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Enzymatic Trends of Fructooligosaccharides Production by Microorganisms

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Abstract Fructooligosaccharides are influential prebiotics that affect various physiological functions in such a way that they promote positive impact to health. They occur naturally in many fruits and vegetables in trace amounts. However, they are mainly produced commercially by the reaction of microbial enzymes with di- or polysaccharides, such as sucrose or inulin as a substrate. For maximum production of fructooligosaccharides on an industrial level, development of more enzymes with high activity and stability is required. This has attracted the attention of biotechnologists and microbiologists worldwide. This study aims to discuss the new trends in the production of fructooligosaccharide and its effect on numerous health qualities through which it creates great demand in the sugar market.

Keywords Prebiotic · Fructooligosaccharides · Inulin · Microorganisms · Fructosyltransferase · Immobilisation

Introduction

Enzymes produced by microorganisms have been antecedently used for making essential foods, such as cheese, bread, wine, beer, etc. The design of food products for health benefit is now relatively becoming a popular trend which helps in disease prevention, treatment, and general well-being [1, 2]. The advancement of these functional foods led to the development of many biotechnological and pharmacological companies. Among them, fructooligosaccharides (FOS) create great demand in global food market and are generally recognised as safe (GRAS)

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from the Food and Drug Administration, USA. The synthetic process of FOS was first performed in 1980 in Japan by Meija Seika Kaisha Limited under the trade name Meioligo. Later on, other countries like France, the USA, Indonesia, Korea and China starts its production at tremendous rate. These countries sold FOS under different trade names, such as Actlight (Beghin-Meiji Industries France), Nutraflora (USA) and Meioligo (Meiji Seika Kaisha Limited Japan).

FOS contain several distinguishing qualities because of its usage as ingredient combination which make it most plentiful as an alternative sweetener in the food market. It is water soluble and one third as sweet as sucrose [3]. However, its viscosity and thermal stability is higher than that of sucrose. Its stability lies in a pH range of 4.0–7.0 and can be refrigerated for a period of 1 year. Moreover, it provides high moisture-retaining capacity, preventing excessive drying and low water interacting activity which is convenient in controlling microbial contamination [4]. The problem with consuming carbohydrates, such as sucrose, is that many bacteria feeds in the mouth such as *Streptococcus mutans*, *Lactobacillus acidophilus*, etc., forming insoluble β-glucans that serve as matrix for plaque formation which causes dental cavities [5]. For this evasion, FOS is presently used as non-cariogenic sugar substitutes in confectionary, gums, drinks, etc. Considering the β-configurations of anomeric carbon, C_2 in the fructose monomers, FOS become nondigestible by human digestive enzymes which are mostly specific for α-glycosidic bonds and hence are not utilised as energy source in the body [6]. FOS acts as a prebiotic food, because it is fed by many Bifidobacteria and *Lactobacillus* sp. which are resistant to acidic pH but is harmful to those bacteria which are foes of colon like C*lostridium* sp. FOS decreases not only the level of triglycerides, serum, cholesterol and lipid but also increases absorption of prominent ions like Ca^{2+} and Mg^{2+} [7]. Hypotriglyceridemia occurs because of a decrease in hepatic synthesis of triglycerides, and hypercholesterolemia results in antagonistic effect of short-chain fatty acids especially propionate on cholesterol metabolism [8]. It has also been found that FOS has no effect on blood glucose level of type 2 diabetes patients due to short-chain fatty acids produced by saccharolytic fermentation [9].

Structurally, FOS are short-chain oligomers of monosaccharide units containing kestose (GF2), nystose (GF3) and 1-β-fructofuranosyl nystose (GF4) in which fructosyl units (F) are bound by β(2→1) position of sucrose with the last one attached to a terminal glucose (G) moiety. Depending on the type of linkage between the monosaccharide units, several studies also showed production of neo-FOS in which fructosyl units are bound at β(2–6) position of sucrose forming neokestose and 6-kestose, respectively (Fig. 1) [10–12].

Synthesis of FOS is a two-stage process in which enzyme is cultivated in the foremost stage and susequently the required enzyme is used for biotrasnsformation process to yield FOS under controlled conditions [13, 14]. The enzymes employed for FOS production are fructosyltransferase (FTase; 2.4.1.9), β-fructofuranosidase (FFase; 3.2.1.26) and endoinulinase (3.2.1.7). FTase possess transfructosylating activity, act on sucrose by cleaving β-1,2 linkages and transferring the fructosyl group to an acceptor molecule such as sucrose and FOS thereby releasing glucose as a by-product [15–17].

$$GF_n + GF_n \rightarrow GF_{n+1} + GF_{n-1} \quad n = 1-3$$

where GF is sucrose and n is number of fructosyl units.

In case of FFase, it possesses hydrolytic activity in low sucrose concentration and transfructosylating activity on high sucrose concentration [18]. However, the endoinulinase undertakes distinct quality; it acts randomly and hydrolyse internal linkages of inulin $(GF)_n$ to yield FOS [16, 19].

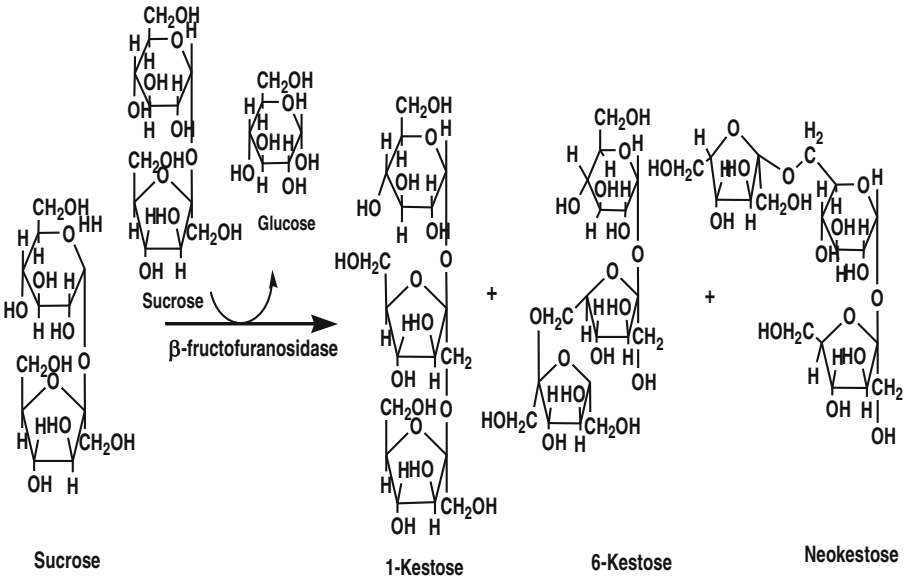

Fig. 1 Structure of FOS formation of various linkages

Screening and Optimisation of FTase and FFase Producing Microorganisms for the Production of FOS

Screening and selection of microorganisms for enzymatic biotransformation of sucrose to FOS has been investigated from the last three decades. Nearly about 30 microorganisms have been accounted for transfructosylating activity but only a few of them have been exploited for the production of FOS at industrial level (Table 1). Microbial strains such as species of *Aspergillus niger* (ATCC 26011), *Aspergillus japonicus* (ATCC 20236), *Aspergillus oryzae* (CFR 202) and *Aureobasidium pullulans* (CFR 77) have immense characteristics for commercial status regarding transformation of sucrose to FOS [13, 19–21]. The foremost attempt of screening was performed by Sangeetha et al. [7] which selected six microorganisms for transfructosylating activity. Among them, *A. pullulans* CFR 77, *A. oryzae* CFR 202 and *Aspergillus flavus* executed high transfructosylating activity resulting to higher yield of FOS; whereas *A. niger, Penicillium citrinum* and *Mucor miehei* did not give satisfactory yield. Using extracellular FTase from these microbial sources, the maximum FOS formation was evaluated at 44 % (*w*/*w*) by *Aureobasidum pullulans*, 25 % (*w*/*w*) by *A. oryzae* and 27 % (*w*/*w*) by *A. flavus* from sucrose 55 % (*w*/*v*) in enzyme–substrate reaction. However, when their intracellular enzymes were employed on the same substrate concentration, FOS yield was elevated up to 55 % (*w*/*w*) by *A. pullulans*, 50 % (*w*/*w*) by *A. oryzae* and 48 % (*w*/*w*) by *A. flavus,* respectively.

FOS producing enzymes are exuded both by extracellular as well as intracellular by various microorganisms. For emancipating intracellular enzyme, utrasonication was performed by Lateef et al. [21] which yielded 59 % of FOS from the cells *A. pullulans* CFR 77. Studies carried out by Fernandez et al. [18] screened 17 different strains of filamentous fungi grown in batch cultures to compare their abilities for the production of FFase. Among them, *A. oryzae* IPT-301, *A. niger* ATCC 20611 and strain IPT-615 showed high FTase activity of more than 12,500 U/l using 2^2 factorial experimental design on pH and temperature variables. The investigated pH vvalues

Table 1 List of reported microorganisms possessing transfructosylating activity for FOS formation

Name of microorganism	Reference
Arthrobacter sp.	[83]
Arthrobacter globiformis IFO-3062	[84]
Aspergillus aculeatus	[85]
Aspergillus flavus CFR 203	[7]
Aspergillus foetidus St-0194	[86]
Aspergillus japonicus ATCC 20236	[29]
Aspergillus niger	[55]
A. niger ATCC 20611	[87]
Aspergillus sp. N74	[88]
Aspergillus oryzae CFR 202	[7]
A. flavus NFCC 2364	[2]
Aspergillus terreus NFCCI 2347	[2]
Fusarium solani NFCCI 2315	[2]
A. pullulans DSM 2404	[89]
A. pullulans ATCC 20524	[20]
A. pullulans KFCC 10524	[90]
A. pullulans	[80]
A. pullulans CCY 27-1-94	[91]
A. pullulans ATCC 9348	[79]
A. pullulans KFCC10254	[92]
A. pullulans KFCC10524	[17]
A. pullulans CFR 77	[7]
A. pullulans 20524	[3]
Bacillus cereus	[24]
Bacillus macerans EG-6	[93]
B. macerans EG-7	[26]
Bacillus subtilis	[94]
Candida sp. LEB-13	[56]
Cryptococcus sp. LEB-V2	[56]
Penicillium citrinum	[95]
P. citrinum	[96]
Penicillium expansum MUM 02.14	[97]
Pencillium islandicum MTCC 4926	[2]
Penicillium purpurogenum	[33]
Penicillium rugulosum	[28]
Penicillium spinolosum	[98]
Rhizopus stolonifer LAU 07	[99]
Rhodotorula sp. LEB 10	[100]
Saccharomyces cerevisiae	[101]
Scopulariopsis brevicaulis	[102]
Sporotrichum thermophile ATCC 28811	[103]
Streptococcus salivarius 25975	[58]
Xanthophyllomyces dendrorhous 269	[104]
Zymomonas mobilis ATCC 10988	[105]

influenced both transfructosylating activity (U_t) and hydrolytic activity (U_h), whereas no significant difference of U_t and U_h was observed at a temperature of 40 and 50 °C, respectively. In another study, research contributed by Maugeri and Hernalsteens [22] obtained 495 yeast strains from fruits and flowers of Brazilian tropical forests. Of these isolated strains, only *Candida* sp. LEB-13, *Rhodoturula* sp. LEB-U5, *Cryptococcus* sp. LEB-V2 and *Rhodotorula* sp. substantiate for FOS formation. The data evaluated after qualitative and quantitative analysis culminate 45 % (*w*/*w*) of FOS from *Rhodotorula* sp. LEB-V10 in 48 h of reaction time while the other three strains credited FOS yield below 40 %.

Furthermore, a bacterium isolated from infected sugar cane identified as *Bacillus cereus* was used for production of FTase. The extracellular FTase from this bacterium gave high enzyme yield of 37.40 U/ml in submerged fermentation when grown on 16 % (*w*/*v*) sucrose and also harvested FTase yield of 29.1 U/g in solid-state fermentation (SSF) [23]. Recently, experiments performed by Ganaie et al. [2] observed seven new molds viz. *A. flavus* NFCCI 2364, *A. niger* (SI), *A. flavus* (NFCCI 2785), *Pencillium islandicum* (MTCC 4926), *Aspergillus terreus* (NFCCI 2347) and *Fusarium solani* (NFCCI 2315) from 20 screened microorganisms. Among these seven investigated isolates, *A. flavus* (NFCCI 2364) proved to be potent producer holding remarkable yield of FOS 63.4 % (*w*/*v*) in 24 h of enzyme substrate reaction.

Investigations carried out by Yoshikawa et al. [24] have observed five types of FFase (I, II, III, IV and V) secreted by cell wall of *A. pullulans* DSM 2404 while grown on sucrose-containing medium. The crude extract at 1st, 2nd and 3rd days were applied to anion exchange chromatography, and result of peaks indicate that FFase I was intensive in the 1st day of culture while FFase (II–V) show much expression on the 2nd and 3rd days of culture. U_t was found maximum to FFase I and was considered FOS producing period while as U_h was found highest to FFase IV regarded as FOS degrading period. In the 1st day, only FFase I was expressed in culture media and large amount of FOS and glucose were accumulated. As the glucose was completely consumed, FFase II–V were expressed on days 2nd and 3rd, indicating that these FFase were repressed by glucose.

Preliminary investigations of screening occasionally lead to poor yield of FOS, but optimisation of culture composition and reaction parameters is exhibited to increase conversion competence of sucrose to FOS. As such interactions influenced U_t of the enzyme, Sangeetha et al. [7] initially harvested 25 % (*w*/*v*) of FOS by *A. oryzae* CFR 202, but this yield was subsequently intensified up to 58 % (*w*/*w*) using response surface methodology (RSM). The combined action evaluated by Plackett–Burman and Doehlert experimental shell design persuaded the production of FOS in both stages [25]. A crude FTase from *Bacillus macerans* EG-7 reduced lag period from 25 h to 30 min by addition of ammonium nitrate at 10 mM which enhanced enzyme activity by 15-fold [26]. A newly isolated strain, TIT-90076 identified as *A. japonicus* produced FFase with high titres of FTase possessing high transfructosylating activity. The optimal conditions for the enzymatic transfructosylating reaction occurred at pH 5.0 and temperature of 55 °C. Sucrose, the best energy carbon source and yeast extract, the best nitrogen source were used for enzyme production. Addition of $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 changed the morphology of the fungal growth from filamentous to pellet form. However, these salts did not affect FFase production [27]. An investigation was carried out by optimising two reaction parameters, pH and temperature for FOS formation by *Pencillium rugulosum* at sucrose concentration of 775 g/l. The best results were obtained at temperature of 55 °C and pH 5.5 with yield conversion of FOS 83.8 % (*w*/*w*) [28]. Other studies, such as those carried out by Ganaie et al. [29], culminate pH 6.0 which is most appropriate for production of FTase in *A. flavus* (NFCCI 2364). The sensitivity of hydrogen ion concentration in cultivation medium which alters growth and enzyme activity of microorganism attributed to the fact that many fungal strains have their acidic pH optima in submerged

condition [30, 31]. The accumulation of FTase in submerged fermentation is intensely affected by constituents of several ingredients especially employment of several carbon and nitrogen sources. The results of these carbon sources, such as maltose, corn starch, fructose, glucose and sorbitol were used for observing growth and FTase production in *A. foetidus* TIT-90076 [27]. By applying such energy sources, the microbial growth was imparted besides the indigent production yield of FTase. Furthermore, their experiment deduced sucrose as a sole carbon source both for growth and production of FTase enzyme. Survey carried out by FTase production demonstrates sucrose as most influential sole carbon source which are rapidly taken by microorganism instead of using polysaccharides which are broken down slowly during fermentation process [32]. The optimal concentration of sucrose as sole carbon source usually ranged from 10 to 25 % (*w*/*v*), and below this concentration, large portion of sucrose is used for growth of microbial cells. However, as presented above, this concentration results in higher enzyme induction and also more amount of sucrose in cultivation media decreases oxygen transfer rate because of the increase in viscosity [29]. In addition to carbon and nitrogen sources, other studied variables that affect FTase production include agitation, time of cultivation, fermentation temperature, aeration rates, addition of different mineral salts and supplement of some amino acids. Studies carried out by Ganaie et al. [29] also concluded 200 rpm as the most suitable rpm for formation of rounded pellets and those above whereby pellet size decreases because of shear stress and abrasive forces. Addition of amino acids like lucine had slight inductive effect on extracellular FTase production by *Pencillium purpurogenum*, where as histidine and lucine had slight inductive effect on intracellular FTase production [33]. Organic salts such as K_2HPO_4 depicted cell growth and buffering reagent and usually ranges from 4 to 5 g/l [13]. However, other microelements such as $MgSO_4{\cdot}7H_2O$, $NaNO_3$, $KH_2PO_4{\cdot}NH_4Cl$ and NaCl are used from 2 to 3 g/l [2, 7, 33].

During the synthesis of FOS, one sucrose molecule acts as donor and another as a recipient. The liberation of a large number of glucose molecules acts as a competitive inhibitor thus preventing FOS formation. A mixed enzyme has been employed to eliminate glucose by glucose oxidase. The conversion of glucose to gluconic acid in the reaction mixture was further precipitated to calcium gluconate [34]. This system increases productivity of FOS at more than 90 % (*w*/*w*). Sometimes addition of additives like glucose isomerase causes structural change in certain compounds leading to increase the production of particular compound. A crude enzyme FFase from *A. pullulans* DSM 2404 was reported to yield 62 % (*w*/*w*) of FOS, but addition of commercial glucose isomerase increased its yield to 69 % (*w*/*w*) [24].

Production of FTase by SSF

SSF is a process in which microorganisms are grown on solid substrate except free water. SSF is comparably similar to other microbiological processes like compositing, ensiling, etc., and it holds tremendous output in food industries for production of enzymes in controlled manner to harvest required products [35]. This process is advantageous in terms of volumetric productivity, low capital cost, energy consumption and less chance of contamination. However, its main drawback is mass and energy transport, moisture, temperature, cell growth and controlling pH [36]. The FOS-producing enzymes are continuously increased due to change in fermentation systems which lead to increased product formation. Many interesting studies have been practised on different value-added products to increase FOS yield in low cost, when SSF conditions were established.

Many agricultural by-products like cereal bran, corn products, sugarcane bagasse, cassava bagasse (tippi), coffee and tea were used for FTase production of *A. oryzae* CFR 202 [37]. By

using these products, the selected fungus showed plentiful growth when cultivated on cereal bran, rice bran, wheat bran, oat bran, corn germ and corn meal. Coffee and tea by-products showed maximum growth on spent coffee and spent tea, but least growth on coffee husk and coffee pulp. However, FOS production was carried out by incubating 2.5 ml FTase of *A. oryzae* CFR 202 cultivated on these by-products with 7.5 ml of sucrose 60 % (*w*/*v*). The highest yield was observed in rice bran, corn germ and wheat bran, at nearly 51 % (*w*/*w*).

Critical analysis of literature claimed by many authors indicate that most strains are usually effective for production of FTase in submerged fermentation (SmF) as compared with SSF. However, an outstanding work, performed by Mussatto et al. [38], maximises the production of FOS under SSF by 2^3 central composite design. Fermentation was carried out using coffee silverskin as solid matrix moistened with 60, 70 and 80 % with 240 g/l of sucrose. The moisture content did not influence FOS productivity, but temperature at 26–30 °C and inoculum rate of 2×10^7 spores/g dry matter increased the yield of FOS to 208 g/l with a productivity of 10.44 g l^{-1} h^{-1} and FFase to 64.12 U/ml with productivity of 4.0 U ml^{-1} h^{-1}. This work was remarkable from the industrial view point to increase both FOS and FFase in a simultaneous manner.

More recently, Lateef et al. [39] reported a local isolate of *A. niger* for the production of FTase in both submerged and solid substrate media. Maximum enzyme activity of 24.49 U/ml was obtained in SmF after 48 h of fermentation in chemically defined medium, while maximum enzyme activities of 20.77 and 27.77 U/g were obtained in SSF using ripe plantain peel and kola nut pod, respectively. The enzyme was used to prepare FOS, with the maximum yield of 33.24 % FOS, consisting of kestose and nystose produced by FTase of kola nut pod fermentation. In a similar study, there have been reports of production of FTase by a strain of *Rhizopus stolonifer* LAU 07 in cassava-based media [40]. The authors reported high titers of enzyme of more than 20 U/g when 5–15 % inoculum sizes were used with minimal supplementation of cassava peels with yeast extract, while maximum FTase yield of 32.87 U/ml was obtained after 96 h of fermentation in cassava steep liquor which was not supplemented with any nutrient. The FTase yielded 34 % of FOS (1-kestose (GF_2); nystose (GF_3)) using 60 % (*w*/*v*) sucrose as the substrate. *R. stolonifer* LAU 07 also possessed antioxidant quality when some agricultural waste products like cocoa pod husk, cassava peel and palm kernel cake were practised for the production of FTase [35]. The protein content of these fermented substrates was increased whereas crude fat and fibre components of the substrates were decreased, respectively. Cassava peel contains higher concentration of cyanogenic glycoside which makes it unfit for animal consumption. Its cyanide concentration was reduced (1.39 %) due to the fermentation process.

Production of FOS by Inulin

Inulin $(C_6H_{10}O_5)_n$, a heteropolysaccharide of plant origin consists of a linear chain of β-2, 1-linked polyfructose units ending with a glucose residue through sucrose-type linkage at the reducing end (Fig. 2). It is a reserve carbohydrate of many plants and vegetables. However, its abundance is seen in roots and tubers of dahlia, chicory and Jerusalem artichoke [41]. Inulin is a potent substrate both for the production of high fructose syrups like inulobiose (F2) inulotriose (F3) inulotetrose (F4) and FOS $(GF)_{2-5}$ [42–44]. The obvious candidate of these oligosaccharides are performed by inulinase enzymes and usually these enzymes are classified as exo-and endo-acting based on cleavage of β-2,1 linkage in inulin. Exoinulinases (EC 3.2.1.80) cleave β-2,1 linkages sequentially starting from the non-reducing end of inulin and split off terminal fructosyl units, releasing fructose with a molecule of glucose, whereas endoinulinase (EC 3.2.1.7) act randomly and hydrolyse internal linkages of inulin to yield

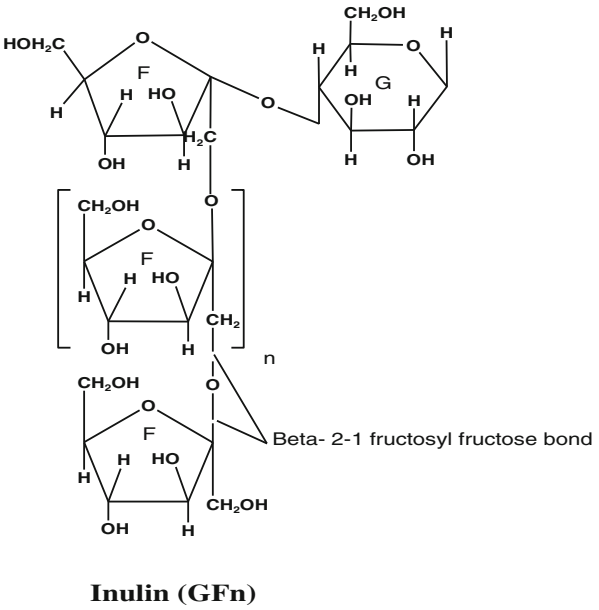

Fig. 2 Structure of inulin

FOS. Fructose is considered as a safe alternative sweetener as compared to sucrose because it has beneficial effects in diabetic patients, increases the iron absorption in children, and has a higher sweetening capacity [6, 42]. Most of the world's population use edible parts of plants containing inulin. However, plants did not contain sufficient amount of inulin to be exploited for commercial purpose. For this, microorganisms are the preferred source of inulinase production in sufficient amounts [43]. Many microorganisms have been extensively reported for the production of inulinase but moulds are regarded as preferential candidates for inulinase activity due to their thermal stability up to 60 °C which makes enzyme unaltered despite high temperature and less chance of contamination. Filamentous fungi such as *Aspergillus* sp. [45], *Penicillium* sp. [46] and yeast *Kluyveromyces* sp. [47] are high inulinase producers. From bacteria, *Bacillus* sp. [48], *Pseudomonas* sp. [49] and *Streptomyces* sp. [50] have been reported as high-yielding inulinase strains [51].

Both FOS and inulin are prebiotics as they are non-digestible food ingredient because of the β configuration of anomeric carbon (C_2) in their fructose monomers makes them to be resistant to hydrolysis. Many plants, e.g. Jerusalem artichoke, chicory roots and dahlia tubers contain high content of inulin, and various methods have been applied for extraction of inulooligosaccharides [51]. Implementation of two methods, simple diffusion and wet milling were carried out for inulin extraction from Jerusalem artichoke [52]. The inulinase practiced was used from *A. niger* and two crude enzyme solutions A and B were obtained. For A enzyme solution, the medium contained 3 % Jerusalem artichoke powder, 0.23 % NH_4NO_3, 0.37 % $(NH_4)_2HPO_4$, 0.1 % K_2HPO_4, 0.05 $MgSO_4$, and 0.15 % peptone. For B enzyme solution, the medium contained 3 % fibruline-long chain (98.5 % inulin with average DP=20), 1.5 % peptone and Czapeck salts. The pH of both culture mediums was adjusted to 5–5.5 and the resultant hydrolysis yield after 60 min was 50.6 % and 6.07 mg fructose/ml from (A) enzyme solution and from (B) solution it was 50.2 % hydrolysis yield and 6.02 mg fructose/ml from enzyme in 90 min.

Production of FOS from inulin in batch process has been carried out by *Pseudomonas* sp. using soluble and immobilised endoinulinase. Inulin was completely hydrolysed and yields FOS 72 % and 83 % respectively, under optimal conditions. The product composition was considerably affected by inulin concentration and enzyme form. The enzyme reactor was successfully run for 28 days at 55 °C achieving a yield of 82 % without any significant loss of enzyme activity [53]. An experimental factorial design has been developed for FOS production from *Kluyveromyces marxianus* var *bulgaricus*. The studied variables were temperature, pH, sucrose and enzyme concentration, respectively. The amount of FOS production was 50.2 g/l by stirred reactor and 44 g/l by packed bed reactor by using immobilised enzyme [54].

Purification and characterisation of microbial FTase and FFase

Purification is a prominent aspect to deduce factual characteristic of an enzyme. Most investigations have been carried out in respect of screening and optimisation process. But only few of their FTase or FFase have been purified and characterised (Table 2). An *A. niger* isolated from sugar cane field harvested both extracellular and intracellular FTase enzyme. The supernatant of 900 ml contains 6,600 units of transfructosylase activity while 118 ml from 150 g wet mycelia contain 2,924 units of transfructosylase activity; 900 ml of extracellular enzyme were fractionated with ammonium sulphate (80 % saturation) and precipitate obtained after centrifugation were sequentially dialysed against deonised water and 0.5 M citrate/phosphate buffer, pH 6.0. The dialysates were further purified by DEAE-cellulose and CM-cellulose column chromatography. Purification of FTase led 138 fold by U_t and 88 fold by U_h. The ratio of U_t/U_h of crude intracellular FTase was 5, corresponding to extracellular FTase of 4 respectively. However, purified U_t/U_h ratio of extracellular FTase was elevated upto 6 which revealed that impeding factors were removed during purification process [55].

Several agricultural by-products like cereal bran, corn products, sugarcane bagasse, cassava bagasse (tippi), coffee and tea processing for FTase production by *A. oryzae* CFR 202 [37]. The enzyme source from SSF using wheat bran was purified 107-fold by ammonium sulphate precipitation (30–80 %), DEAE cellulose chromatography and Sephadex G-200 chromatography. The molecular mass of the purified FTase was 116.3 kDa by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and the enzyme was stable at 30 and 40 °C holding 91 % of its activity after 2 h of incubation. Above this temperature enzyme loses its stability by 80 % and its optimum for FTase activity was found at 60 °C. However, its pH was stable over 5–7 and 100 % activity was retained at pH 7. The optimum pH for FTase activity was studied 6, whereas 84 and 77 % activity were retained at pH 5 and 7, respectively.

Study executed by Hernalsteens and Maugeri [56] use extracellular enzyme from cells of *Rhodotorula* sp. LEB-V10 and partitioned by centrifugation at 5 °C (4,000×*g*) for 10 min. The enzyme was recovered by ethanol precipitation, and the precipitate was recovered by centrifugation by adding ethanol to the final concentration of 70 % (0.4 °C) in 50 mM sodium acetate buffer (pH 4.5) further stored at −18 °C. Two-step ethanol fractionation at 95 and 70 % to cell-free supernatant was also carried out and enzyme-rich precipitate were again collected by centrifugation. Nearly about 75 % of the U_t and 40 % of U_h was recovered with 70 % ethanol, however two step fractionation leads recovery of 25 % of U_t with purification factor of 2. The native-PAGE analysis showed only single band of protein after purification equivalent to 140–150 kDa. However, SDS-PAGE with 7.5 % polyacrylamide gel revealed two bands 77 and 124 kDa representing that the native enzyme exists as a dimer. Both FTase and FFase activities were stable at temperature of 66 °C while FFase show stability at pH 4.0 and FTase at pH 5.0, respectively. The yield of FOS synthesis from purified enzyme was 48 % (*w*/*w*) from

Table 2 Characteristic features of FOS producing purified enzymes from various microorganisms

Microorganism	Enzyme	Molecular weight (kDa)	Optimum pH	Stability pH	Optimum temperature (°C)	Stability temperature (°C)	Reference
Aureobasidium pullulans (C-23)	Fructosyltransferase	190	5.0	2.5–12.0	55	30–70	[106]
A. pullulans (CFR77)	Fructosyltransferase	147 and 170	5.0	3.5–10	55	30–70	[57]
A. pullulans (DSM 2404)	β-fructofuranosidase	430	5.0	6.0–10.0	50	30–60	[89]
Aspergillus niger (AS0023)	Fructosyltransferase	81–168	5.8	4.5–11.0	50	30–50	[107]
A. niger	Fructosyltransferase	–	5.5–6.5	5.5–6.5	50	Not Reported	[55]
Aspergillus oryzae	β-fructofuranosidase	89	6.0	5–9	40	Up to 40	[71]
A. oryzae CFR 202	Fructosyltransferase	116.3	6.0	5–7	60	40	[37]
Rhodotorula dairensis	β-fructofuranosidase	172	5.0	3-7	55	55–60	[108]
Cryptococcus sp. LEB-V2	Fructofuranosidase	90 and 130	4.0	4.5	65	60	[23]
Bacillus macerans EG-6	Fructosyltransferase	66	5.0	5.0–7.0	50	20–50	[93]
Streptococcus salivarius ATCC 25975	Fructosyltransferase	125.4	6.0	Not reported	37	Not reported	[58]

50 % (*w*/*v*) of sucrose. However, there was no addition of FOS when amount of enzyme was increased and reaction by-products like glucose and fructose were lessened.

An intracellular FTase obtained by wet milling from *A. pullulans* CFR 77 produced 59 % of FOS after 9 h of reaction time. The enzyme source was precipitated by gradual fraction of ammonium sulphate from 0 to 30 %, 30 to 60 % and finally 60 to 80 %. After analytical polyacrylamide gel electrophoresis, the purified enzyme revealed two bands 147 and 170 kDa. The molecular markers used were carbonic anhydrase (29,000), ovalbumin (egg albumin, 45,000), bovine albumin (66,000), phosphorylase b (97,400) and alcohol dehydrogenase (150,000). The specific activity of the final purified material was 42 U/mg proteins, representing a purification factor of 79.44 and yield of 43 % [57].

A recombinant FTase of *Streptococcus salvarius* 25975 expressed in *Escherichia coli* resulted in electrophoretically homogeneous band with specific activity of 58 U/mg of proteins. This fraction represented only 0.2 % of total protein and 3 % of original activity of purification factor 35-fold. The enzyme was purified by electrophoretic homogeneity after a combination of adsorption ion exchange and gel filtration chromatography. The purified enzyme showed maximum activity in presence of Ca^{2+} but was inhabited by metal ions like Cu^{2+}, Zn^{2+}, Hg^{2+} and Fe^{2+} at pH 6.0 and temperature of 37 °C [58].

Synthesis of neo-FOS (neokestose and neonystose) from extracellular purified FFase of yeast *Xanthophyllomyces dendrorhous* (ATCC-MYA-131) was accomplished by Linde et al. [12]. The purified protein (20 μg) treated with peptide-*N*-glycosidase F and SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 8 % polyacrylamide) deduce enzyme as a glycoprotein with content of 59–67 % N-linked carbohydrate. The molecular mass was estimated at 160 kDa with overall yield of 18 % and Coomassie-stained SDS-PAGE hydrolase activity with an overall yield of 10 %. Production of FOS was much higher 120 g/l at 70 °C than below temperatures. The ratio of U_t/U_h activity was 2.7 times higher at 60–70 °C than 40–50 °C. Neokestose was the predominant product formed by *Xanthophyllomyces*.

Cloning and Functional Analysis of FTase Gene for the Production of FOS

Production of FOS by microbial process leads to cloning of genes encoding FTase to obtain microorganisms more profitable and reliable for better improvement of yield. Industrial Microbiology and Biotechnology have made advancement regarding recombinant DNA technology for characterisation of new FTase genes and enzymes so as to express similar or dissimilar proteins [36, 59]. The availability of genome sequences of some microorganisms, especially *Aspergillus* can provide an important contribution for understanding production of enzymes and metabolites [60]. *Aspergillus* genes are more homogenous, with size ranging from 1.6 to 2.2 kb and encoding enzymes of about 600 amino acids. However, bacterial FTase genes vary from 1.2 kb in *Thermotoga maritime* and 4.4 kb in *Leuconostoc citreum* producing enzymes with 432–1,466 amino acids. FOS-related genes of plants and enzymes are about 2.0 kb and 680 amino acids, respectively [61–64].

A PCR-cloning strategy to clone *A. oryzae* N74 FTase gene for production of recombinant enzyme was done by Rodriguez et al. [65]. The size of FTase was 1,630 bp size with 99 % resemblance with other *A. oryzae* strains and between 1 and 68 % identities with other *Aspergillus* strains. This gene encodes 525 amino acids protein with 99 % similarity with other *A. oryzae* strains and between 11 and 69 % similarities with other *Aspergillus* strains. Eventually, tertiary structure model of *A. oryzae* N74 FTase was similarly anticipated with other glycoside hydrolase of 32 family members. The investigations carried out show elevated level of sequence conservation between *A. oryzae* strains and initial step regarding development of FOS by industrial sector by recombinant microorganism carrying FTase gene from *A. oryzae* N74.

The purification and characterisation of FTase of *Aspergillus sydowi* was performed, and further sequence of cDNA was pursued. This enzyme had molecular weight of 75 kDa, which revealed that it is different from other FTases [66]. The gene was expressed in *E. coli*, *Saccharomyces cerevisiae* and *Solanum tuberosum*. Crude extracts of recombinant microorganisms produced FOS in vitro; however, *S. tuberosum* produced molecules of inulin with more than 40 units of hexose. The FTase of *A. sydowi* is particularly interesting because it synthesises different products under different experimental conditions. Suspensions of fungal conidia synthesise a high molecular weight inulin. However, when the same gene was expressed in *E. coli* or *S. cerevisae*, it produced a mixture of oligosaccharides with a low degree of polymerisation [66]. The strategy of cloning enzyme genes in the methylotrophic yeast, *Pichia pastoris*, has been reported by Trujillo et al. [67] which showed the production of FOS (1-kestose) with a conversion efficiency of about 70 % from sucrose. The enzyme was originally obtained as levansucrase of *Glucoacetobacter*. Seibel et al. [68] described the FTase as levansucrase or inulosucrase, using the same code E.C. 2.4.1.9.

Recently, complete genomic studies through gene prediction based on homology of known genes made possible the discovery of new genes. In the genome programme of *A. oryzae* RJB4O, the gene sequence of FTase was identified [69]. In the same way, a gene encoding a FTase of *S. mutans* UA159 was found within the genome sequence of this organism [70].

Two types of FFase F1 and F2 in *A. oryzae* KB strain were observed [71]. FI possess transfructosylating activity which produces kestose, nystose and fructofuranosylnystose, whereas F2 possess hydrolytic activity producing glucose and fructose. N-terminal amino acid sequences of purified FFase performed by Automatic Edman degradation using Procis 491 protein sequencing system of F1were DYNAAPPNLST and for F2 it was YSGDLRPQ, respectively. Investigations carried out by real reverse transcription polymerase chain reaction using primers F1 (forward 5′-TTACACTGATCCTGACAC-3′ and reverse: 5′-ACCGAAGA CGTTACCGGT-3′) and for F2 (forward: 5′-CTTGCGGCAGTTGCACAAGC-3′ and reverse: 5′-TACCACTGAGCCGCATAG-3′) was designed from sequencing data of *A*.oryzae strain. Analysis of agarose gel, marked expression of F2 gene smaller at high sucrose concentration than F1 gene which is higher than F2 gene in low sucrose (0.5 %) concentration contained medium.

Production of FOS by Immobilisation

The exploitation of enzymes for large-scale production increases advancement in food industry. In order to promote catalytic activity of such enzymes, behaviour and stability led by immobilisation process changes kinetic pattern of resultant product [72]. Immobilisation of microbial cells has been of interest for nearly 30 years, and its accomplishment of practical application strongly rely on the properties of carrier employed by forming large number of bonds such that each unit of carrier can immobilise large amount of enzymes if needed [73–75]. There are various methods by which enzymes can be localised, ranging from covalent chemical bonding to physical entrapment. However, the attractions of immobilised enzymes from analytical standpoint are primarily their reusability, cost saving, greater efficiency and control of their catalytic activity (e.g., potentially longer half-lives, predictable decay rates and more efficient multi-step reactions). Immobilisation technique has been applied in the production of FOS from the last three decades which enable economical utilisation and cost preclusive enzyme.

An *A. pullulans* cell mass mixed with sodium alginate was employed by developing spherical gel beads. About 5.2 g of beads was reacted with 55 % (*w*/*v*) of sucrose which

culminate 56 % (*w*/*w*) of FOS formation in 42 h of reaction [76]. In a related study, many substances such as polyurethane foam, stainless steel sponge, vegetal fibre, pumice stones, zeolites and foam glass have been accustomed for cell immobilisation. Vegetable fibre was investigated as the most possible immobilised carrier which efficiently cultivated *A. japonicus* ATCC 20236. The outcome yield of FOS was obtained at 116.3 g/l conceding exalted activity of FFase activity. The media containing zeolites gave the best results; however, cells in this media were mostly free and only meagre amount was immobilised on the carrier. Although the fungus was remarkably immobilised on stainless steel sponge (1.13 g/g carrier), only a smaller extent of the fungus were found on polyurethane foam (0.48 g/g), zeolite (0.19 g/g) and pumice stones (0.13 g/g) carrier. However, no cell adhesion was observed on foam glass [77].

Several polymers, such as polyacrylamide, gelatine, alginate and k-carrageenan have been employed forming gel beads through needle or disk automisation system into $Cacl_2$ solution [78]. The feasible utility may be obtained despite reduction of both volume and weight of beads, as formed beads contain 90–95 % water and only 10 % of solid matter. The application of this system was to reduce both weight and volume of the required biocatalyst which possibly pack significantly much into fixed volume bioreactor leading to potential increase of productivity at nearly twofold. For this intention, potential improvement of bioreactor performance was asserted by Zherebtsovn et al. [79] on the development of hydrated and dehydrated beads to investigate kinetic characterisation of FOS by *A. pullulans* (ATCC 9348) cells. The gel beads were dehydrated by placing them at −15 °C for 6–24 h to induce freeze dehydration. The volume of beads reduced due to ice formation outside the beads which lowered the vapour pressure and hence pull water from inside the beads. The shrinked beads reduced bead volume by 82 % and bead weight by 85 % which were further successfully used for the production of FOS. The FOS yield of dehydrated beads (23 %, *w*/*w*) decreased in contrast with the hydrated beads (43 %, *w*/*w*) due to some diffusional resistance to substrate permeation.

A successful attempt for continuous production of FOS was performed by Jung et al. [80] with packed bed at plant-scale reactor. The reactor was designed by positioning a thousand of needles on the bottom of a stainless steel vessel, and height-to-diameter ratio was adhered constant (3:1) so as to form uniform radial temperature within the plant bed reactor. The calcium alginate mixture was spilled into vessel and pressure was applied to drive the mixture through the needles. The resulting drops entered $CaCl_2$ solution and formed alginate beads. The storage tank contained calcium alginate gel was slightly fluidised with air to avert crush or breakage of bead by hydrostatic pressure within the tank. The enzyme activity was maintained over 100 days without recharging the immobilised cells. The FOS productivity of reactor was 180 g l^{-1} h^{-1}.

Recently, Ganaie et al. [81] immobilised mycelial cells of *A. flavus* NFCCI 2364 by two polymers chitosan and sodium alginate for continuous production of FOS (Fig. 3). The composition of sodium alginate and $CaCl_2$ were optimised and best bead formation was observed of sodium alginate 0.3 % (*w*/*v*) cross linked with $CaCl_2$ solution 0.1 % (*w*/*v*). A successful continuous production of FOS, averaging at 63.5 % (*w*/*w*) was observed by FTase entrapped alginate beads for up to 7 days without much losing activity. However, FTase-containing chitosan beads lose its efficiency after the 3rd cycle and produced FOS at an average of 40.74 % (*w*/*w*) in three successive cycles.

FTase of *A. pullulans* KFCC 10524 immobilised on highly porous anion exchange resin (Diaion HPPA 25) equilibrated with 0.1 M citrate buffer (pH 5.5) for 24 h and packed into glass column (25×12 cm) with bed volume of ca. 50 cm^3 was operated [82]. This condition was successfully performed for continuous production of FOS 1,174 g l^{-1} h^{-1} in 30 days

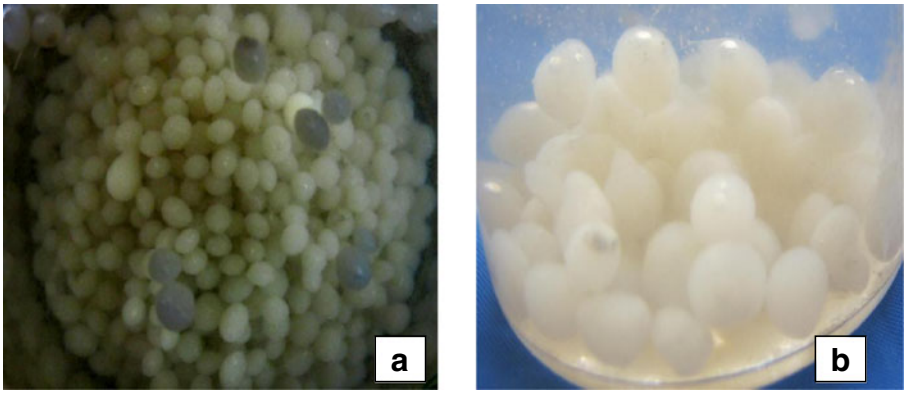

Fig. 3 Morphology of FTase-entrapped alginate beads (**a**) and chitosan beads (**b**)

following 8 % loss of activity was observed. Similar study carried out by Hayashi et al. [20] performed an outstanding procedure by immobilised FTase on salanised porous glass, and they successfully operated immobilised enzyme column up to 30 days without loss of initial enzyme activity. However, their system has least application regarding industrial objective because of low sucrose concentration up to 40 % (*w*/*v*) and temperature at 30 °C favoured chance of contamination problem in prolonged operation.

Conclusions

FOS-type prebiotics are nutritional compounds which have a wide range of applications in human health. In order to extend the frontiers of its production, a large number of microorganisms have been screened for transfructosylating activity for production of FOS. Among the tested strains, thermostable fungi are highly favoured to harvest FOS on large scale. The progressive movement of prominent processes like optimisation, immobilisation, purification and ultrasonication increase the kinetic properties of transfructosylating activity to increase its yield efficiency. Production of FTase from agricultural wastes by SSF is advantageous with respect to simple operation and provides less chance of contamination. The strategy of using purified cloned genes by several microorganisms leads to express new approaches for the better improvement of FOS production.

Future Prospect

Biotechnology is a key factor in the developmental process of producing a number of foods, seldom thought of by consumers, which are tasty, nutritious, safe, and convenient for society. The application of bioprocess technology have enabled quantitative investigations of empirical qualities with food grade oligosaccharides especially FOS which still holds its impact on account of its health qualities. Many microorganisms up to date have been employed for FOS production, but screening some novel source of microorganisms with high conversion efficiency in shorter period of time remains challenging, as well as removing FOS by-products like glucose and unreacted sucrose. The creation of transgenic microorganisms possessing high transfructosylating activity is a key work to progress in this area, and the awareness of additional FOS benefits and less cost production is importantly needed.

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