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Screening of biocatalysts for transformation of sucrose to fructooligosaccharides

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ABSTRACT

Twenty microorganisms comprising of sixteen molds, two yeasts and two bacteria were evaluated for their ability to produce fructosyltransferase (FTase) and generate fructooligosaccharides (FOS) from sucrose. FTase production by these microorganisms was studied over a period of 120 h on medium containing 20% (w/v) sucrose as the sole carbon source. High FTase levels (35–31 U/ml) were observed in culture filtrates of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* and *Penicillium islandicum*. Higher concentrations of FOS were generated from 50% (w/v) sucrose using culture filtrates of *A. flavus* NFCCI 2364 (63.40%, w/w), *A. niger* SI 19 (54.94%, w/w), *A. flavus* NFCCI 2785 (44.61%, w/w), *P. islandicum* MTCC 4926 (43.56%, w/w), *A. terreus* NFCCI 2347 (24.17%, w/w) and *Fusarium solani* NFCCI 2315 (15.25%, w/w). Kestose, nystose and 1-fructofuranosyl nystose were the predominant oligosaccharides as revealed by HPLC analysis.

1. Introduction

Recent awareness regarding the nutritional and health benefits of prebiotic oligosaccharides has lead to the development of several biotechnological endeavors for their generation. FOS consists of a mixture of fructo-oligomers with two or three fructose units bound to the β-2, 1 position of sucrose. Conventionally, these are mainly understood to be composed of 1-kestose, 1-nystose and 1-fructofuranosyl-nystose [1]. FOS have been particularly studied because of their specific physiological effects on human health especially growth stimulation of beneficial bifidobacteria in the digestive tract by inhabiting growth of pathogens [2,3], protonation of potentially toxic ammonia and amines [4], relief of constipation [5], diminution of total blood cholesterol, triglyceride, phospholipids and general improvement of human health [2,6]. They also confer secondary beneficial effects like non cariogenicity, safety for diabetics, absorption of Ca^+, Mg^+ and Fe^+ ions [7]. In food industries, FOS are also used as stabilizers or bulking agents for the production of sweeteners [8].

Commercial production of FOS relies upon enzymatic transformation of sucrose by microbial fructosyltransferase (FTase). Fructosyltransferase (FTase, EC 2.4.1.9) and β-fructofuranosidase (FFase, EC 3.2.1.26) with high transfructosylating activity are the two enzymes known to catalyze FOS generation from sucrose. FTase possess only transfructosylating activity, cleave the β-1, 2 linkage of sucrose and transfer fructosyl group to an acceptor molecule leading to formation of fructooligosaccharides and release of glucose. FFase catalyze both hydrolytic and transfructosylating reactions, however, latter is evidenced only with higher sucrose concentrations [9–11]. Efforts are being made to explore and identify microorganisms that produce FTase with desired activity, stability and end products to suit industrial conditions [12].

Owing to these benefits considerable efforts on the isolation and screening of microorganisms for FTase production with high transfructosylating activity are being made. Microorganisms like *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus japonicus*, *Aureobasidium pullulans* are regarded as potential producers of FOS [13–15]. The most investigated microorganisms in this subject are fungi belonging to genera *Aureobasidium*, *Aspergillus* and *Penicillium* [1,16]. Fructosyltransferase production from bacteria (*Lactobacillus*) and yeasts (*Rhodotorula*, *Candida*, *Cryptococcus* sp) has also been reported to some extent [17,18]. However, there is a strong need to explore the microbial diversity for identification of strains with high transfructosylating activity containing more oligosaccharides and less of monomeric sugars [14]. The main objective of the screening scenario was to find new microbial strains having high transfructosylating activity for biotransformation of sucrose to FOS production.

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2. Materials and methods

2.1. Microorganisms and culture conditions

Twenty microbial strains including sixteen molds, two yeasts and two bacteria were investigated for transfructosylating activity. Fifteen microorganisms were obtained from culture collections viz. National fungal culture collection of India (NFCCI, Pune, India), American type culture collection (ATCC, USA) and Microbial type culture collection (MTCC, Chandigarh, India). Five molds were isolated from sugarcane field plantations of Sagar district, Madhya Pradesh and apple orchids of Wakura Ganderbal Kashmir. Soil isolates (SI) of *Aspergillus flavus* (NFCCI 2783, NFCCI 2785) and *Fusarium* sp. (NFCCI 2784) were deposited in NFCCI, Pune while *A. niger* (SI 19) and *Trichoderma* sp. (SI 27) are deposited in culture collection of Department of Applied Microbiology, Dr. Harisingh Gour University, Sagar. *A. flavus* (NFCCI 2364), *Aspergillus versicolor* (NFCCI 2025), *Aspergillus fumigatus* (NFCCI 2452), *A. niger* (ATCC 2601), *Fusarium solani* (NFCCI 2315), *Aspergillus awamori* (NFCCI 1560), *Aspergillus terreus* (NFCCI 2347), *Rhizopus oryzae* (NFCCI 2282) and *Penicillium purpurogenum* (MTCC 1786) and aforesaid five soil isolates were grown and maintained on PDA (Potato dextrose agar). *Penicillium chrysogenum* (MTCC 161) and *Penicillium islandicum* (MTCC 4926) were grown on Czapek extract agar (CEA). Yeasts *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 2601) were grown on SYPA (sucrose, yeast extract, peptone and agar) whereas bacterial strains, *Bacillus cereus* (ATCC 117) and *Bacillus subtilis* (ATCC 6621) were cultured on nutrient agar media (NAM). The temperature for incubation for molds and yeasts was 28 °C while bacteria were incubated at 35 °C.

2.2. Chemicals

FOS standards 1-kestose (GF_2), 1-nystose (GF_3), 1-fructofuranosylnystose (GF_4) and sugar standards sucrose, glucose, fructose, were obtained from Wako Pure Chemicals Japan and Sigma Aldrich USA. Other analytical grade chemicals and media ingredients were purchased from Hi-media and Merck (India).

2.3. Inoculum development

Inoculum of molds was prepared by transferring full loop of spores harvested from five days old cultures of strains grown on PDA into 100 ml of sucrose-yeast extract (SYE) broth (1% sucrose and 0.2% yeast extract, pH 5.5) and incubating at 28 °C for 24 h on rotatory shaker (Lark Innovata, Germany) at 200 rpm. Yeast inoculum was prepared by adding a loopful of five days old yeast culture in the SYE medium and incubating it at 28 °C for 24 h at 200 rpm. For bacterial cultures, loopful of 2–3 days old culture was transferred in the NA medium and flasks were incubated at 37 °C for 24 h at 200 rpm.

2.4. Production of fructosyltransferase enzyme

Cultivation medium used for enzyme production contained (w/v): sucrose 20%, yeast extract 0.5%, $NaNO_3$ 1%, $MgSO_4.7\ H_2O$ 0.05%, KH_2PO_4 0.25%, NH_4Cl 0.5%, and NaCl 0.25% having an initial pH of 5.5. Aliquots of 100 ml of this medium were dispensed in 250 ml Erlenmeyer flasks and autoclaved at 121 °C for 15 min. 10 ml from 24 h old inoculum was transferred into 100 ml media and the flasks were incubated at 28 °C (molds and yeasts) or 35 °C (bacteria) in shaker incubator at 200 rpm for 120 h. Flasks were withdrawn at regular time intervals of 48, 72, 96, and 120 h. Contents were filtered through Whatman filter paper No. 2 and the cell-free culture filtrate was used as a source of extracellular enzyme without further purification. The cell mass obtained after filtration was washed with distilled water and dried at 105 °C for 4 h and was used for biomass determination as dry cell weight per volume (g/l).

2.5. Enzyme assays

Extracellular fructosyltransferase (FTase) and β-fructofuranosidase (FFase) were assessed by incubating 250 μl of enzyme sample with 750 μl of sucrose solution as described earlier [19,20]. For transfructosylating activity (Ut) 50% (w/v) sucrose in 0.1 M citrate buffer (pH 5.5) was incubated with enzyme sample at 55 °C for 1 h in a water bath and the reaction was arrested by keeping the reaction mixture in boiling water for 10 min. FTase activity was analyzed in appropriately diluted reaction mixture (1/50) with commercial GOD-POD kit. The absorbance of released glucose was read at 505 nm by UV/visible spectrophotometer (Hitachi Techcom India). Hydrolytic activity was determined following the same procedure except the substrate used was 0.5% (w/v) sucrose. The conversion of sucrose by FFase yields glucose and fructose. However, presence of FTase directs the addition of fructose for generating fructan polymer [15]. The estimated glucose (G) and reducing sugar (R) in the reaction mixture (F = fructose, F′ = transferred fructose) are explained in Eqs. (1) and (2) below.

$$R = G + F \rightarrow F = R - G \quad (1)$$

$$F' = G - F = 2G - R \quad (2)$$

One unit of FFase is defined as the amount of enzyme required for the hydrolysis of 1 μmole of sucrose per minute. One unit of transfructosylating activity is defined as amount of enzyme required to transfer one 1 μmole of F′ per minute.

2.6. Production and analysis of FOS

FOS production was carried out by adding 1 ml of enzyme samples collected at various time intervals to 3 ml of 50% (w/v) sucrose dissolved in 0.1 M citrate buffer (pH 5.5) for period of 24 h at 55 °C. The amount of FOS formation in the samples was analyzed by high performance liquid chromatography (HPLC, Waters) with sugar-pak column (6.5 × 300 mm) and refractive index (RI) differential detector (RI 2414). The samples were filtered using 0.45 μm membrane filters (Millipore) before injecting through the 20 μl valve. The temperature of the column was maintained at 70 °C by column oven (Dyna, Mumbai). The RI detector was operated at 30 °C and water was used as the mobile phase at a flow rate of 0.2 ml/min. The retention times of FOS components were compared with the standards for identification and their concentration was quantified from the peak area. Calculations and analysis were performed using Empower 2 software, Build 2154 (Waters).

3. Results and discussion

3.1. Change in pH and biomass during fermentation

All the test microorganisms were able to colonize sucrose-yeast extract medium producing different amounts of biomass and changes in pH of the medium. Maximum biomass formation (41.6 g/l) was recorded in native soil isolate, *A. niger* (SI 19) after 120 h. Other microorganisms like *A. fumigatus* (NFCCI 2452), *A. flavus* (NFCCI 2785), *A. flavus* (NFCCI 2783) *A. awamori* (NFCCI 1560), *A. terreus* (NFCCI 2347), *F. solani* (NFCCI 2315), *P. islandicium* (MTCC 4926) showed cell mass production in the range of 20–40 (g/l) (Table 1). The rest of molds, yeasts and bacteria showed relatively poor growth and produced biomass only upto 10 (g/l). Least biomass production 0.63 (g/l) was seen in case of *A. versicolor* NFCCI 2025 at the end of 120 h. *A. flavus* 2364 produced 36.2 (g/l) biomass

Table 1
Changes occurring in pH of medium and biomass (g/l) formation during 120 h of fermentation (culture conditions: 200 rpm, 28 °C). Data has been represented as mean ± standard deviation (average of 3 repetitions).

Microorganism	48 (h)		72 (h)		96 (h)		120 (h)	
	pH	Biomass	pH	Biomass	pH	Biomass	pH	Biomass
A. versicolor (NFCCI 2025)	4.48 ± 0.64	0.32 ± 0.10	4.96 ± 0.19	0.45 ± 0.23	5.10 ± 0.37	0.57 ± 0.21	5.45 ± 0.28	0.63 ± 0.19
A. fumigatus (NFCCI 2452)	5.87 ± 0.49	28.41 ± 0.61	6.05 ± 0.49	30.19 ± 0.48	6.25 ± 0.43	30.85 ± 0.51	6.46 ± 0.39	32.03 ± 0.10
A. flavus (NFCCI 2364)	5.81 ± 0.11	36.24 ± 0.60	5.70 ± 0.25	31.26 ± 0.80	5.62 ± 0.23	25.39 ± 1.24	5.56 ± 0.16	20.46 ± 1.27
A. flavus SI (NFCCI 2785)	4.45 ± 0.76	4.45 ± 1.12	4.64 ± 1.23	4.64 ± 1.43	5.34 ± 0.89	5.34 ± 0.56	5.75 ± 1.23	5.75 ± 1.00
A. flavus SI (NFCCI 2783)	4.28 ± 1.45	13.66 ± 1.09	4.89 ± 0.78	16.99 ± 0.78	5.46 ± 0.49	22.64 ± 0.86	5.86 ± 1.07	23.86 ± 1.78
A. awamori (NFCCI 1560)	4.08 ± 0.23	23.74 ± 1.45	3.88 ± 0.26	26.15 ± 1.70	3.54 ± 0.54	30.20 ± 2.07	3.36 ± 0.47	31.86 ± 1.48
A. terreus (NFCCI 2347)	4.93 ± 0.21	15.27 ± 0.67	3.81 ± 0.25	18.49 ± 1.50	3.17 ± 0.16	21.82 ± 1.43	2.51 ± 0.29	17.34 ± 1.09
A. niger (SI 19)	3.35 ± 0.58	21.63 ± 1.44	2.76 ± 0.73	33.81 ± 1.45	2.45 ± 0.70	37.85 ± 1.01	1.82 ± 0.47	41.61 ± 0.60
A. niger (ATCC 2601)	2.98 ± 0.67	15.74 ± 1.56	2.54 ± 1.03	20.53 ± 1.50	1.98 ± 0.65	25.67 ± 0.78	1.75 ± 0.98	13.67 ± 0.46
Trichoderma sp. (SI 27)	4.29 ± 1.02	11.53 ± 0.56	5.50 ± 0.67	15.65 ± 0.43	5.80 ± 0.76	17.72 ± 0.88	6.37 ± 0.99	19.42 ± 0.53
F. solani (NFCCI 2315)	6.35 ± 0.38	22.98 ± 1.36	5.50 ± 0.40	24.15 ± 0.55	5.19 ± 0.16	26.61 ± 1.18	4.63 ± 0.24	27.64 ± 0.58
Fusarium sp. SI (NFCCI 2784)	3.18 ± 0.55	2.17 ± 0.06	3.33 ± 0.97	3.11 ± 0.01	3.32 ± 0.50	3.91 ± 0.05	3.55 ± 0.61	3.52 ± 0.05
R. oryzae (NFCCI 2282)	3.14 ± 0.36	17.33 ± 0.70	3.04 ± 0.45	20.0 ± 0.81	3.39 ± 0.49	23.79 ± 0.65	3.47 ± 0.43	27.42 ± 0.61
P. purpurogenum (MTCC 1786)	2.15 ± 0.63	4.29 ± 0.34	2.35 ± 0.27	5.98 ± 0.61	2.37 ± 0.23	6.14 ± 0.71	2.52 ± 0.32	5.95 ± 0.41
P. chrysogenum (MTCC 161)	3.53 ± 0.53	26.53 ± 0.56	4.17 ± 0.53	27.16 ± 1.02	4.23 ± 0.55	29.87 ± 0.62	4.49 ± 0.41	31.22 ± 0.45
P. islandicum (MTCC 4926)	2.55 ± 0.40	1.30 ± 0.62	3.14 ± 0.16	1.77 ± 0.49	3.77 ± 0.62	2.26 ± 0.39	5.26 ± 0.47	2.45 ± 0.44
C. albicans (ATCC 10231)	2.34 ± 0.45	3.46 ± 0.67	2.71 ± 0.33	5.59 ± 1.00	2.73 ± 0.64	5.33 ± 0.79	2.93 ± 0.28	5.66 ± 1.10
S. cerevisiae (ATCC 2601)	3.39 ± 0.60	2.15 ± 0.82	3.13 ± 0.87	2.75 ± 0.79	3.11 ± 0.80	3.01 ± 0.18	2.98 ± 0.78	3.82 ± 1.44
B. cereus (ATCC 117)	7.50 ± 0.34	0.90 ± 0.28	7.39 ± 0.52	1.07 ± 0.49	7.15 ± 0.60	1.17 ± 0.59	6.94 ± 0.26	0.77 ± 0.57
B. subtilis (ATCC 6621)	7.90 ± 0.17	0.66 ± 0.22	7.16 ± 0.59	0.67 ± 0.35	7.15 ± 0.40	0.84 ± 0.25	6.85 ± 0.75	0.64 ± 0.30

in 48 h followed by sharp decrease indicating rapid growth of the fungus in first two days followed by autolysis.

A sharp decrease in pH from initial set value of 5.5–2.0 by many molds and yeasts was noticed during the cultivable length of time. This may be due to the production of some organic acids by molds. However, other strains did not show any major changes in their medium pH. Since the pH was not controlled during the whole fermentation process and the enzyme extracted was first settled by buffer system before use. Previously published results also indicate a sharp decrease in pH in case of *Rhizopus stolonifer* [19].

3.2. Transfructosylating and hydrolytic activities

Aliquots withdrawn from cultures at regular intervals from 48 h to 120 h of incubation were analyzed for the transfructosylating activity (Ut) and Uh activities (Table 2). Higher Ut levels were noticed in *A. flavus* NFCCI 2364 (33.73 U/ml) after 120 h of incubation while *A. niger* (SI 19) produced 35.64 U/ml of Ut in relatively short span of 72 h incubation. This value was not maintained on subsequent periods due to gradual decrease of transfructosylating activity which reduced Ut/Uh ratio. Noticeably, *A. terreus*, *A. awamori* and *P. islandicum* produced approximately 32.0 U/ml of transfructosylating activity in 48 h of incubation but their enzyme activity was sharply reduced on subsequent fermentation periods. Strains of yeasts and bacteria show significant levels of FTase activity during course of 120 h fermentation. However higher hydrolytic activities (Uh) were only noticed in *A. terreus* (21.5 U/ml) and *A. awamori* (17.45 U/ml) which indicate presence of β-fructofuranosidase enzyme. The pattern of FOS formation in these two microorganisms is different because FFase transforms sugar to FOS (Fig. 1b and c) and simultaneous releases of monomeric sugars like glucose and fructose respectively [8]. The Ut/Uh ratio is preferable for indicating relative intensity of transfructosylating activity for efficient formation of FOS and this ratio is most important parameter for screening of microorganisms for FOS production.

Table 2
Transfructosylating activity (Ut) and hydrolytic activity (Uh) of twenty selected microorganisms grown on medium containing 20% (w/v) sucrose as C-source over a period of 120 h. Culture filtrate obtained at different time intervals was used as source of enzyme and was incubated for 1 h at 50 °C with 50% (w/v) and 0.5% (w/v) sucrose respectively. Data represent mean ± standard deviation of three independent experiments.

Microorganism	48 (h)		72 (h)		96 (h)		120 (h)	
	Ut	Uh	Ut	Uh	Ut	Uh	Ut	Uh
A. versicolor (NFCCI 2025)	3.97 ± 0.39	2.16 ± 0.41	7.63 ± 0.87	3.10 ± 0.53	10.76 ± 0.81	6.76 ± 0.53	11.90 ± 0.68	4.97 ± 0.46
A. fumigatus (NFCCI 2452)	3.67 ± 0.46	1.90 ± 0.25	6.07 ± 0.25	2.97 ± 0.21	7.40 ± 0.24	3.50 ± 0.24	7.52 ± 0.43	4.16 ± 0.50
A. flavus (NFCCI 2364)	18.91 ± 0.97	5.02 ± 0.11	24.09 ± 1.41	5.24 ± 0.23	28.08 ± 0.83	5.04 ± 0.15	33.73 ± 0.74	4.79 ± 0.34
A. flavus SI (NFCCI 2785)	14.36 ± 0.67	4.56 ± 0.74	18.87 ± 0.99	4.98 ± 0.53	20.09 ± 1.02	5.01 ± 0.44	23.76 ± 0.56	5.28 ± 0.33
A. flavus SI (NFCCI 2783)	11.87 ± 1.09	4.08 ± 0.28	13.98 ± 0.78	4.21 ± 0.74	19.86 ± 0.67	6.98 ± 0.65	22.84 ± 1.00	7.89 ± 0.44
A. awamori (NFCCI 1560)	20.46 ± 2.41	17.45 ± 1.04	22.60 ± 2.38	14.67 ± 0.73	21.07 ± 1.64	13.62 ± 0.57	13.32 ± 0.82	10.53 ± 0.67
A. terreus (NFCCI 2347)	31.85 ± 0.98	21.51 ± 0.80	12.94 ± 0.44	9.21 ± 0.99	15.47 ± 0.41	8.44 ± 0.90	16.42 ± 0.39	8.31 ± 0.41
A. niger (SI 19)	24.76 ± 1.28	11.34 ± 1.03	35.64 ± 1.25	13.05 ± 2.04	28.05 ± 1.16	13.38 ± 2.66	22.77 ± 1.65	11.19 ± 1.23
A. niger (ATCC 2601)	12.87 ± 0.56	8.98 ± 0.83	15.76 ± 0.98	9.89 ± 0.57	17.98 ± 1.22	11.73 ± 1.08	19.63 ± 1.09	10.93 ± 0.68
Trichoderma sp. (SI 27)	8.98 ± 0.76	6.63 ± 0.53	10.74 ± 0.54	6.89 ± 0.73	12.95 ± 0.87	7.98 ± 0.45	14.36 ± 0.33	8.63 ± 1.06
F. solani (NFCCI 2315)	5.97 ± 0.60	2.94 ± 0.77	9.10 ± 0.82	3.40 ± 0.41	12.78 ± 0.43	5.28 ± 0.50	15.67 ± 0.70	3.76 ± 0.51
Fusarium sp. SI (NFCCI 2784)	4.91 ± 0.47	2.36 ± 0.86	5.11 ± 0.82	2.89 ± 0.85	5.93 ± 0.84	3.17 ± 0.82	6.02 ± 0.80	3.18 ± 0.87
R. oryzae (NFCCI 2282)	6.61 ± 1.07	3.20 ± 0.47	6.12 ± 1.15	4.72 ± 0.61	9.00 ± 0.61	7.44 ± 0.64	11.30 ± 0.78	9.24 ± 0.96
P. purpurogenum (MTCC 1786)	13.48 ± 0.85	8.55 ± 0.50	14.70 ± 0.66	9.26 ± 0.75	15.56 ± 1.06	8.10 ± 1.30	16.33 ± 0.52	6.68 ± 0.67
P. chrysogenum (MTCC 161)	32.65 ± 1.42	12.67 ± 0.86	29.07 ± 2.77	11.78 ± 0.62	24.89 ± 1.22	10.39 ± 1.31	19.78 ± 1.39	10.60 ± 0.87
P. islandicum (MTCC 4926)	8.78 ± 0.75	6.22 ± 0.73	10.89 ± 0.44	6.95 ± 0.45	13.78 ± 0.90	9.03 ± 0.65	15.98 ± 1.16	11.41 ± 1.25
C. albicans (ATCC 10231)	12.81 ± 1.23	9.72 ± 0.84	12.22 ± 0.80	7.42 ± 0.82	11.83 ± 0.77	9.21 ± 0.79	12.70 ± 1.74	8.90 ± 0.55
S. cerevisiae (ATCC 2601)	13.04 ± 1.25	9.05 ± 1.20	12.97 ± 0.92	8.39 ± 1.59	15.76 ± 1.91	9.50 ± 2.41	6.30 ± 1.92	3.39 ± 1.29
B. cereus (ATCC 117)	16.87 ± 0.95	9.14 ± 0.27	13.68 ± 0.48	8.99 ± 0.59	12.85 ± 0.77	9.53 ± 0.23	8.41 ± 0.50	3.66 ± 0.58
B. subtilis (ATCC 6621)	4.05 ± 0.44	2.61 ± 0.40	8.86 ± 0.25	2.59 ± 0.25	7.29 ± 0.56	2.96 ± 0.54	4.67 ± 0.77	4.24 ± 0.96

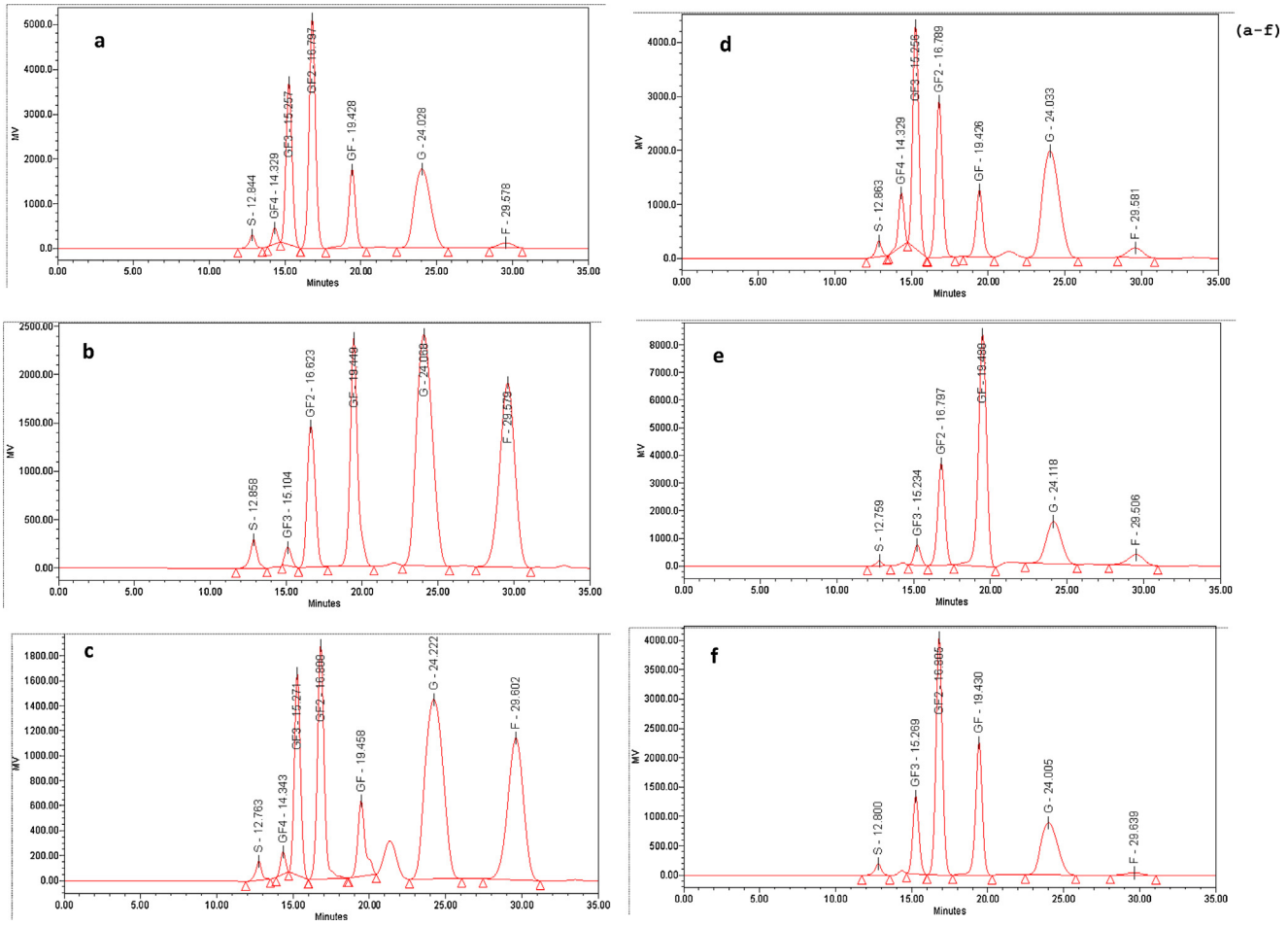


Fig. 1. HPLC chromatograms of reaction product (FOS) formed by incubating culture filtrates obtained from different fungi with 20% (w/v) sucrose (incubation time: 24 h, pH 5.5, 50 °C). (a) *A. flavus* NFCCI 2364, (b) *A. awamori* NFCCI 1560, (c) *A. niger* SI 19, (d) *A. terreus* NFCCI 2347, (e) *F. solani* NFCCI 2315 and (f) *P. islandicum* MTCC 4926 (abbreviations: S = solvent, G = glucose, F = fructose, GF = sucrose, GF_2 = kestose, GF_3 = nystose, GF_4 = fructofuranosylnystose).

However this ratio varies by optimizing cultural parameters like pH and temperature due to dependence of FTase and FFase [9]. In our study the maximum Ut/Uh was achieved in three strains of *A. flavus* NFCCI (2364, 2785, 2783), *A. niger* (SI 19), *P. islandicum*, *F. solani*, *A. terreus* and *A. awamori* in distinct incubation periods. The value of FTase are comparable by previously published reports by [19,21] which accounted maximum activity of FTase in 120 h of incubation in *A. oryzae* CFR 202 and *R. stolonifer* LAU 07 respectively. The corresponding increase of FTase activity from 48–120 h in *A. flavus* NFCCI 2364 may be releasing of intracellular FTase by disintegration of

Table 3
FOS formation % w/w (weight by weight) of selected microorganisms from enzyme substrate (FTase-sucrose) reaction by culture filtrates of different fermentation time.

Microorganism	48 (h)	72 (h)	96 (h)	120 (h)
A. versicolor (NFCCI 2025)	0.59 ± 0.12	1.25 ± 0.52	1.38 ± 0.24	1.5 ± 0.25
A. fumigatus (NFCCI 2452)	0.74 ± 0.11	0.85 ± 0.21	0.89 ± 0.63	1.20 ± 0.22
A. flavus (NFCCI 2364)	36.14 ± 1.08	48.20 ± 1.92	55.51 ± 1.54	63.40 ± 2.58
A. flavus SI (NFCCI 2785)	19.89 ± 0.67	33.64 ± 0.99	40.56 ± 0.53	44.61 ± 1.09
A. flavus SI (NFCCI 2783)	11.86 ± 0.43	17.97 ± 0.74	23.86 ± 0.53	28.98 ± 0.97
A. awamori (NFCCI 1560)	3.66 ± 0.48	4.34 ± 0.59	7.29 ± 1.31	10.47 ± 0.67
A. terreus (NFCCI 2347)	24.38 ± 1.35	21.08 ± 0.91	20.13 ± 1.54	18.07 ± 1.03
A. niger (SI 19)	26.69 ± 1.26	55.81 ± 1.71	43.83 ± 2.08	37.22 ± 1.52
A. niger (ATCC 2601)	1.78 ± 0.63	2.35 ± 1.08	4.18 ± 1.35	5.89 ± 0.54
Trichoderma sp. (SI 27)	0.98 ± 0.56	1.53 ± 1.08	1.78 ± 0.97	2.10 ± 1.03
F. solani (NFCCI 2315)	4.03 ± 0.63	8.51 ± 0.72	12.45 ± 1.44	15.12 ± 0.71
Fusarium sp. SI (NFCCI 2784)	0.67 ± 0.41	1.08 ± 1.16	1.82 ± 0.66	2.88 ± 0.23
R. oryzae (NFCCI 2282)	0.68 ± 0.39	1.77 ± 0.74	2.41 ± 1.53	3.55 ± 1.24
P. purpurogenum (MTCC 1786)	4.54 ± 0.59	5.57 ± 0.91	5.84 ± 1.27	8.56 ± 2.41
P. chrysogenum (MTCC 161)	42.51 ± 1.83	18.19 ± 1.25	14.96 ± 1.31	10.21 ± 1.05
P. islandicum (MTCC 4926)	0.49 ± 0.44	1.06 ± 0.29	1.32 ± 0.65	1.15 ± 0.70
C. albicans (ATCC 10231)	2.28 ± 1.75	5.16 ± 1.44	2.67 ± 1.28	3.42 ± 1.67
S. cerevisiae (ATCC 2601)	1.53 ± 0.67	1.45 ± 0.93	2.83 ± 0.70	2.76 ± 0.60
B. cereus (ATCC 117)	1.88 ± 0.74	2.58 ± 0.43	1.29 ± 0.51	0.78 ± 0.39
B. subtilis (ATCC 6621)	0.93 ± 0.22	0.95 ± 0.64	1.42 ± 0.58	0.56 ± 0.31

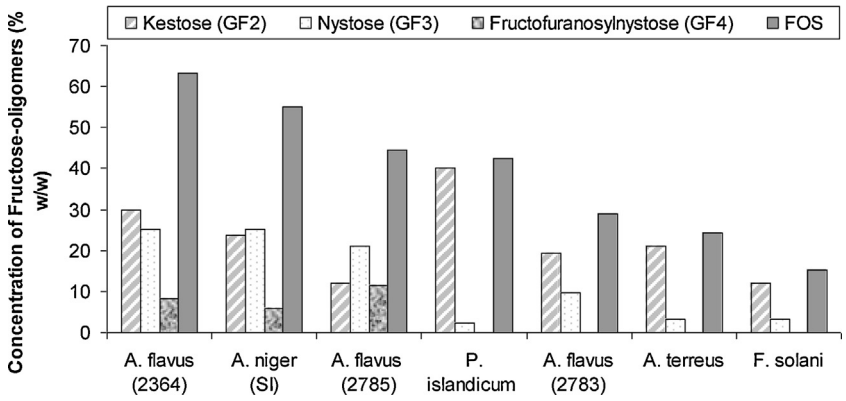
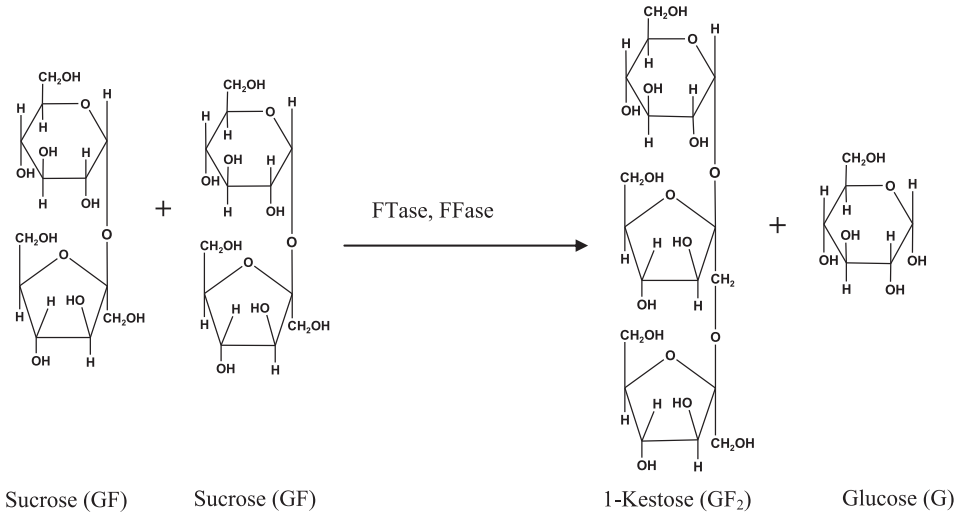


Fig. 2. Maximum production of kestose (GF_2), nystose (GF_3), 1-fructofuranosylnystose (GF_4) during production of FOS by microorganisms.



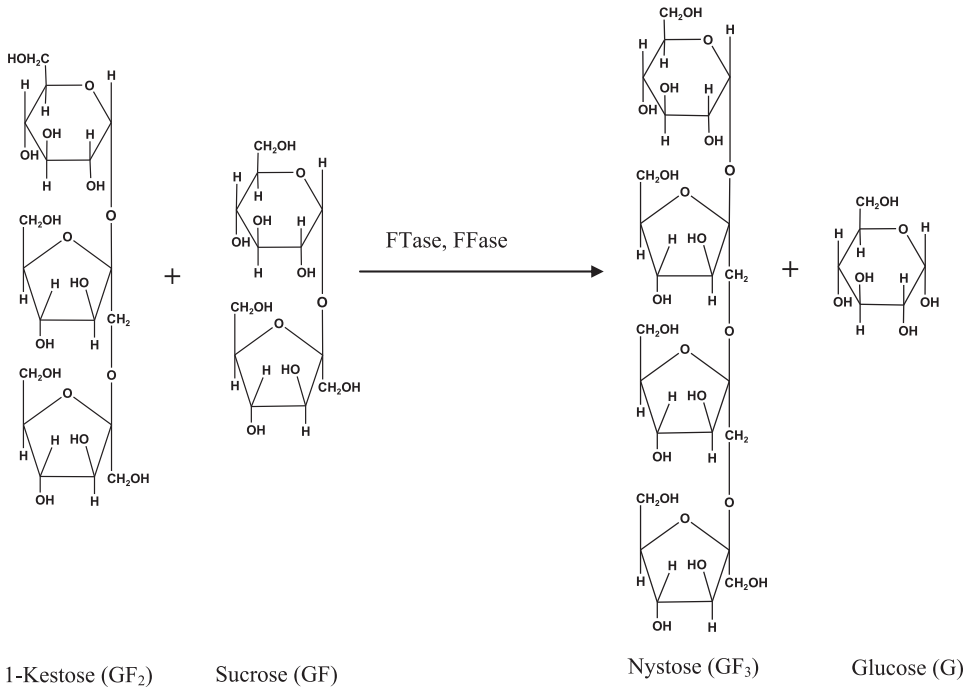


Fig. 3. Reaction scheme of FOS formation by enzyme catlyzed reaction of diaccharide (sucrose) with fructosyltransferase (FTase) and fructofuranosidase (FFase) enzyme.

hypae in consequence of prolonged agitation. While as decrease in enzyme activity after 48–72 h of incubation in other strains may be releasing of some metabolic by-products such as ethanol and acetic acid which tend to decline the FTase activity [22].

3.3. Production of FOS using cell-free culture filtrates

Evaluation of FOS formation by cell-free culture filtrate collected at different time intervals (48, 72, 96, and 120 h) was studied by incubating enzyme samples with 50% (w/v) sucrose as substrate for period of 24 h. The results of FOS production expressed as % (w/w) of the substrate (sucrose 50%, w/v) are summarized in Table 3. Among 20 screened microorganisms for FTase activity, *A. flavus* (2364) proved to be potent producer of FOS in all fermentation periods. Its outcome yield was highest quantified 63.40% (w/w) in which kestose (GF_2) was the predominant product formed in initial reaction time while high molecular weight oligomers like nystose (GF_3) and 1-fructofuranosyl nystose (GF_4) was formed at later stage of 24 h of reaction (Fig. 1a). The estimated concentration of kestose (GF_2) was observed maximum 29.89% (w/w) followed by nystose (GF_3) 25.26% (w/w) and 1-fructofuranosyl nystose (GF_4) 8.25% (w/w). In case of *A. niger* (SI 19) highest FOS formation was observed from 72 h culture filtrate. However, the concentration of nystose (GF_3) 25.31% (w/w) was more than kestose (GF_2) 23.74% (w/w) and 1-fructofuranosyl nystose (GF_4) 5.94% (w/w) (Fig. 1b). High nystose (GF_3) formation in late reaction period can be attributed to the fact that the newly produced kestose (GF_2) serves as acceptor for further oligomerization. This is in accordance with observation by [23] where the enzyme follows disproportionate type reaction, i.e. $GF_n + GF_n = GF_n - 1 + GF_n + 2$.

The pattern of FOS formation in *P. islandicum* (MTCC 4926) was quite distinctive than other screened microorganisms. The harvested product mainly contains immense amount of kestose (GF_2) (40.12%, w/w) and much less amount of nystose (GF_3) (1.39%, w/w) which implies that this peculiar microorganism did not able to use much amount of kestose (GF_2) for elongation of glycosidic chain (Fig. 1f). This implies that there is possibility of only sucrose as fructosyl donor and acceptor for formation of FOS. It may be that FTase might have not big enough acceptor site to adapt nystose (GF_3) or may be strong efficiency of kestose (GF_2) to bind its sub sites for prolonged time [24]. This is the primary report of such interesting biocatalyst which can be attributed for the formation of kestose (GF_2) in large amount.

Apart from these, other strains also produced significant amounts of FOS. *A. flavus* (NFCCI 2785), *P. islandicum*, *A. flavus* (2783), *A. terreus* and *F. solani* showed 44.61%, 42.51%, 28.98%, 24.38% and 15.12% (w/w) FOS generation, respectively (Fig. 1, Table 2). The order of FOS producing microorganisms tested in the present study can be summed up as *A. flavus* (NFCCI 2364) > *A. niger* (SI) > *A. flavus* (NFCCI 2385) > *P. islandicum* (MTCC 4926) > *A. flavus* (NFCCI 2383) > *A. terreus* (NFCCI 2347) > *F. solani* (NFCCI 2315).

The general mechanism of sucrose transformation into FOS by FTase is performed by multistep reactions with different saturation and inhibition effects (Fig. 3). The accumulation of monomers by competitive inhibitor such as glucose halts the further process of FOS formation as evidenced by the efficient production of FOS under comparatively low sucrose concentration by previously published reports [17,21,25]. The excellent profile of kestose formation than other oligomers especially *A. flavus* (2364) and *P. islandicium* (Fig. 2) is beneficial for health assets due to its sweetening strength. It is due to the fact that increased fructose chain decreases sweetening power of FOS [26]. Thus it would be interest to utilize such type of strains in industrial process which has high capability of kestose formation.

4. Conclusion

The present effort of screening programme resulted in identification of some new microbial strains showing high transfructosylating activity for FOS production. A good attainment of their favourable yield clearly signifies their vast ability for commercial application in food and feed industry. However more extensive work is needed to optimize the cultural parameters and purifying the FTase to enhance the productivity of FOS. The HPLC analysis deduce *A. flavus* (NFCCI 2364), *A. niger* (SI 19) and *P. islandicum* (MTCC 4926) the most prosperous strain for synthesizing high amount of FOS to be captivated on industrial level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.07.008.

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