-mannan) hydrolysis well established as a feed additive in the agriculture industry,[63] *β*-MOS can be derived from mannans that represent one of the major constituents (around 50%) of hemicellulose within variety of

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plants - primarily softwood, plant seeds and legumes. Also, certain agricultural by-products (for example copra meal or palm kernel meal)[64] and waste materials such as spent coffee grounds[65] proved to be a great mannan sources (up to 60% of mannans). Plant mannans may appear as homo- (linear mannan) or hetero-polymers (galactomannan, glucomannan and galactoglucomannan) of mannose depending on a source, and therefore, *β*-MOS are primarily generated throughout hydrolytic activity of *β*-mannanase (EC 3.2.1.78) leading to *β*-MOS of varying degree of polymerization (DP) generation. Different *β*-mannanases showed different preferences in terms of potential substrates and potential products, thus, achieved yields and DP of obtained MOS proved to be highly dependent upon the utilized *β*-mannanase. For example, with *β*-mannanase from *Aspergillus oryzae* (ManAo) under the same conditions, MOS were obtained from various agricultural byproducts (locust bean gum (LBG), guar gum (GG), konjac gum (KG), palm kernel cake (PKC) and copra meal (CM)) in wide range of yields 9-56 %,[46] clearly showing specificity towards particular substrates or more specifically types of mannans within these substrates. Magengelele et al. examined potential of recombinant *Aspergillus niger* endo-mannanase (Man26A) expressed in *Saccharomyces cerevisiae* Y294 for conversion of three substrates - ivory nut linear mannan and two galactomannan substrates with varying amounts of galactosyl substitutions (GG and LBG).[67] The enzyme exhibited high substrate specificity towards locust bean gum and ivory nut mannan with major products DP 2-4, while specificity towards guar gum was rather low and these reactions generated MOS of higher DP. However, when considering the quantity of obtained MOS, the results showed discrepancy, since higher yields were achieved when using galactomannans (4.91 mg/mL total reducing sugars for guar gum and 3.89 mg/mL total reducing sugars for locust bean gum) as substrates than linear mannan (2.24 mg/mL total reducing sugars) during extended periods of mannan hydrolysis. Unlike these

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enzymes, some β-mannanases show an obvious preference towards unsubstituted regions of linear mannan, and therefore utilization of different auxiliary enzymes such as β-glucosidase (EC 3.2.1.21), β-mannosidase (EC3.2.1.25), as main-chain mannan-degrading enzymes, and α-galactosidase (EC 3.2.1.22), and acetyl mannan esterase (EC 3.1.1.6), that provide removing of side-chain substituents and breaking down the complex structure of substituted mannans, might be taken into consideration.[68] Yang et al. showed that the hydrolysis efficiency of the β-mannanase from *Trichoderma reesei* is greatly affected by the side chain of galactoses that introduce steric hindrance for enzyme. They have examined potential of α-galactosidase to enhance hydrolysis of galactomannan from Sesbania seeds (26 % w/w), by adding enzyme separately (firstly treated with α-galactosidase), sequentially, and simultaneously. Finally, the enzymatic hydrolysis by simultaneous addition of α-galactosidase significantly improved obtained MOS yields (from 17% to 31%).[68] However, it must be noted here that apart from the enzyme and substrate selection the careful optimization of process conditions should be performed in order to achieve satisfactory product yields, and some of these examples are presented within several review papers. [64,67] Besides the achieved yields, determination of DP of obtained products plays an important part in development of MOS synthesis process, since their prebiotic function, as well as their antioxidant, anti-inflammatory, cryoprotectant, anti-stress and anti-diabetic potential, is highly dependent upon this property. Arunrattanamook et al. used gut microbiota model microorganism *Lactobacillus reuteri,* to examine the prebiotic potential of synthesized β-MOS.[69] This study showed that medium length MOS (DP 4 and DP 5) exhibited the highest prebiotic potential, since higher MOS were poorly utilized and on the other hand, excessive hydrolysis of mannans resulted in the loss of selectivity toward beneficial bacteria. Therefore, in terms of obtaining the most promising MOS prebiotic mixture and increase

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selectivity toward production of medium length MOS, different strategies have been proposed, such as changing the sources of mannan or optimizing enzymatic hydrolysis conditions, manipulation with *β*-mannanase.[66-68] In their work, Arunrattanamook et al. [69] improved the specificity of *β*-mannanase from *Aspergillus niger* (ManF3) toward the desired product size through rational-based enzyme engineering. Namely, they have replaced tyrosine (Tyr 42 and Tyr 132) within the enzyme active site with glycine, which being smaller amino acid, enabled formation of extended substrate-binding site and consequently, increased the possibility for higher molecular weight MOS to bind to the enzyme. This mutation finally resulted in additional space for the enzyme to accommodate larger hydrolysis products. Mutations of tyrosine into glycine resulted with enhanced yields of medium chain MOS (DP4 and DP5), however these mutations have reduced hydrophobic interactions within the enzyme molecule and thus, negatively affected thermal and conformational stability of the mutant enzymes, why it was impossible to obtain double mutant enzyme despite being successfully constructed.

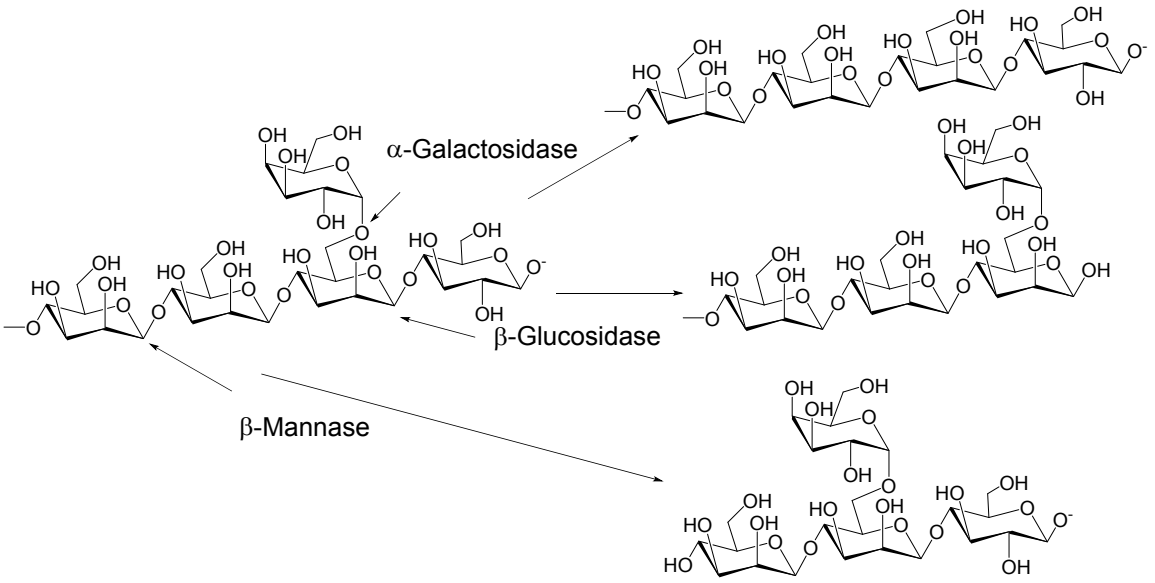

Fig. 6. Hydrolysis of mannans.

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Up to date, numerous β-mannanases have been tested for MOS synthesis, prevalently from genera *Aspergillus* and *Bacillus*.[67] However, recent studies have brought to our knowledge a previously scarcely explored group of β-mannanolytic enzymes originated from gut microbiota. For example, Bhattacharya et al.[70] have explored potential of a cell-surface exposed β-mannanase (BoMan26B) from the abundant gut bacterium *Bacteroides ovatus* for MOS synthesis from galactomannan abundant in legumes and acetyl-galactoglucomannan abundant in softwoods. The reaction yielded partially acetylated linear and galactosyl containing β-MOS (MOS/GMOS) that had approximate degree of polymerization (DP) between 2 and 6, which was revealed using newly developed high-resolution anion-exchange chromatography procedure. Abundance of MOS derived from galactomannan was in the following order: prevalently DP 5 followed by DP 6 = DP 2 and DP 4 and finally DP 3, while profile of MOS derived from acetyl-galactoglucomannan was slightly different: prevalently DP 5 followed by DP 4 and DP 2 = DP 3 and finally DP 6. Under the optimal conditions, yields of obtained oligosaccharides were 33% (w/w) and 30% (w/w), respectively. Prebiotic potential of obtained oligosaccharides was confirmed by means of measuring production of short-chain fatty acids using the human gut bacteria *Bifidobacterium adolescentis* ATCC 15703 and *Roseburia hominis* DSMZ 6839 as acetate and butyrate producers, respectively.[70]

IMO (Fig 3d) are defined as a mixture of oligosaccharides composed of several (usually 2-10) glucose units linked with α-(l⟶4) and α-(1⟶6) glycosidic bonds. They are mainly comprised of isomaltose, panose, isomaltotriose, isomaltotetraose, isopanose, and higher branched oligosaccharides. Great deal of IMO is nowadays synthesized throughout different chemical and enzymatic process, but they could be found in honey and soy based fermented products, such as miso and soy sauce, in small quantities. Apart from their controversial prebiotic activity due to existence of readily digestible α-l,4

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linkages, which places them in the emerging prebiotics category, they are characterized by high temperature and pH stability, low viscosity, low water activity, that enabled approval of various health claims making them interesting for application in food and feed sector.[71] IMO are primarily synthesized by enzymatic conversion of starch from natural and sustainable plant sources (Fig 7). Cereal crops (wheat, rice, barley, corn), pulses, and tubers (cassava and potato) are considered major sources of starch to produce IMO.[72] This process involves multimeric enzymatic action of α-amylase (EC 3.2.1.1) and β-amylase (EC 3.2.1.2) that hydrolyze the long and internal starch branches, together with the starch debranching pullulanase (EC 3.2.1.41) and transglycosylating α-glucosidase (EC 3.2.1.20) as shown at Fig. 7. During the first phase, thermo-tolerant α-amylase liquefies the starch by random cleavage α-1,4 linkages, and thereafter α-amylase together with β-amylase, and pullulanase provide starch conversion to maltooligosaccharides. Subsequent reaction is catalyzed by α-glucosidase, enzyme with primary hydrolytic function that acts on the non-reducing terminal of α-glucosides, but alternatively can transfer a glucosyl residue to another glucose, maltose, isomaltose or isomaltotriose molecule by formation of α-1,6 linkages. Studies have shown that this step is crucial in IMO synthesis, and that the right choice of α-glucosidase and reaction conditions may greatly influence functional activity of obtained products throughout increment of panose and isomaltose share in oligosaccharides.[72] For example, it is determined that simultaneous processes of saccharification and transglycosylation are more likely to produce increase in yield and productivity of IMO from starch compared to the conventional process enabling lower glucose accumulation through greater transglycosylation. Duong Hong et al., for instance, developed a simple two-step procedure for IMO production using sweet potato starch as a low-cost substrate.[73] Firstly, they have examined the effect of several commercial α-amylase preparations (Spezyme

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Xtra, Liquozyme SC DS, Spezyme Alpha, and Termamyl SC DS) on the process of starch liquefaction (25 % w/v) after 30 minutes with aim to find out the most suitable α-amylase from the aspect of obtained mixture of oligosaccharides. The targeted oligosaccharide mixture of first step was DP 2-6, since it was presumed that it would shorten the simultaneous saccharification and transglycosylation time required for maximum concentration of future IMO with DP between 2 and 4. Accordingly, Spezyme Xtra preparation (1.0 CU/g) was chosen for liquefaction reaction since the highest concentration of wanted oligosaccharides (49.24% w/w) without any generation of free glucose and low generation of starch residue (1.98% w/w) was achieved.

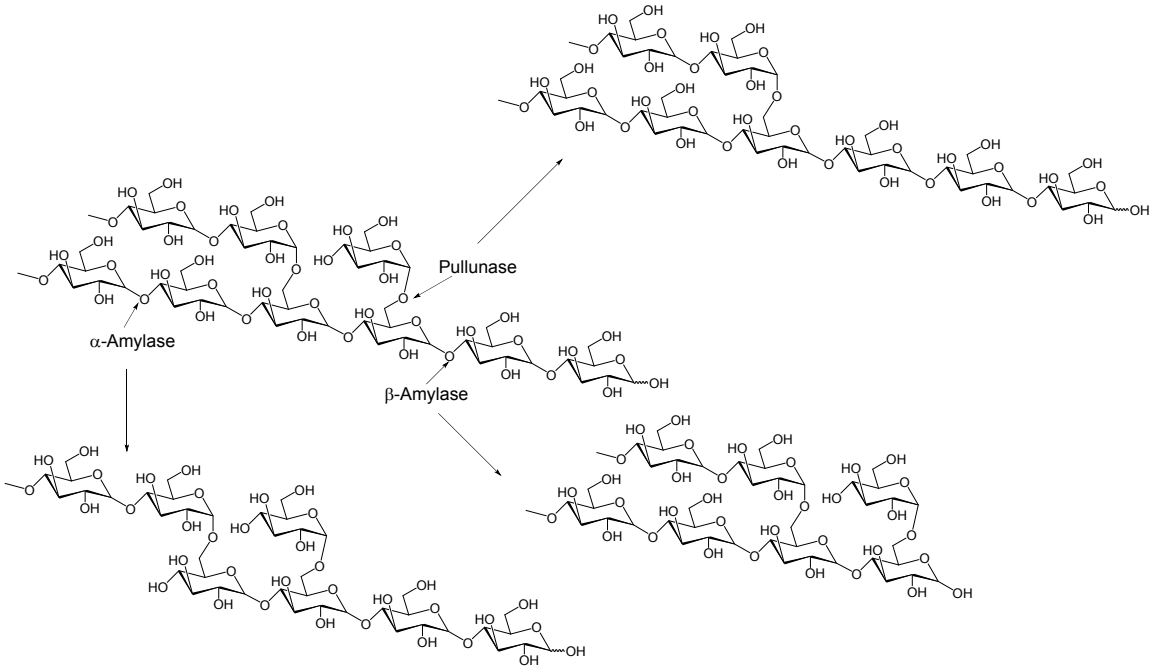

Fig. 7. Hydrolysis of starch by different enzymes.

Thereafter the barley *β*-amylase, pullulanase M2 from *Bacillus licheniformis*, and transglucosidase from *Aspergillus niger* were used for simultaneous saccharification and transglycosylation upon the detailed optimization of enzyme dosage and reaction conditions. Finally, *β*-amylase (3 U/g), pullulanase (0.8 U/g), transglucosidase (10 U/g)

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were applied leading to generation of 68.85 g/L of IMO at total reaction time three times shorter than in previous studies.[73]

Another approach toward the development of IMO rich in panose involves the isolation of new α-glucosidase enzyme preparation that would possess advanced kinetic characteristics, primarily increase in the ratio of transglycosylation to hydrolytic activity. Kumar et al. have isolated for the first time *α*-glucosidase from non-niger *Aspergillus* isolate with high transglycosylation potential.[73] The new soil isolate was identified as *Aspergillus neoniger.* After purification using DEAE Sepharose-CL6B column, *α*-glucosidase was found to possess molecular mass of 145 kDa and some structural similarities with commercially available α-glucosidase from *Aspergillus niger,* although their gene sequence are considerably different. When comparing their activities, it must be noted that maltose consumption profiles for both enzymes were similar, even though the initial rate of consumption is slightly slower in case of the new enzyme. Likewise, the hydrolysis rate of maltose to glucose is lower when compared to the commercial enzyme, which is quite important since low generation of undesirable by-product can be expected. Additionally, the new enzyme showed higher potential for panose synthesis in comparison to commercial enzyme, as well as reduction of secondary hydrolysis of panose rate.[74] Therefore, it can be concluded that this enzyme shows a great potential for IMO synthesis. Successful maltose conversion into desirable IMO (panose, isomaltose and isomaltotriose) can be also achieved using *Saccharomyces cerevisiae* cells with α-glucosidase activity.[73] Namely, aglA gene that encodes α-glucosidase from *Aspergillus niger,* known for its transglycosylating activity, was expressed in *Saccharomyces cerevisiae* in a manner that yeast cells can be used directly as the catalytic agent. The aglA gene was fused to glycosylphosphatidylinositol anchor sequences, from the yeast

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SED1 gene, which enabled stable attachment of the resulting hybrid enzymes to the cell surface. Thereafter, the potential of obtained enzyme preparation to perform maltose conversion into IMO was examined. After 8 h incubation, the only reaction product present at a significant concentration was panose. Despite the fact that panose and glucose are produced at equimolar concentrations in the transglycosylation reaction, the glucose is most likely consumed by the yeast during this initial period, since it was not detected in the reaction mixture. As the reaction progresses, the glucose concentration increases, becoming available as the acceptor to produce isomaltose. Therefore, after 24 h, the main transglycosylation product is isomaltose, with lower but significant amounts of panose and isomaltotriose, and low concentrations of higher IMO.[74]

Alternative way of IMO production is based on sucrose as substrate for synthesis of dextran throughout the activity of dextransucrase (EC 2.4.1.5), and subsequent conversion of dextran into IMO using activity of dextranase (EC 3.2.1.11). Similarly, to IMO production from starch, the highest interest of scientific community was in development of efficient transglycosylation process.

Chitin is a polymer composed mostly of GlcNAc units linked by β-1→4 bonds, along with small amounts of GlcN. It is one of the most abundant polymers in nature, along with cellulose. Natural sources of chitin include exoskeletons of arthropods, mollusks, marine crustaceans, and various microorganisms.[75-76] The large quantities of shell and exoskeleton waste generated by these sources contribute to ocean and environmental pollution. As a result, numerous studies have been conducted to explore the potential applications of chitin and its derivatives. Thus, these polymers have been used as food stabilizers, dietary fiber, and in healthcare.[77-78] However, their crystalline structure, resulting from a high proportion of hydrogen bonds, makes them poorly soluble

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in most solvents. Therefore, the deacetylated product has also garnered interest due to its improved solubility in water and greater chemical modifiability, which often results in higher physiological activity. Chitosan is the deacetylated product of chitin. It is typically produced using chemical methods on a large scale, which consume significant amounts of acids and energy, making them environmentally unsustainable. However, enzymatic hydrolysis methods offer many advantages over chemical methods, including high efficiency in environmentally benign conditions. Biosynthetic methods are used to prepare various oligosaccharides, including those derived from natural and semi-synthetic sources. These carbohydrates, known as CHOS (Fig 3e), possess beneficial biological properties, such as antibacterial,[79] immunoprotective,[80] antitumor,[81] and improvements in intestinal health.[82] As a result, they have significant applicability, particularly in the food industry.

CHOS can be enzymatically produced through chitin hydrolysis processes or synthesis processes from monomeric or short-chain molecules. Chitin hydrolysis processes are catalyzed by the enzyme chitinase, which has endo- and exo-chitinase activity (EC 3.2.1.14 and 3.2.1.29, respectively) (Fig.8). Other enzymes, such as lysozyme, lipase, pectinase, glucanase, or papain, have also been described to catalyze the hydrolysis of chitin.[83] Yhamabai et al. described the use of chitinase from an extract of *Bacillus chitosanase*, BsCsn46A, which was capable of producing oligosaccharides of 2-3 sugar units after a 48-hour reaction. The study also demonstrated the anti-inflammatory activity of these oligosaccharides.[84]

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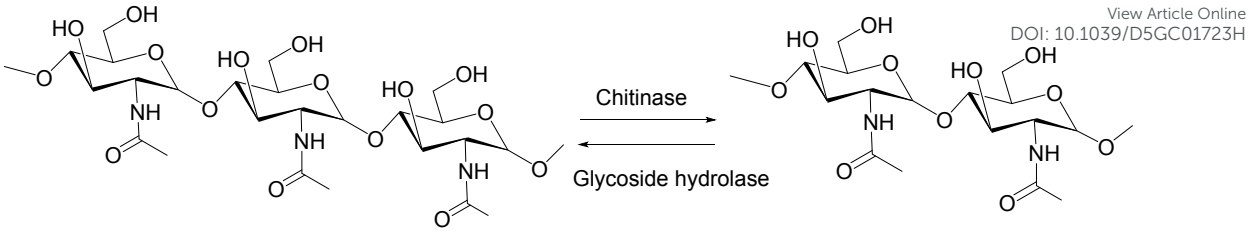

Fig. 8. Hydrolysis/synthesis of chitin oligosaccharides catalyzed by chitinase/glycoside hydrolase.

Oligosaccharides can be obtained from monomer units through a hydrolytic process. This reaction is typically catalyzed by the enzyme glycoside hydrolase, either through reverse hydrolysis or transglycosylation.

2.2. Polyphenolic emerging prebiotics

According to the latest definition of prebiotics by the ISAPP in 2017, a compound is considered a prebiotic if it positively impacts the structure and activity of the microbiota, thereby enhancing host health.[85] This broader definition has spurred increased and more intensive research into the synthesis and evaluation of potential prebiotics for two main reasons: it expands the range of compounds that meet this criterion, and it acknowledges that the targeted microbiota includes not only the gut but also the skin, thereby involving different microorganisms that respond to different prebiotics.

Polyphenols are among the most thoroughly researched emerging prebiotics. These compounds are widely known for their beneficial bioactivities, such as antioxidant, antiatherogenic, anti-inflammatory,[86-87] and nowadays effect of different polyphenol structures on human microbiota is analyzed to select those with prebiotic properties and thereby novel field of application. Based on reports of polyphenols as compounds which

simultaneously exhibit stimulatory effect on beneficial human microbiota and antimicrobial activity against pathogens conferring a health benefit, there is a suggestion of novel classification of polyphenols as emerging class of ”duplibiotics”.[88] Polyphenols act as prebiotics by influencing the gut microbiota through complex interactions with microorganisms. Some studies have shown that they uniquely support the growth of beneficial commensal microorganisms, while inhibiting the growth of pathogens. [87,89] The most representative studies dealing with polyphenol-rich extracts as potential prebiotics are presented in the Table 1. Evidences on prebiotic activity are available for plant extracts rich in certain types of flavonoids, however straightforward relationship between specific structures and prebiotic activity is established only in a limited number of human studies.

Polyphenolic compounds can be classified into various groups based on their chemical structures, including flavonoids, phenolic acids, tannins, coumarins, quinones, stilbenes, and lignans and different types of polyphenol structures are presented in Fig. 9. [86]

Table 1. Review of the studies of examining polyphenol-rich extracts as potential prebiotics.

Polyphenols	Microbes stimulated in the gut	Microbes inhibited in the gut	Dosage of the polyphenol	Kind of techniques for the analysis used	Ref.
Orange juice rich in hesperidin and naringenin	*Lactobacillus*, *Akkermansia*, and *Ruminococcus*	-	300 ml/day orange juice for 60 days	*In vivo* study using human faces, qPCR	90
Mango rich in gallotannins and gallic acid	*Lactococcus lactis*	*Clostridium leptum Bacteroides thetaiotaomicron*	400 g of mango/day for 6 weeks	*In vivo* study using human faces, qPCR	91
Blackcurrant extracts	Bifidobacteria, Lactobacilli	*Bacteroides*, Clostridia	2 ml of berry extracts/day for 4 weeks	*In vivo* study using the cecal of rats, FISH	92
Blackcurrant extract powder	Bifidobacteria, Lactobacilli	*Clostridium* spp. and *Bacteroides* spp.	672 mg of blackcurrant powder/d	*In vivo* study using human faces, FISH	93

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Mixture of anthocyanins from blueberry, black currant and black rice extracts.	Bacteroidetes	Firmicutes and Actinobacteria	215 mg anthocyanins/d for 8 week	*In vivo* study using human faces	94
Cranberry powder rich in anthocyanins	*A. muciniphila, Muribaculaceae, D. newyorkensis, Angelakisella, Coriobacteriaceae, Eggerthellaceae*	-	200 mg of polyphenols/kg of body weight in mice for 8 weeks	*In vivo* study using the faces of HFHS-fed mice	95
Concord grape polyphenols rich in anthocyanins	*Akkermansia muciniphila*	reduction in the ratio of Firmicutes to Bacteroidetes	1% Concord grape polyphenols for 13 weeks	*In vivo* study using the faces of C57BL/6J mice.	96
Bilberry extract rich in anthocyanins	*Lactobacillus*	reduction of the Firmicutes/Bacteroidetes ratio	10, 20 or 40 mg extract/kg body weight/day for 70 days	*In vivo* study using the faces of Sprague-Dawley rats	97
Green tea extract rich in Epigallocatechin in gallate Dark tea extract rich in Gallocatechin	*Lactococcus and Akkermansia* *Lactococcus* and *Akkermansia*	*Turicibacter and Romboutsia* *Turicibacter,Parasutterella, Lachnoclostridium*	5 mg/kg bodyweight/day for 4 weeks	*In vivo* study using the faces of C57BL/6J female mice	98
Gallic, ellagic acid, chebulinic acid from triphala	*Bifidobacterium spp. Lactobacillus spp.*	*E. coli*	2% of triphala extract for 3 weeks	In vivo study using *Drosophila melanogaster*; qPCR	99
Fermented papaya juice rich in gallic and caffeic acids	-	Firmicutes, *Clostridium scindens/ Eggerthella lenta*	9 g fermented papaya juice/day for 30 days	*In vivo* study using human faces, qPCR	100
Chlorogenic acid	*Bacteroidaceae, Lactobacillaceae*	*Desulfovibrionacea, Ruminococcaceae, Lachnospiraceae, Erysipelotrichaceae*	150 mg/kg/day for 6 weeks	*In vivo* study using the cecum of mice	101
Ripened Pu-erh tea extract rich in catechins	*Roseburia and Akkermansia*	reduction of the Firmicutes/Bacteroidetes ratio	0.4% of water extract for 8 weeks	*In vivo* study using male C57BL/6N mice	102
Dicaffeoylquinic acids from *Ilex kudingcha*	*Bifidobacterium and Akkermansia*	-	3.3 or 10 mg/mouse for 8 weeks	*In vivo* study using male *C57BL/6 mice*	103

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They are commonly extracted from these raw materials by conventional extraction methods which involve utilization of organic solvents. However, novel environment-friendly extraction techniques, which relies on the use of microwaves, ultrasound and enzymes, along with greener media such as deep eutectic solvents, water or supercritical fluids, are nowadays being extensively examined. [104-105]

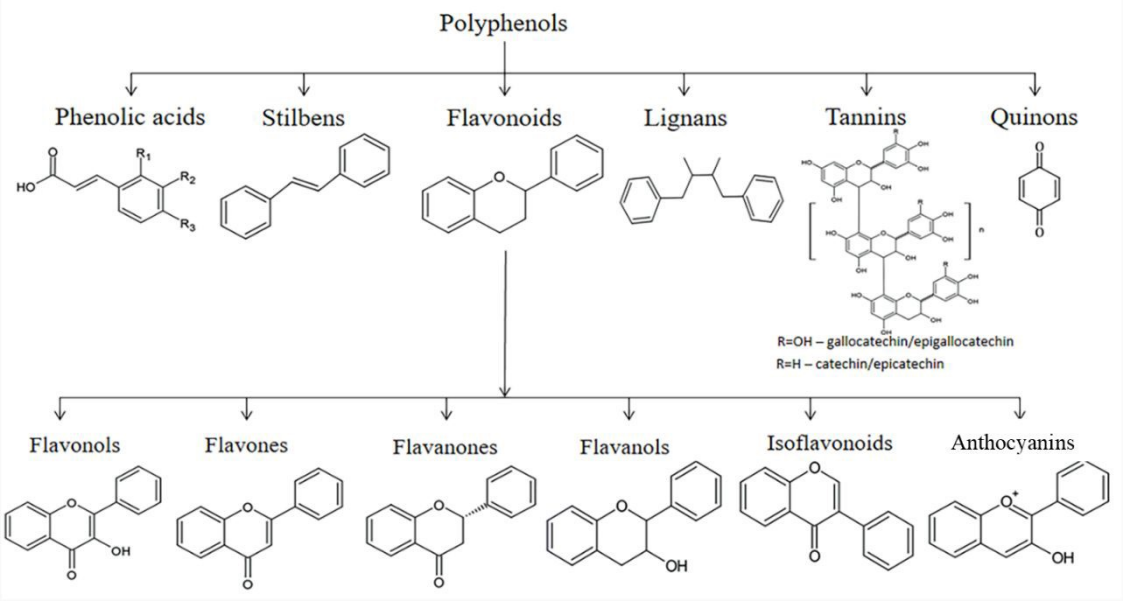

Fig 9. Classification of polyphenolic compounds.

Enzyme-assisted extraction procedures have emerged as very promising since, in addition to the ability of cell wall degrading enzymes to improve polyphenol extraction efficiency and extract composition, they can also serve as a tool for modifying naturally occurring phenolic compounds, thereby altering their bioavailability and bioactivity, including prebiotic potential (Fig. 10).[12] The following literature examples will give insight into how different enzyme preparations were previously used for obtaining high yields of specific phenolics with emerging prebiotic potential.

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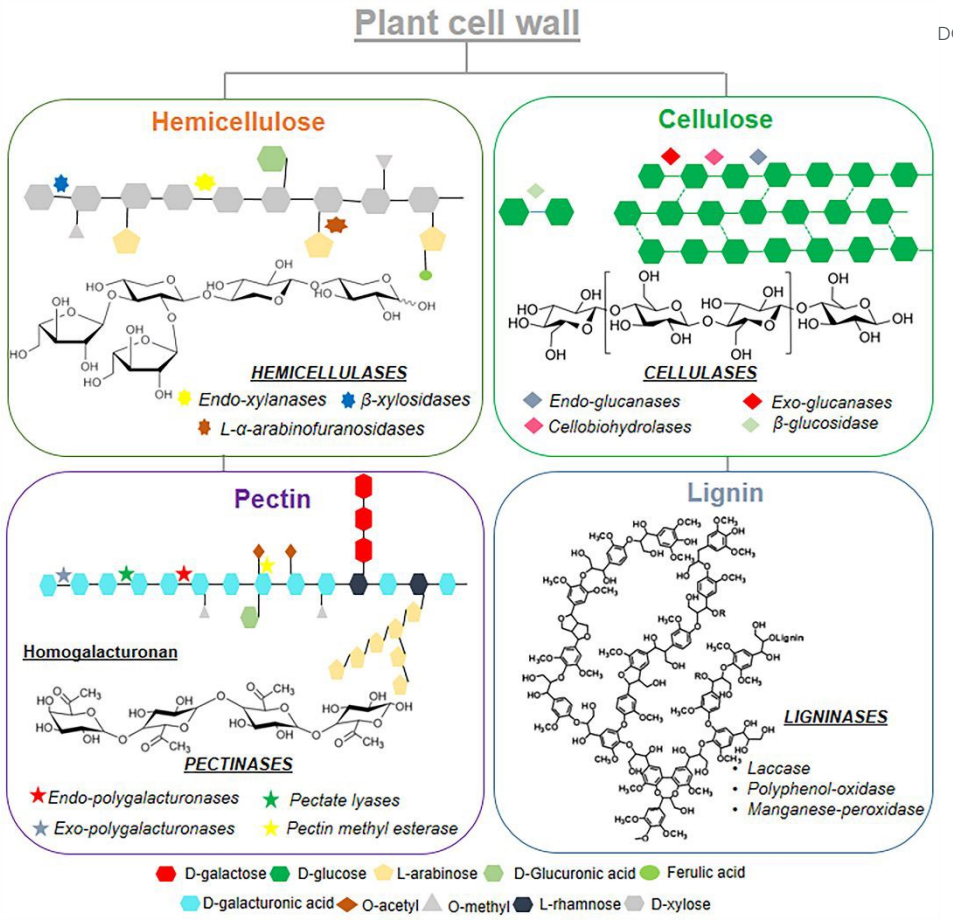

Fig. 10. Schematic representation of enzyme-catalyzed hydrolysis of plant cell wall structures.

One of the polyphenols most thoroughly examined as potential prebiotic is quercetin. Several animal and *in vitro* studies found that quercetin had positive effect on intestinal microbiota composition. [107-110] It is widely distributed in various fruits, vegetables, grains and leaves usually as glycoside derivatives, hence *β*-glucosidase-based modification processes were often applied for obtaining this compound. In a study performed by Lindahl *et al.* enzymatic hydrolysis of quercetin 3,4'-*O*-diglucoside (Fig. 11a) was combined with continuous-flow hot water extractor which resulted in high quercetin yields from yellow, red and shallot onions. [111] Onion skin waste, widely available industrial waste which originates from onions processing, was also used as raw material for quercetin production. For example, Choi and coworkers obtained increase of quercetin

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extraction yield of 1.61 times, in comparison with conventional method, using mixture of cellulase (Cellulclast® 1.5L), pectinase (Pectinex® SP-L) and xylanase (X2629 endo-1,4-*β*-D-xylanase).[112] Other plant materials have also been used for quercetin production in enzyme-based processes. In study with guava leaves as raw material, enzyme-aided extraction enabled significantly higher yields of soluble phenolics, including quercetin, compared to conventional extraction.[113] While xylanase-assisted extraction did not influence composition and yield of extracted polyphenols, process with cellulase or *Trichoderma reesei β*-glucosidase enhanced content of soluble phenolics around two times, while enhancement of quercetin was even higher (3.5-fold).

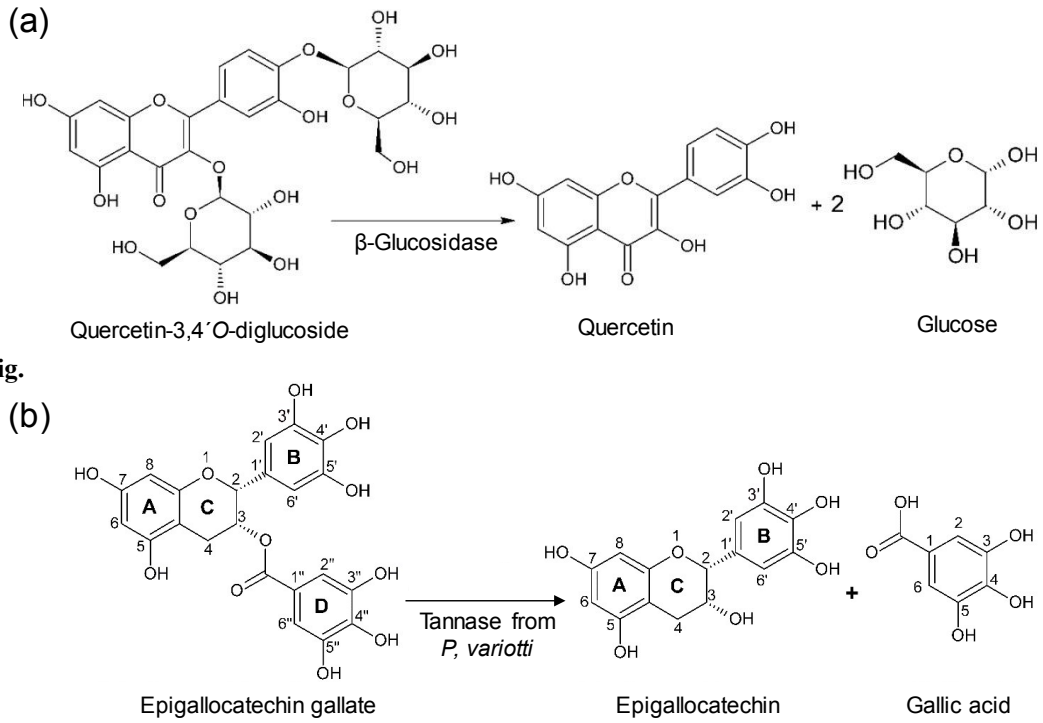

Fig. 11 A) Hydrolysis of quercetin 3,4'-*O*-diglucoside catalyzed by *β*-glucosidase (TnBgl1A N221S/P342L). B) 5BHydrolysis of epigallocatehin gallate catalyzed by tannase from *P. variotii*.

β-glucosidase activity is generally useful for transforming wide range of naturally occurring phenolic glycosides other than quercetin into their aglycons. One such example is a sequential process of enzymatic hydrolysis and supercritical fluid extraction of

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flavonols and dihydrochalcones from apple pomace.[114] Commercial enzyme mix snailase readily removed 96% of sugar moieties from quercetin glycosides, kaempferol glycosides, phloridzin and 3-hydroxyphloridzin by exhibiting *β*-glucosidase activity and enabled production of flavonoid aglycones (quercetin, kaempferol, phloretin and 3-hydroxyphloretin) with 90% extraction yield after sCO_2 extraction. A simultaneous process of enzyme-assisted supercritical fluid extraction was also successfully performed. Phenolic acid rich extracts (Table 1) and individual phenolic acids (e.g. sodium ferulate, gallic acid etc.)[115-116] were also widely assessed as potential intestinal prebiotics. Enzyme-assisted extraction processes have shown good potential for obtaining increased yields of these compounds from various plant materials. For example, very high yields of three phenolic acids (*p*-coumaric acid 8 times, ferulic acid 4 times, and caffeic acid 32 times) in extract was achieved, in an extraction of unripe apples with enzyme preparation Viscozyme® L. [117] Another illustrative study was focused on grape seed extract and grape pomace as known sources of polyphenols. By applying commercial *Aspergillus ficum* tannase with grape seed extract and grape pomace and pectinolytic preparation Pektozyme® with grape pomace galloylated form of catechin was modified into its free form, releasing gallic acid which led to increase antioxidant activity. [118] Meini and coworkers applied enzymatically-assisted process for the extraction of grape pomace polyphenols and proved that application of pectinase, cellulase and tannase could significantly improve extraction efficiency (by 66%) and antioxidant activity (by 80%), but also serve as a tool for obtaining extracts of different compositions. [119] Hence, using tannase from *A. oryzae* particularly influenced gallic acid extraction, while *p*-coumaric acid and malvidin-3-*O*-glucoside were extracted more efficiently with the aid of cellulase from *A. niger*.

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Flavan-3-ols are structurally very diverse derivatives of flavans with numerous reports of prebiotic potential of various monomeric, oligomeric and polymeric flavan-3-ols, both in forms of extracts rich in these compounds (Table 1) or as individual molecules.[120-122] *A. niger* cellulase and pectinase were used for extraction of catechins from black tea (Assam tea variety, S3A3 tea cultivar) leaves and enabled catechin and epigallocatechin gallate content increase by 51.26 and 15.36%, respectively, compared to conventional hot water extraction process.[123] Another study combined enzymatic treatment with cell wall-degrading enzymes with sequential tannase hydrolysis of extracted catechins from green tea leaves, which led to increased extraction efficiency and free radical scavenging activity. [124] Among all tested enzymes, Viscozyme® L stood out as most effective, since after sequential treatment with Viscozyme® L and tannase, more than 95% of epigallocatechingallate and epicatechingallate was hydrolyzed to epigallocatechin and epicatechin and high amounts of gallic acid were released increasing antioxidant activity. Battestin *et al.* also applied tannase-catalyzed hydrolysis for obtaining epigallocatechin and gallic acid by the degalloylation of epigallocatechin gallate green tea extract using *Paecilomyces variotii* tannase (Fig. 11b). [125] By Since epigallocatehin gallate, unlike degalloylated derivatives and gallic acid, was not able to revert gut microbiota dysbiosis in previous researches, these examples show high potential of using tannase treatments for increasing not only antioxidant, but also prebiotic capacity of plant extracts naturally rich in galloylated catechins.

Anthocyanins are also very thoroughly investigated, because not only growth of beneficial gut microbiota is being stimulated, but also by metabolizing anthocyanins microbiota act as the ‘mediator’ that increases bioavailability and therapeutic potential of anthocyanins against different chronic diseases, such as obesity, type II diabetes, cardiovascular disease, neurodegenerative disease, inflammatory bowel disease, cancer,

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fatty liver disease, chronic kidney disease and osteoarthritis. [126] Number of relevant literature data are showing gut microbiota modulatory activity of anthocyanin rich extracts prebiotic-like effect of extracts rich in specific anthocyanins (Table 1). [127-129] Enzyme-assisted extraction of anthocyanins from mulberry wine residues and eggplant peel enabled significant increase of total anthocyanin and polyphenol content in comparison with conventional process. Dominant anthocyanins of mulberry wine extract were cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside, while eggplant peel extract was reach in delphinidin, petunidin and malvidin. [130-131]

Evident effect of type of carbohydrate moiety of polyphenols on their prebiotic properties indicate that enzymatic modification of this part of molecule can be used as biotechnological tool for fine tuning of their activity. Unlike *β*-glucosidase, which liberates aglycon forms of corresponding phenolic glycosides, rhamnosidase was previously used for their partial deglycosylation which often led to increased prebiotic activity. Good example is study by Pan *et al.*, in which comparison of effects on human gut microbiota and its metabolism of different aglycons (hesperetin (flavanon), naringenin (flavanon) and quercetin (flavonol)), their diglycosides (hesperidin, naringin and rutin) and enzymatically derived (Fig. 12) monoglycosidic forms (hesperetin-7-*O*-glucoside, prunin and isoquercitrin) was analyzed by *in vitro* simulated fermentation.[132] Results showed that among nine tested flavonoids, enzymatically derived monoglycosides exhibit most significant beneficial effect on the human gut microbiota composition. *Bifidobacterium* and *Lactobacillus* abundance was significantly enhanced by three monoglycosides and rutin, while potential pathogens (e.g., *Lachnoclostridium* and *Bilophila*) were inhibited by three monoglycosides, naringin and rutin.

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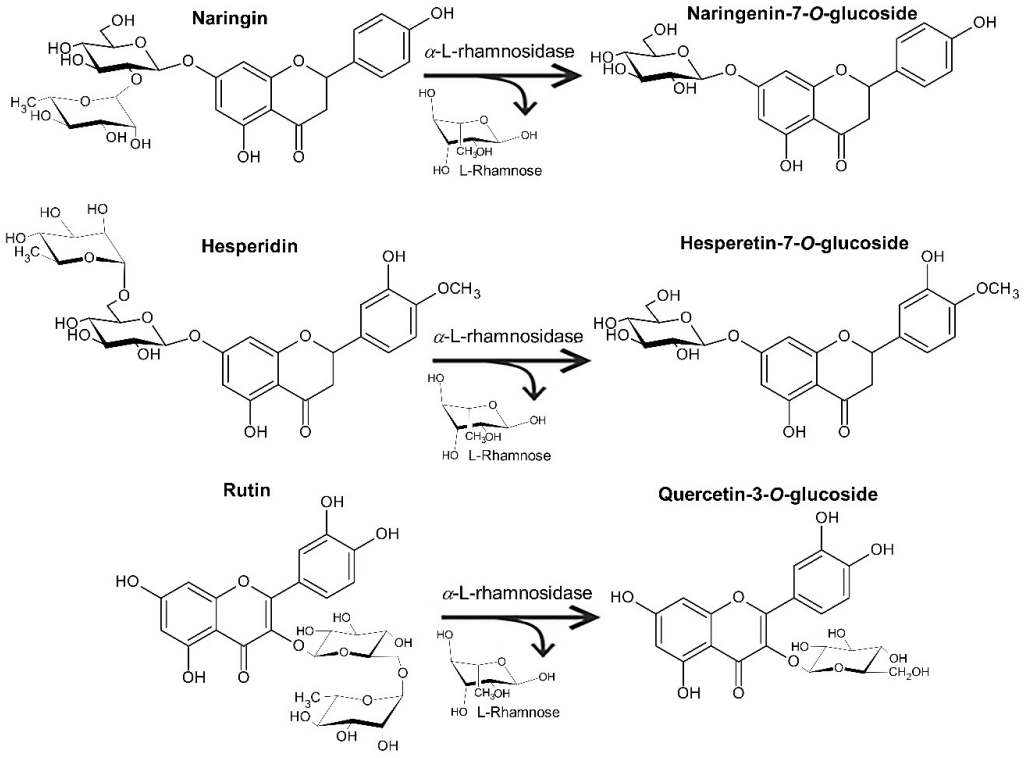

Fig. 12. *α*-L-rhamnosidase derived monoglycosides, hesperetin-7-*O*-glucoside, naringenin-7-*O*-glucoside, and quercetin-3-*O*-glucoside, demonstrate most significant effect on the human gut microbiota composition.

Importance of transformation of polyphenols to monoglycosidic form for their prebiotic activity was observed in another study where rutin, hesperidin, naringin and narcissin/rutin mixture were used and 14 tested probiotic strains showed substrate specific rhamnosidase activity which ranged up to 27 % for hesperidin and 56 % for narcissin after four days and 80% for hesperidin and 97 % for narcissin after 10 days. [133]

Beside gut, skin microbiome is attracting increasing attention of scientists, consumers and industry. Nowadays, it is known that certain skin commensal strains, such as coagulase negative *Staphilococci*, are considered as probiotics against pathogenic species, primarily *S. aureus*. [134-136] Frequent application of antibiotics, weakened immune system, genetic and external factors are often leading to development of various skin diseases (e.g.

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psoriasis, atopic dermatitis) accompanied by skin microbiome disbalance characterized by decreased microbial diversity and decreased population of commensal bacteria. Beside oligosaccharides and certain sugar alcohols (e.g. xylitol), polyphenols were assessed for their skin prebiotic potential.[137-140] Study with blackcurrant extract revealed that enzymatic processes can provide improvements in development of novel skin prebiotics, since it was shown that enzymatically derived extract, obtained using mixture of commercial preparations Viscozyme® L and Rohapect® MC, exhibited higher prebiotic capacity compared to conventionally obtained one, since it promoted beneficial *S. epidermidis* growth to higher extent and inhibited harmful *S. aureus* more strongly. [141] Chromatographic analysis revealed differences in anthocyanin composition caused by partial cyanidin rutinoside and delphinidin rutinoside hydrolysis in enzymatic extracts, implicating that formed monoglycosides could be responsible for increased prebiotic activity, which is in line with some of the results obtained with gut microorganisms. It should be noted that enzymatically derived extracts were rich in cell wall polysaccharides degradation products, since preparations with cellulolytic and pectinolytic activity were applied, which could also contribute to improved prebiotic properties.

At the end, it should be noted that, although promising results were obtained with different polyphenol-rich plant extracts or their constituents obtained by conventional and enzymatic processes, dose-dependent inhibitory effect of various polyphenols against probiotic strains and stimulation of pathogens was previously reported as well, indicating that further investigations are needed for better understanding of their gut/skin microbiome modulatory activity and prospective oral/topical application. [142-144]

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3. Technologies for improving enzymes in oligosaccharides synthesis

3.1 Protein engineering modification

Protein engineering at the genetic level have been an interesting approach to improve enzymes.[145] This approach has been very useful to improve the production of oligosaccharides by different ways. One approach is to introduce mutations in the sequence of hydrolytic enzymes to improve their activities. For example, in production of xylooligosaccharides, Wang et al. have proposed a method for improving the catalytic performance of XynLC9 xylanase from *Bacillus subtilis* by mutation of the N-terminal residues 5-YWQN-8 leading to development of two mutants named W6F/ Q7H and N8Y that possess a 2.6- and 1.8-fold higher catalytic activity than wild type XynLC9, respectively. The double mutant W6F/Q7H with highest catalytic activity was selected to hydrolyze corncob-extracted xylan in comparison to wild type enzyme. During the whole reaction course, W6F/Q7H (substitutions at positions 6 and 7 with Phe and His) exhibited superior performance in XOS synthesis and after 14 h the concentration of XOS reached 10.6 mg/ml, which was about 1.6-fold higher yield than that of wild type XynLC9. Additionally, both mutants showed enhanced thermostability.[146]

A second approach is focused to eradicate the hydrolytic capacity of the enzymes by punctual amino acid modification in the protein structure to get a synthase, the so-called glycosynthases. Thus, the concept is based on the exchange of the catalytic residue (usually an aspartate or glutamate) responsible for promoting a nucleophilic attack on the substituted anomeric carbon (Fig. 13) by a non-functional residue (usually alanine or glycine). As a result of that, they become hydrolytically incompetent.[147-148] Then, the enzymatic ability of the enzyme goes through the transfer of an activated glycosyl donor

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(typically a glycosyl fluoride in anomeric position) to a suitable acceptor to catalyze the formation of a glycosidic bond at high yields.[147]

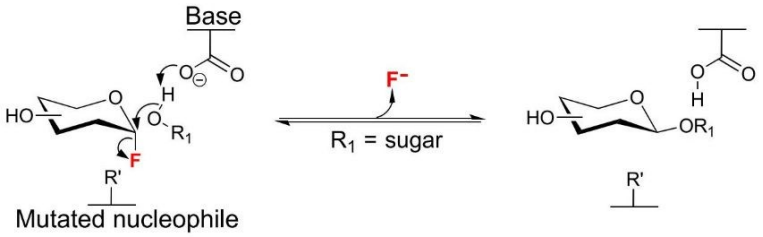

Fig. 13. Trans-Glycosylation process catalyzed by β-Glycosynthases.

This synthetic potential has an enormous impact in the oligosaccharide synthesis.[129] As prebiotic compounds, complex human milk oligosaccharides are gaining increased attention.[150-151] A microorganism engineered for the synthesis of oligosaccharides from nutrients is considered the most promising system for process development.[152] Therefore, K. Schmölzer *et al* [153] developed a glucosynthase (variant D746E) from *Bifidobacterium bifidum* β-N-acetylhexosaminidase JCM1254 through β-glycosylation of activated N-acetyl-D-glucosaminyl donors by 1,2-oxazoline (Fig. 14a). This represents an important synthetic strategy towards oligosaccharides.[154] The authors emphasized enzymatic chemoselectivity as it is decisive for highly efficient glycosylation of lactose (~90%) of NAG-oxa lactose giving rise to lacto-N -triose II (LNT II), a central building block of human milk oligosaccharides (HMOs). The wild-type enzyme, in contrast, hydrolyzes both the NAG-oxa donor and the trisaccharide product with significantly higher activity than glycosynthase. This makes the wild-type enzyme quite unsuitable for synthetic application. This involves chemically prepared NAG-oxa in 40% yield from N-acetyl-D-glucosamine (GlcNAc). Using equivalent amounts of NAG-oxa and lactose at their solubility limit (600 mM), LNT II was obtained (515 mM; 281 mg mL−1; ~90% yield; ≤1 h reaction time), and could be immediately recovered from the biocatalytic reaction

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with 85% purity. These process efficiency metrics reveal the remarkable potential of glycosynthase for chemical process application and highlight it as far superior to alternative synthetic options for trisaccharide production.

BbhI is an exo-acting β-N-acetylhexosaminidase and belongs to the GH-20 family of glycoside hydrolase families. Enzymes of the GH-20 family use the participation of the neighboring group of the 2-acetamido group of the substrate in catalysis. The enzymatic reaction is promoted by a highly conserved triad of residues (Glu, Asp, Tyr; Fig 14b) and proceeds through an intermediate 1,2-oxazolinium ion. A close-up of the active site of a modeled structure of BbhI is shown in Fig. 14c. Based on evidence for endo-β-N-acetylglycosaminidases from the GH-18 and GH-85,[155-156] families, a promising design for the BbhI glycosynthase was to substitute residues (Asp746, Tyr827) involved in the stabilization of the oxazolinium intermediate. The structure model (Fig. 14c) corroborates the evidence for the sequence alignment (Fig. 14b) by suggesting that Asp746 and Tyr827 are positionally conserved at the BbhI active site. Within the GH-20 family, there is limited precedence in the development of glycosynthases. A variant D313A of β-hexosaminidase from *Streptomyces plicatus* (GH-20) and Tyr470 (Phe, His, Asn) variants of β-hexosaminidase from *Talaromyces flavus*[157] (GH-20) reflect conceptually similar enzyme design strategies to the one applied here to BbhI. Four site-directed variants (D746E, D746A, D746Q, Y827F) of BbhI were prepared (Fig.14d). Asp746 variants involve loss of electrostatic stabilization (D746A) of the intermediate, a likely steric conflict in substrate/intermediate positioning (D746E), or both (D746Q).

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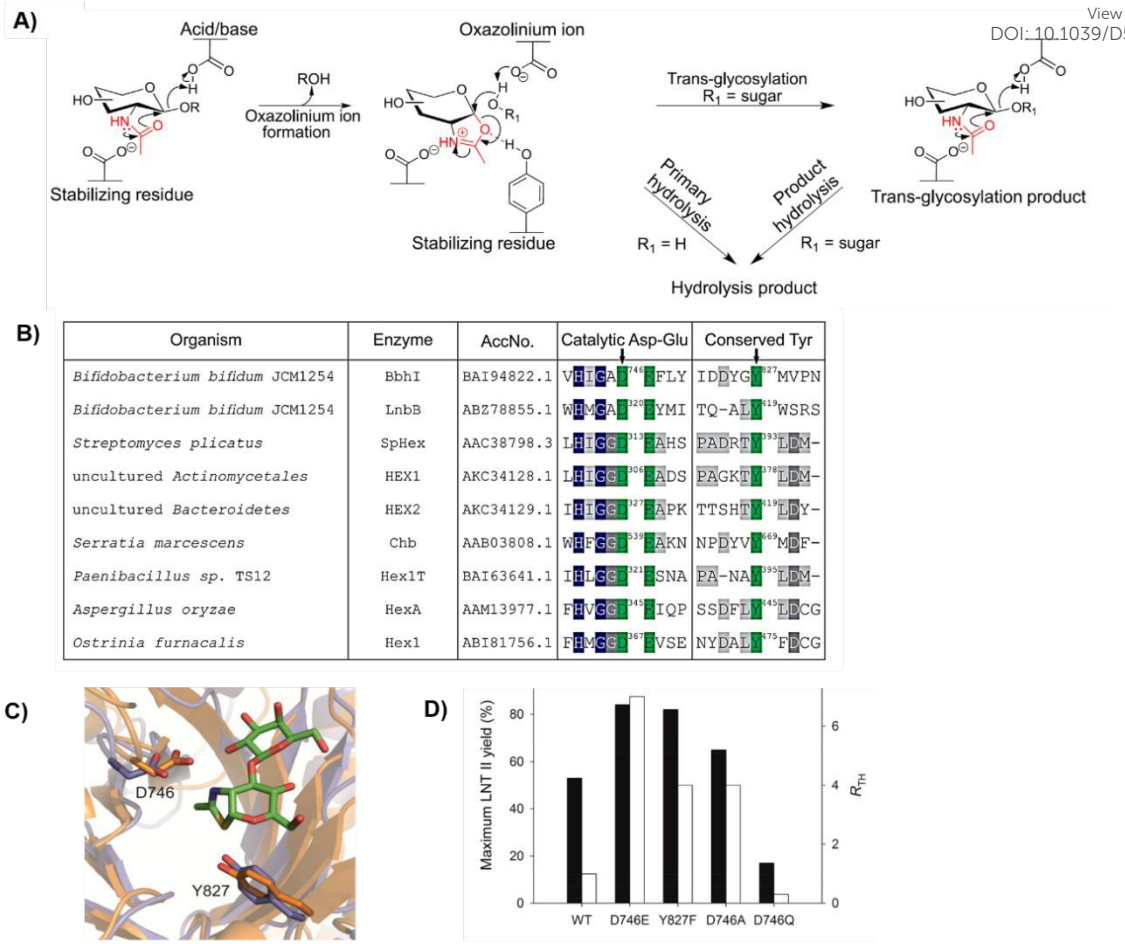

Fig.14 A) Trans-Glycosylation by β-Glycosynthases and β-N-Acetylhexosaminidases; **B)** Partial sequence alignment of pro- and eukaryotic GH-20 β-N-acetylhexosaminidases displaying residues critical for substrate-assisted catalysis. Critical amino acids (green), mutation sites (arrows); **C)** Close-up view of the modeled active site of BbhI. The homology model (shown in blue). Key active-site residues of BbhI used for mutagenesis dare drawn as sticks (Asp746; D746, polarizing residue; Tyr827; Y827, stabilization of reaction intermediate by hydrogen bonding). The LNB-thiazoline bound in lacto-N-biosidase is shown with green-colored carbon atoms; **D)** Comparison of enzymes regarding the maximum yield of LNT II (black bars) and the selectivity parameter RTH (white bars). Copyright® 2019 American Chemical society.

The Y827F variant involves removal of a hydrogen bond for substrate binding and catalysis.[158] However, they are predicted to have low activity for LNT II hydrolysis, but

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these BbhI variants could utilize 1,2-oxazoline of N-acetyl-D-glucosamine (NAG-oxa) as a donor for the β-1,3-glycosylation of lactose.

3.2 Chemical Modification of protein surface: controlling selectivity in the synthesis of short-oligosaccharides.

Selectivity represents one key important characteristic in the enzymatic synthesis. Structural modifications could influence in the capacity to recognize one regioisomer of a particular carbohydrate over another. Thus, recently modifications by introducing chemical molecules on the protein surface have been sucessfully developed to enhance this.[159-163] One example has demonstrated the selectivity of levansucrose in controlling the polymer size formation.[159] Authors performed a chemical modification on the tyrosine of Levansucrease from *B. megaterium*, which preferentially happened at residues Y196 and Y247 (Fig. 15). The modification was based on a luminol derivative which contein a alkyne group, which represent an orthogonal group. Then, by click chemistry reaction using an azide-anomeric activated glucose, regiselectivity monosaccharides are incorporated on the protein. This modification allowed to alter regioselectivity of the enzyme, and the bioconjugate was able to produce mainly larger polymers (Fig. 15).

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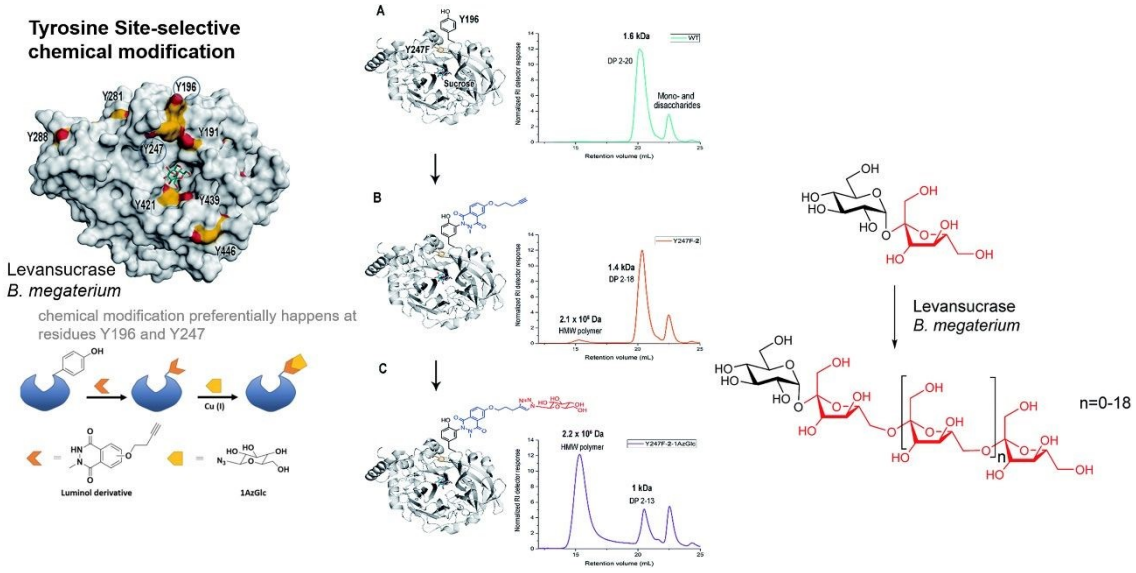

Fig 15. Polymer-elongation specificity by chemial modification of levansucrase from *B. megaterium.* Figure adapted from ref. 159 with permission from RSC publishing, copyright 2018.

Another new alternative in the production of new carbohydrate molecules has been the *in situ* cascade metallo-enzymatic reaction. In this approach, a new type of artificial metalloenzyme containing two active sites, one from the enzyme an another artificial created by enzyme-induced *in situ* generation of copper nanoparticles (CuNPs) has been developed.[162-163] A green process in the production of the new biocatalyts allowed the production of a previous lipase supported on graphene sheets, enabling the selective formation of CuNPs on the enzyme surface, exclusively where the process involved coordination of the metal with the enzyme aminoacids in aqueous media. This CuNPs modified lipase conserved the excellent enzymatic regioselectivity against monodeacetylation of peracetylatd glucal in C-3 and combined with the oxidative capacity of the CuNPs for disaccharides synthesis by a epoxy-intermediate formation (Fig 16). This chemical modification allow to obtain a very robust biactive catalysts with excellent reciclability.[162-163]

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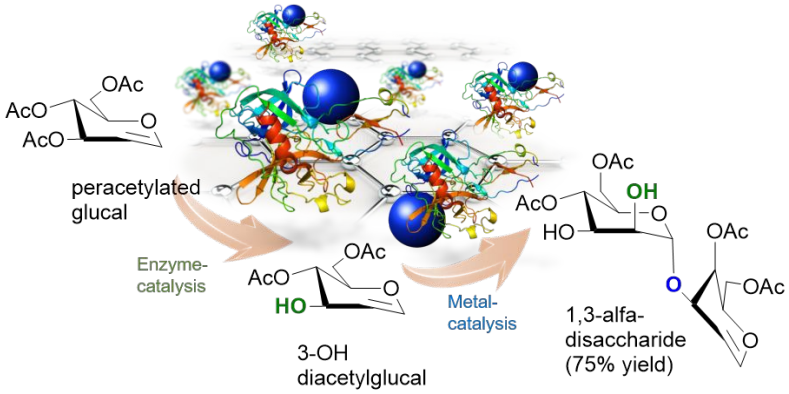

Fig. 16. Synthesis of new dissacharides by bioconjugate CuNPs-enzyme hybrid. Adapted from ref. 162 with permission from the American Chemical Society, copyright 2023.

3.3 Immobilization technology to obtain oligosaccharides for industrial application

The use of immobilized enzymes in chemical processes offers several advantages over processes catalyzed by soluble enzymes. One of the most significant advantages is the ability to reuse the catalyst during different reaction cycles. This eliminates the need for purification processes caused by the mixture of the soluble enzyme and the different components of the reaction in the case of homogeneous catalysis processes.[164] In addition to these advantages, other benefits have been described, such as the ability to use immobilization processes to enhance various enzyme properties, including activity, stability and selectivity.[165-168] In the field of oligosaccharide production, enzymes are often improved to enhance their properties and enable the reuse of catalysts in successive reaction cycles. Immobilization has been shown to improve enzyme properties, as demonstrated by the immobilization of a xylanase from *Aspergillus niger* on porous agarose supports using covalent attachment methods. The derivatives resulting from this

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process were 1100 times more stable than the soluble enzyme. This allowed for the production of 60% of oligosaccharides ranging from X2 to X6 xylose units.[169]

The polygalacturonase from *Aspergillus aculeatus* was immobilized by entrapment in sol-gel systems, resulting in highly active derivatives (94.6% of its initial activity) and improved stability. The immobilized enzyme retained 57% of its catalytic activity after incubation at 55ºC for 2 hours, compared to only 17% for the free enzyme. Furthermore, antibacterial activity of the obtained POS using standard methods was confirmed for POS within DP2–DP4 range. This immobilization method allowed for enzyme reuse, with 65% activity retained after 6 batch reactions.[170]

A β-mannanase from Konjac glucomannan preparation was immobilized by entrapment in alginate gels. The enzyme extract, with a purity of 95%, retained 68.3% of its catalytic activity after immobilization. The immobilized catalysts were more stable against extreme pH and high temperatures, with optimal pH and temperatures of 6 and 75ºC, respectively. These catalysts could be reused in the production of MOS for 8 cycles, retaining 70.3% of their catalytic activity. In the oligosaccharide production assay, MOSs was generated at a concentration of 8 mg/mL, consisting of 5 mg/mL of mannobiose and 3 mg/mL of mannotriose.[171]

In order to make the process of MOS synthesis more cost-effective and economically viable by enhancement of enzyme stability, Suryawanshi et al. carried out LBG hydrolysis by utilization of immobilized preparation from a newly isolated newly isolated *Aspergillus quadrilineatus* RSNK-1.[172] Namely, the mixture of hemicellulotytic enzymes comprised of endo-*β*-mannanase, endo-*β*-xylanase, *β*-xylosidase, *β*-glucosidase and *α*-galactosidase generated by SSF fermentation on low-cost copra meal was covalently immobilized on aluminum oxide pellets (AOP) after glutaraldehyde 1% (v/v) activation.

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After the immobilization procedure optimization using statistical method of response surface methodology, MOS production was performed in column bioreactor. The reaction finished in 20 min, and thereafter the obtained immobilized preparation (Man-AOP) was successfully recycled 10 times, without significant activity loss. The average MOS generation within the 10 successive cycles was 0.95 mg/cycle (1.50, 5.15 and 2.84 mg/mL of DP4, DP3 and DP2 MOS, respectively).

Chalane et al.[173] described an endodextranase system (D8144) from *Penicillium sp*. immobilized on an epoxy-activated monolithic Convective Interaction Media (CIM®) disk to produce on-line IMO from Dextran T40. The system retained more than 80% of its residual activity after 5000 column volumes, demonstrating the high stability of the immobilized endodextranases.

Hooda co-immobilized chitinase and glucoseaminidase onto polyurethane nanoparticles coated with zinc oxide.[174] The co-immobilized catalyst demonstrated greater activity under optimal pH and temperature and higher stability against temperature than soluble enzymes. After incubating the soluble enzymes at 75ºC, there were a 75% loss of activity, while in the case of the co-immobilized catalyst, the loss was only 40%. The catalyst properties were improved, enabling its reuse for at least 50 reaction cycles, with a 50% loss in catalytic efficiency.

Another interesting example of immobilization has been described by Ruzic *et al*,[175] employing the same variant, i.e., *Bifidobacterium bifidum* β-N-acetyl hexosaminidase variant D746E was developed using glucosynthase immobilized on Cu^{2+}- agarose beads (4%) (~30 mg/g) packed in a fixed bed (1 mL), for stable continuous production of LNT II (145–200 mM) with a quantitative yield of donor substrate (Fig. 17a). Wild-type β-N-acetyl-hexosaminidase used under exactly comparable conditions gives mainly (~85%)

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the hydrolysis product D-glucosamine. By allowing short residence times (2 min) that are difficult to establish for mixed-pot reactor types, the glycosynthase flow reactor achieves effective uncoupling of LNT II formation (~80–100 mM/min) from slower side reactions (decomposition of donor substrate, enzymatic hydrolysis of LNT II) for optimal synthetic efficiency (Figure 17b-c). Thus, this study provides a strong case for the application of flow chemistry principles to glucosynthase reactions and thereby reveals the important synergy between enzyme and reaction engineering for biocatalytic oligosaccharide synthesis.

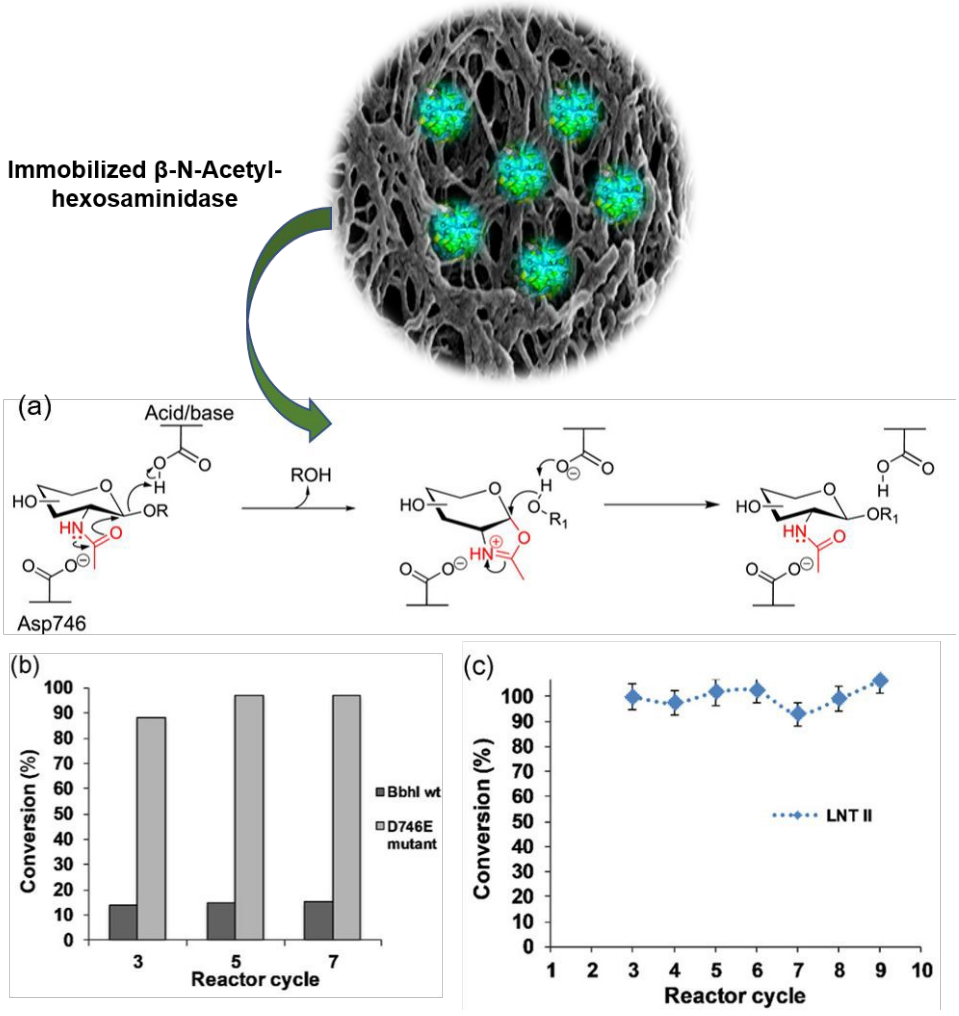

Fig. 17. A) Asp746 of BbhI facilitates the formation of the oxazoline intermediate in the enzymatic reaction.;B) Conversion of GlcNAc-oxa into LNT II.; C) Recyclability process using immobilized D746E glycosynthase.

3.4. Process intensification in oligosaccharides synthesis

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Process intensification (PI) concepts and methods can deliver significant benefits to biochemicals and green chemicals production. Most promising PI concepts for enzymatic bioproducts synthesis are transition from batch to continuous processing in novel types of reactors (e.g. micro/milli reactors, oscillatory flow reactors, spiral reactors, rotating disk reactors, etc.), integration of reaction and separation in multifunctional units (e.g. membrane reactors, chromatographic reactors, etc.) and utilization of alternative energy sources (e.g. microwaves, sonication, etc.). Application of mentioned methods in reactor and process design can result in radical improvements of productivity, higher desired product selectivity, reduction of reactors volumes and other fixed costs, lower energy and utilities costs, better process operability and control, and reduced waste generation. Recent reviews[176-178] are shedding light on versatile and ample possibilities of PI for biochemical applications and therefore proposing a new term: Bioprocess Intensification (BPI / BI).

Regarding emerging prebiotics synthesis, research and utilization of PI is still in early stage. Expectedly, more examples in the literature can be found for established prebiotics, e.g. galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). Fig. 18 presents process intensification methods and devices used for prebiotics synthesis, including the main results achieved (Fig. 18). For transfer from batch to continuous processing, classical tubular reactors with fixed bed of immobilized enzymes have been investigated (Fig 18a). Shin *et al.*[179] have used continuous fixed-bed reactor with *β*-galactosidase adsorbed at chitosan particles and obtained good GOS yield of 55% with stable operation for 15 days. Warmerdam *et al.*[180] reported they achieved the same GOS yield in a fixed-bed bioreactor, as in a comparable batch one, but the volumetric productivity was

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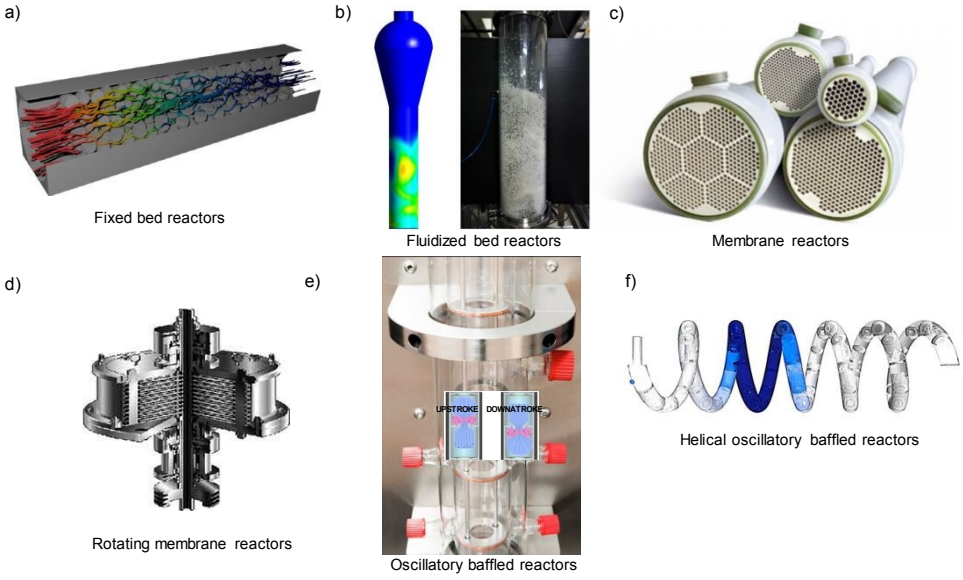
increased 6 times in the continuous reactor. Lorenzoni *et al.*[181] used both fixed-bed and fluidized-bed reactors for continuous production of FOS. They reported similar FOS yields (54-59%) in both reactors, although immobilized enzymes were more stable in the fixed-bed (no changes in activity for 40 days). Zambelli *et al.*[182] showed for FOS synthesis that the reaction time of 72 h in batch can be reduced to 10 h in fixed-bed reactor, resulting in much higher volumetric productivity. Downsides of fixed-bed reactors are relatively high pressure drop and possible mass transfer limitations, both external due to low velocities used (long residence times needed), and internal due to interparticle diffusion effects (in case of porous particles with immobilized enzymes) (Fig 18b). Those negative effects can be somewhat reduced in fluidized-bed reactors. Membrane reactors have also demonstrated potential for process improvement, as *in situ* separation of components can lead to higher conversions of substrate and yields of the prebiotic products. Petzelbauer *et al.*[183] used membrane reactor for GOS synthesis and gain GOS concentration which was 3 to 4 times higher than in batch under the same conditions. Córdova *et al.*[184] have designed and optimized an ultrafiltration membrane bioreactor. By using high concentrations of lactose, the amount of generated GOS per unit mass of the catalyst is increased by 2.44 times compared to batch systems. Pottratz *et al.*[185] investigated the production of GOS by immobilizing the enzyme β-galactosidase on a methacrylic macroporous monolith that was used as a membrane reactor. In this reactor, the fluid flows unhindered through the pores, which reduces the mass transfer limitations (that occur when conventional supports are used). The results showed that during continuous production, the GOS yield was up to 60% higher compared to the batch. These reactors are suitable for industrial production due to the simple process scale-up achieved by adding monoliths. Sen *et al.*[186] compared a rotary disk membrane bioreactor (Fig 18d) with a batch reactor in terms of GOS production. The research

showed that the product yield and purity were higher in the rotating disk membrane bioreactor, with yields 80.2% and 77%, respectively. When comparing product purity, the obtained purity is twice as high when using a rotary disk membrane bioreactor (67.4%) than when using a batch reactor followed by membrane filtration (32.4%). Disadvantages of membrane reactors are the use of high pressures that can reduce enzyme activity by damaging its activation centers, possibility of membrane clogging and fouling; all of which can lower the yield and productivity.



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PI methods in continuous flow synthesis				Reactor types	OS	Main results - [Reference]
Immobilized enzymes				Fixed bed reactors	GOS	Yield 55% - [97]
					GOS	Productivity 6x batch - [98]
					FOS	Productivity 7x batch - [100]
					XOS	Yield 80%, Conversion 3x batch - [110]
				Fluidized bed reactors	FOS	Yield 55-59%, High stability - [99]
Combined reaction and separation				Membrane reactors with immobilized enzyme	GOS	Yield 60% higher than batch - [103]
				Membrane reactors	GOS	Concentration 3-4x batch - [101]
					GOS	Yield per enzyme mass 2.4x batch - [102]
					POS	Yield 83%, productivity 17.5 g/L/h [111]
Intensified mixing and transfer				Rotating membrane reactors	GOS	Yield 80% and purity 2x higher than batch [104]
				Oscillatory baffled reactors	GOS	Yield per enzyme mass 30-40% higher than batch [105, 106]
				Helical oscillatory baffled reactors	GOS	Yield 30-50% and productivity 3x batch [107, 108, 109]

Fig 18. Process Intensification strategies for prebiotic synthesis. Figure prepared adapted from ref. 179,181,183,186-189.

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Furthermore, newer types of reactors with special constructions and operation have been proposed for prebiotics synthesis. Those are oscillatory flow reactors with baffles (OBR) (Fig 18e), which exhibit very good mixing due to induced and structured vortices appearing at low net flow rates (needed for longer residence times). Slavnić[187] showed that OBRs can be efficiently used for GOS synthesis, both with free-flowing and immobilized enzymes, obtaining the productivity higher than in the batch reactor. Shear stresses in OBRs are lower than in classical mixing units (with propellers), thus the enzymes maintain their stability.[187] Recently, Todić *et al.*[188-189] proposed a modification of OBR, replacing the straight tube with a curved one in a helical oscillatory baffled reactor (HOBR, Fig18f), which displays even better mixing due to additional Dean vortices. Pravlović *et al.*[190] confirmed that GOS can be produced in 3D printed SOBR with 3-fold increase of volumetric productivity, and higher selectivity per enzyme consumed, in comparison to the batch reactor. This work[189-191] also demonstrates how modeling, optimization and 3D printing can aid rapid development of intensified enzymatic processes and novel reactors for prebiotic synthesis.

It is reasonable to assume that many of the above presented PI methods can be applied also to emerging prebiotic synthesis with comparable success. One should note that the production of emerging prebiotics is more demanding, as it commonly involves complex-structure substrates and multiphase processing. Thus, some of the above concepts would not be suitable or would need considerable modifications. Nevertheless, several research efforts have been made for intensification of emerging prebiotics synthesis, e.g. XOS and POS. A flow-type microreactor (150 x 300 μm, 18.7 μL) was used to intensify and increase the efficiency of enzymatic XOS production from beechwood xylan using purified endoxylanase from *Thermomyces lanuginosus*. The xylan hydrolysis performance was compared with the same reaction performed under batch conditions

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(100 rpm). Results of the experiments showed that XOS synthesis was significantly improved over the batch reactor, namely yield of over 80% was achieved in less than one minute of residence time. The enzymatic xylan hydrolysis performance and xylan to XOS conversion was three times higher under optimized flow conditions in comparison to the examined batch process.[192] The disadvantages of micro-reactors are related to their small-size channels that are not suitable for solid-liquid flows (often required for emerging prebiotic synthesis), as well as high fixed costs per unit of flowrate (costs for industrial level capacity).

Another continuous process was designed for production of POS from sugar beet pulp in a cross flow continuous enzyme membrane reactor achieving stable production for 28.5 h at the optimized conditions, obtaining an average POS yield of 82.9% (w/w) combined with a volumetric productivity of 17.5 g/L/h, and a specific productivity of 8.0 ± 1.0 g/g E/h. This work demonstrated stable continuous production of POS from sugar beet pulp using reactor more suitable for upscaling.[193]

4. Conclusions and outlook

Emerging prebiotics are compounds with high impact, not only through general beneficial effect of prebiotics on human health and well-being, which is very important nowadays due to increasing occurrence of diseases related to food intake and sedentary lifestyles, but also through their contribution to green transition as they are compounds that can be produced by green biotechnological processes. These prebiotics, primarily oligosaccharides and polyphenols, are eco-friendly because they are sustainably sourced since can be derived from raw materials such as industrial by-products or waste (e.g., meals, pomaces, bagasse, biomass) through extraction or hydrolysis from polysaccharide constituents. This review presents the most notable chemical

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compounds identified as emerging prebiotics, along with evidence of their prebiotic activity. Efficient enzymatic processes are essential for the optimal use of raw materials and for fine-tuning the physiological activity of prebiotics. These green catalysts are employed to produce prebiotic oligosaccharides and to enhance the extraction and structural modification of polyphenolic prebiotics. Consequently, the review highlights the significance of enzymatic glycosylation and glycolysis in the production of emerging prebiotics and describes the most relevant enzymes used in these processes. High enzyme costs often pose a significant obstacle to the development of new enzyme-based green technologies, hence this review provides a comprehensive overview of current efforts to maximize the biocatalytic potential of enzymes using engineering tools such as protein engineering, immobilization technology, and process intensification. The share of emerging prebiotics in the rapidly growing prebiotic market is expected to rise from 4.7% to 12.9% over the next decade, hence joint application of different types of scientific expertise presented in this review is necessary to commercialize processes that meet market demands while also adhering to the industry's green transition regulations. Furthermore, Life Cycle Assessment (LCA) studies could be considered for the quantification of the impacts of products and processes on their whole life cycle, adopting a holistic approach.[194-195] This analysis could facilitate the identification of the main environmental hotspots of the production process, the comparison of the different process alternatives, and the suggestion of eco-design options for the improvement of these processes. Moreover, these findings could be re-used in other LCA studies, which would include emerging prebiotics in the production system.

Author contributions

All authors revised and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Conflicts of interest

The authors declare no conflict of interest, financial or otherwise.

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Data availability statements

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- No primary research results, software or code have been included and no new data were generated or analysed as part of this review.