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Research Article

Optimization of Conditions for in vitro Production of Fructooligosaccride using *Aureobasidium pullullans*

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Abstract: Oligosaccharides are very well recognized as 'functional food ingredients' because of their positive effects on human health. This research work focuses on the microbial production of Fructosyl Transferase (FTase) and the production of Fructooligosaccharides (FOS) by transfructosylation using this enzyme. Fructooligosaccharides are industrially produced from sucrose by microbial enzymes with transfructosylating activity. Fructosyltransferase is produced intra- and extracellulraly by several microorganisms including bacteria and fungi. Despite the large number of microbial FTase producers, only a few of them have the potential for industrial application and were focused in several studies about FOS production. The increasing interest in prebiotic compounds opens also possibilities for small-scale use of FTase. In the present study fungus *Aureobasidium pullulans* was used to produce FOS and effect of different physical and chemical parameters on FOS production were studied. The analysis of the product was performed by High Pressure Liquid Chromatography (HPLC).
Keywords: Fructooligosaccharide, FOS, Fructosyl transferase enzyme, HPLC, Probiotics, *Aureobasidium pullulans*

INTRODUCTION

Fructooligosaccharides (FOS) are oligosaccharides of fructose which contain a single glucose moiety; they are produced by the action of fructosyl transferase enzyme (FTase,) from many plants and microorganisms [1]. The FOS formed contains fructosyl units bounded at the β-2, 1 position of sucrose; they are mainly composed by 1-kestose (GF2) 1-nystose (GF3) and 1-fructofuranosylnystose (GF4) [2]. FOS is produced industrially by fermentation of sucrose substrate by microbial enzymes FTase with transfructosylating activity. Most of these enzymes have been found in fungi such as *Aspergillus* sp., *Aureobasidium* sp., *Arthrobacter* sp. and *Fusarium* [2]. Fructooligosaccharides are non cariogenic sweeteners and stimulate the growth of intestine friendly *Bifidobacteria* [3]. They have been considered to contribute towards the prevention of colon cancer and to reduce cholesterol, phospholipids and triglyceride levels in serum. FOS exhibits sweetness levels between 30 and 50 percent of sugar in commercially-prepared syrups. They are about 0.4 and 0.6 times as sweet as sucrose and have been used in the pharmaceutical industry as a functional sweetener [4]. FOS possess properties such as low caloric values, non-cariogenic properties, decrease levels of phospholipids, triglycerides and cholesterol, help gut absorption of calcium and magnesium, are useful for diabetic products and are used as prebiotics to stimulate the bifidobacteria growth in the human colon [3].

FOS can be produced by degradation of inulin, or polyfructose, a polymer of D-fructose residues linked by β(2-1) bonds with a terminal α(1-2) linked D-glucose. The degree of polymerization of inulin ranges from 10 to 60. Inulin can be degraded enzymatically or chemically to a mixture of oligosaccharides with the general structure Glu-$(Fru)_n$ (GF_n) and Fru_m, (Fm), with n, m ranging from 1 to 7. The main components of this class are kestose (GF2), nystose (GF3), fructosylnystose (GF4), bifurcose (GF3), inulobiose (F2), inulotriose (F3), and inulotetraose (F4). The second class of FOS is prepared by the transfructosylation action of a β-fructosidase of *Aspergillus niger* on saccharose. The resulting mixture has the general formula of GF_n with n ranging from 1 to 5 [5-7].

FOS promotes the microflora in the large intestine as substrate, increasing the overall gastrointestinal tract (GI Tract) health [8]. It has also been touted as a supplement for preventing yeast infections. Several studies have found that FOS and inulin promote calcium absorption in both the animal and human gut [9]. In the recent times the research work for the production of fructooligosaccharides using microorganisms especially fungi is much in demand industrially because of their low maintenance and quicker production. In the present work production of FOS from *Aureobasidium pullulans* and effect of

different physical and chemical parameters on FOS production was studied.

MATERIALS AND METHODS

Chemicals

Fructooligosacchrides standard 1-kestose (GF2) 1-nystose (GF3) and 1-fructofuranosylnystose (GF4) were from Wako Pure chemical industries, ltd. (Japan)

Micro organism and Culture Conditions

The fungal strain *Aureobasidium Pullulans (ATCC 20524)* was procured from Imtech.

Revival of Lyophilized *Aureobasidium Pullulans*

After thawing at 4^0C, 1ml of sterile distilled water was added to the culture of *Aureobasidium pullulans* (ATCC 20524). Contents were thoroughly mixed and entire contents were transferred into the sterile test-tube containing 5 ml of sterile distilled water. This mixture was kept at room temperature for 6 – hour for rehydrations

Culturing of *Aureobasidium Pullulans* on Growth Medium [10]

Slants of Yeast extract malt extract agar YEME agar containing Sucrose (0.4gm), yeast – extract (0.4 gm), malt – extract (1gm), and agar (2 gm) per 100, pH 6.5 were used for growth. The inoculums of revived culture of *Aureobasidium pullulans* were streaked on these YEME slants. Slants were incubated at 25°C. After 72 – hours of incubation good puffy growth were observed on the YEME slants of *Aureobasidium pullulans.*

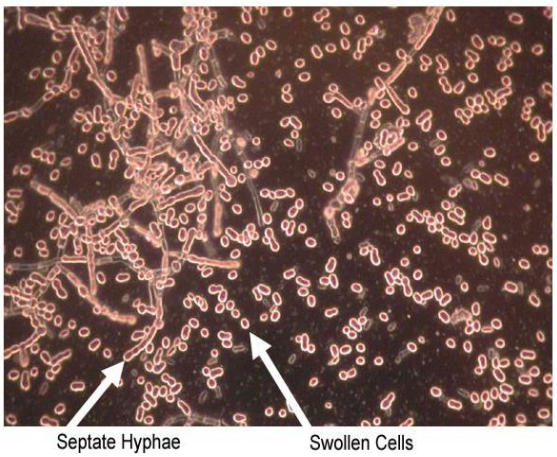


Fig. 1: *Aureobasidium pullulans*

Microscopic Identification of Culture of *Aureobasidium Pullulans* [11]

One loop full of culture were taken and washed with normal-saline solution twice. The cell pellets were suspended in the phosphate – buffer 100µl of culture taken and mixed with 100µl of Methylene blue solution. This mixture was left for 5 minutes at room temperature. The sample were placed on the slide observe the colony was creamy white to pink having black clusters and hyphae were hyaline with septa. Single celled, ovoid shaped conidia produced on short denticles were observed.

Production of Fructosyl transferase Enzyme [12]

The suspended culture for *Aureobasidium pullulans* were prepared by mixing 20 gm of sucrose, 1 gm of yeast – extract, 0.5 gm di-potassium hydrogen phosphate, 1 gm sodium nitrate, 0.05 gm magnesium sulphate dissolved in 100 ml distilled water . The solution were transferred into two 250 ml in conical flask and plugged with non-adsorbent cotton and wrapped with aluminum foil. The flasks were then kept at room temperature in order to cool. pH of the solution was adjusted at 6.5-6.8 by 1N HCl before sterilization. The medium were sterilized in the Autoclave at 121°C for 20 minutes at 15 lb pressure. After sterilization, the seed-culture of *Aureobasidium pullulans* was added to 50 ml sterile shake flask medium in the 250 ml conical flask. These flasks were kept in orbital shaker incubator at temperature (28°C) at 120 rpm for 24 hrs. After 24 hrs good growths were observed in all conical flasks these well grown 50 ml flasks transfer in 150ml of each sterile medium and incubate in shaker incubator at temperature (28°C) at 120 rpm for 24 hrs.

At the end of respective periods of culture it was centrifuged (4^0C, 6000 rpm) using a refrigerated centrifuge, the supernatant and the cells were separated in culture shake flask broth for cell Immobilization.

Cell immobilization of *Aureobasidium pullulans* [13]

The Cell immobilization was done by taking dry cells (20% w/v) from seed culture of a particular micro organism into a solution of sodium alginate at room temperature. The mixture was extruded as small beads into 1% (w/v) of calcium chloride solution. The immobilized cells (hydrated beads) were kept in tubes containing 100 ml of water as one tube containing 30 beads respectively. The beaded tubes were then kept in deepfreeze at -15oC for 24 hours to ensure freeze dehydration process. This helped to study enzyme kinetics by using different techniques [8].

Enzyme assay (Fructosyltransferase activity) [12]

The enzyme activity was determined by measuring the release of glucose into culture medium, it was helpful to measure the turnover of a particular enzyme involved at different reaction conditions of temperature and pH. The whole enzyme assay was done by following the procedure of Yun *et al.* [1] in which 1.5 ml sucrose was prepared in 0.1M Sodium acetate buffer that was added to 0.1 ml enzyme solution. After incubation period of 1 hour at 55^{o} C, 1 ml of dinitro salicylic acid was added to terminate the reaction the absorbance was read at 540nm in spectrophotometer.

Sampling of FOS for Analysis

The samples were taken at 4-hr, 8-hrs, and 12-hr of the reaction time separately and filtered through HYFLOW with 0.45 μm membrane. The levels of different sugars were analyzed using HPLC.

HPLC Analysis [14**]**

The Fructooligosaccharides were analyzed by high performance liquid chromatography (HPLC). Chromatographic Conditions:- Column: - Shodex Asahipak NH_2^{00} P-50 4E, 250 mm X4.6 mm X5.0 μ Flow Rate: - 1.0ml / min Detector: - RI. Injector Volume: - 10 μL. RI Optical Unit Temperature: - 35 C. Column Temperature: - 30 C. Run Time: - 30 mins. Reagent: - Acetonitrile, Water To 700 ml filtered acetonitrile, add 300 ml of filtered water, mix and degas. Standard was prepared by dissolving 10mg each of Fructose, Glucose, and Sucrose, Kestose, Nystose, and GF4 were weighed accurately in 10 ml water. The samples were prepared by dissolving about 1.0 g of the sample in 50 ml water. The column was initially washed with water. It was then treated with acetonitrile (80:20) at a flow of 1ml /min for 30 minutes and then ran mobile phase for 30 minutes.

The blank (water) was injected followed by the standard preparation (six injections) and sample preparation (two injections). The peak responses were recorded for fructose, glucose, sucrose, kestose, (GF2), nystose (GF3) and GF4. The content of fructose, glucose, sucrose, kestose (GF2), nystose (GF3) and GF4 of the FOS sample were calculated

Calculations

$$\% = \frac{AT \times WS \times 50 \times 100}{AS \times 10 \times WT}$$

Where,

AT = Average of the area counts of the peak obtained from the chromatograms of the sample solution.

AS = Average of the area counts of the peak obtained from the chromatograms of the standard solution.

WS = Weight of standard in mg.

WT = Weight of sample in mg.

The enzymatic activity was determinate by the total yield of fructooligosaccharide (Y_{FOS}),which was calculated from the % yield of 1- Kestose (GF2), nystose (GF3), and 1-β-fructofuranosylnystose (GF4),selectively calculated**.**

$$Y_{Fos} = Y_{GF2} + Y_{GF3} + Y_{GF4}$$

Where,

Y_{Fos} = % yield of Fructooligosaccharides

$Y_{GF2,}$ = % yield of 1- Kestose

Y_{GF3} = % yield of nystose

Y_{GF4} = % yield of 1-β-fructofuranosylnystose

Optimization of FOS production:

Otimization of FOS production by *A. pullulans* was performed under following steps: [10]

Effect of temperature on fructosyltransferase activity during the production of FOS

The effect of temperature on fructosyltransferase activity was monitored by assaying the enzyme at 45^0C, 50^0C, 55^0C, and 60^0C under the experimental condition in shaker incubator. Sucrose solutions of 600 gm/l were prepared by dissolving 40gm of sucrose in distilled water and make final volume up to 100 ml with distilled water (four flasks for different temperatures studies). Sucrose solution was transferred in a 250 ml conical flask and the pH was adjusted at 5.5 by 0.1N HCl. 10 gm (immobilized bead of *A. pullulans*) were mixed in sucrose solution and are kept in orbital shaker incubator at above said temperatures at 120 rpm.

Effect of pH on fructosyltransferase activity during the production of FOS

The investigation of the effect of pH was realized in the range of 5.5, 6.0, 6.5, and 7.5 at the temperature of 55^0C. Sucrose solution of 600 gm/l were prepared by dissolving 60gm of sucrose in distilled water and make final volume up to 100 ml with distilled water (4 flasks for different pH). Sucrose solution was transferred in a 250 ml conical flask and the pH was adjusted at 5.5, 6.0, 6.5 and 7.5 in respective flasks by 0.1N HCl or 0.1N NaOH. 10gm (immobilized bead of *A.pullulans*) were mixed in sucrose solution and are kept in orbital shaker incubator at 55°C at 120 rpm.

Effect of different concentration of carbon source during the production of FOS

Fructooligosacchrides production was carried out using sucrose (as substrate) at 3 different concentrations (50 % sucrose solution, 60% sucrose solution, 70% sucrose solution) mixed with beads (immobilized cell). Sucrose solutions 100ml were transferred in a 250 ml conical flasks and the pH was adjusted at 5.5 by 0.1N HCl. 10gm (immobilized bead of *A. pullulans*) were mixed in sucrose solution and are kept in orbital shaker incubator at 55°C at 120 rpm.

Effect of the process of agitation

The effect of agitation was studied by keeping the incubated flask on shaker at 100 rpm, 120 rpm, 150 rpm, and 180rpm during fructosyltransferase activity. 100ml sucrose solutions in four flasks (600 gm/l) were prepared. Sucrose solution was transferred in a 250 ml conical flask and the pH was adjusted at 5.5 by 0.1N HCl. 10gm (immobilized bead of *A. pullulans*) were mixed in sucrose solutions and are kept in orbital shaker incubator at 55°C at 100 rpm, 120 rpm, 150 rpm and 180 rpm.

Effect of different incubation periods: at 4 hrs, 8 hrs, 12 hrs, 16 hrs, 20 hrs and 24 hrs

Sucrose solution of 600 gm/l were prepared by dissolving 60gm of sucrose in distilled water and make final volume up to 100 ml with distilled water. Sucrose solution was transferred in a 250 ml conical flask and the pH was adjusted at 5.5 by 0.1N HCl. 10gm (immobilized bead of *A. pullulans*) were mixed in sucrose solution and are kept in orbital shaker incubator at 55°C at 120 rpm.

RESULTS AND DISCUSSION

Following are the optimized conditions for FOS production using fungus *Aureobasidium pullulans* : 55^0C temperature, 6.0 pH, 60% sucrose as substrate, 150 rpm agitation and 24hrs incubation.

Table1, 2, 3, 4 & 5 show the values of FOS production and left over sucrose as well as all mount different individual constituents GF2, GF3, GF4 and fructose.

Table 1: HPLC analysis report for effect of temperature on FOS production showing presence of different sugars

Reaction time interval		4hrs sample	8hrs sample	12hrs sample
45 ^{0} C	Fructose%	0.34	0.62	0.39
	Glucose%	6.25	7.09	8.99
	Sucrose%	34.08	28.32	24.44
	Kestose%	13.85	16.67	20.28
	Nystose%	2.71	3.35	4.93
	GF4%	0	0	0
	FOS%	16.11	21.00	26.23
50 ^{0} C	Fructose%	0.30	0.21	0.44
	Glucose%	7.41	9.46	11.86
	Sucrose%	35.65	26.88	21.26
	Kestose%	15.01	18.57	22.37
	Nystose%	3.21	4.45	5.78
	GF4%	0	0	0
	FOS%	18.31	22.02	27.99
55 ^{0} C	Fructose%	0.48	0.64	0.83
	Glucose%	9.27	14.53	16.74
	Sucrose%	29.98	13.13	8.39
	Kestose%	20.59	24.35	23.07
	Nystose%	3.70	9.31	12.43
	GF4%	0	0	0.41
	FOS%	25.33	34.45	36.81
60 ^{0} C	Fructose%	0.14	0.22	0.56
	Glucose%	5.05	7.62	9.99
	Sucrose%	48.51	41.98	26.43
	Kestose%	9.90	16.27	21.48
	Nystose%	1.43	2.69	4.57
	GF4%	0	0	1.21
	FOS%	10.23	18.43	25.66

Table 2: HPLC analysis report for effect of pH on FOS production showing presence of different sugars

Reaction time interval		**4hrs sample**	**8hrs sample**	**12hrs sample**
5.5 pH	Fructose%	0.26	0.42	0.68
	Glucose%	9.35	13.33	13.46
	Sucrose%	26.97	15.29	13.96
	Kestose%	21.31	24.66	25.23
	Nystose%	4.74	9.61	10.37
	GF4%	0	0.65	1.31
	FOS%	27.13	37.05	39.01
6.0 pH	Fructose%	0.21	0.53	0.62
	Glucose%	9.46	12.36	13.64
	Sucrose%	26.96	20.77	16.56
	Kestose%	18.57	23.89	25.22
	Nystose%	4.45	6.60	9.31
	GF4%	0	0	0.82
	FOS%	34.25	43.02	49.99
6.5 pH	Fructose%	0.24	0.96	1.66
	Glucose%	10.03	12.97	15.74
	Sucrose%	26.93	18.38	10.75
	Kestose%	21.74	24.33	23.34
	Nystose%	4.06	6.63	9.94
	GF4%	0	0	0.52
	FOS%	23.10	29.33	33.67
7.0 pH	Fructose%	0.34	0.27	0.42
	Glucose%	9.11	12.08	13.60
	Sucrose%	29.75	22.67	19.21
	Kestose%	18.73	22.61	23.49
	Nystose%	2.85	6.41	8.43
	GF4%	0	0	1.59
	FOS%	20.47	29.22	33.25
7.5 pH	Fructose%	0.53	0.46	0.57
	Glucose%	9.40	10.91	13.27
	Sucrose%	34.42	23.13	17.28
	Kestose%	20.14	22.66	25.22
	Nystose%	3.36	5.24	8.10
	GF4%	0	0	0
	FOS%	23.45	27.93	32.21

Table 3: HPLC analysis report for effect of sucrose concentration on FOS production showing presence of different sugars

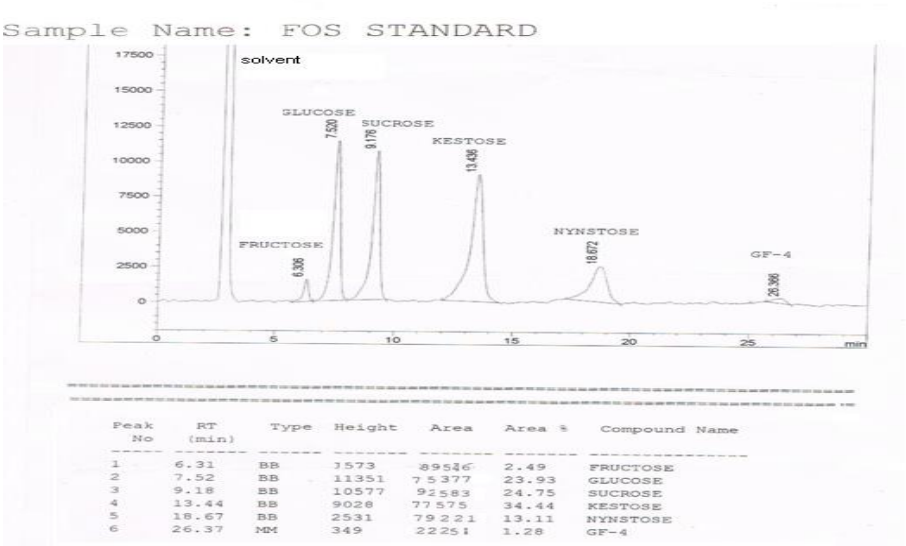
Reaction time interval		4-hrs sample	8-hrs sample	12-hrs sample
50%	Fructose%	0.46	0.59	0.69
	Glucose%	12.01	14.19	15.12
	Sucrose%	21.75	16.87	13.32
	Kestose%	21.89	22.95	22.27
	Nystose%	7.31	9.11	10.36
	GF4%	0	0	0
	FOS%	29.32	32.02	33.63
60%	Fructose%	0.39	0. 62	0.85
	Glucose%	9.62	12.30	16.35
	Sucrose%	25.04	17.23	8.66
	Kestose%	20.29	22.99	22.62
	Nystose%	4.63	7.31	12.48
	GF4%	0	0	2.34
	FOS%	25.92	30.99	38.11
70%	Fructose%	0.36	0.84	0.46
	Glucose%	9.95	14.27	13.08
	Sucrose%	37.54	16.22	13.18
	Kestose%	19.95	25.48	24.44
	Nystose%	4.89	8.93	7.51
	GF4%	0	0	0.54
	FOS%	24.04	34.01	33.34

Table 4: HPLC analysis report for effect of agitation on FOS production showing presence of different sugars

Reaction time interval		4-hrs sample	8-hrs sample	12-hrs sample
100 rpm	Fructose%	0.28	0.57	0.64
	Glucose%	10.37	12.40	14.16
	Sucrose%	25.64	17.70	13.39
	Kestose%	16.70	18.42	19.28
	Nystose%	5.50	7.92	10.27
	GF4%	0	0	0
	FOS%	22.00	26.42	28.55
120 rpm	Fructose%	0.38	0.55	0.88
	Glucose%	11.10	13.89	16.17
	Sucrose%	21.20	15.20	8.98
	Kestose%	17.87	19.22	18.33
	Nystose%	6.49	9.49	13.16
	GF4%	0	0	0
	FOS%	24.36	28.31	30.09
150 rpm	Fructose%	0.33	0.29	0.76
	Glucose%	10.81	11.99	14.61
	Sucrose%	23.76	20.34	14.55
	Kestose%	19.62	21.01	21.59
	Nystose%	5.87	7.30	9.41
	GF4%	0	0	0
	FOS%	25.49	28.33	31.11
180 rpm	Fructose%	0.35	0.33	0.50
	Glucose%	12.14	13.66	15.35
	Sucrose%	23.31	17.86	16.14
	Kestose%	20.31	21.49	21.63
	Nystose%	6.66	7.79	9.19
	GF4%	0	0	0
	FOS%	25.66	29.25	30.02

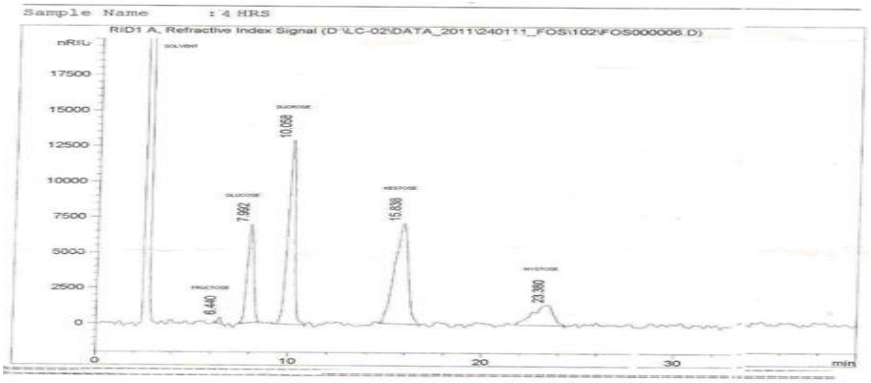
Table 5: HPLC analysis report for effect of incubation time on FOS production showing presence of different sugars

Reaction time interval	Fructose %	Glucose %	Sucrose %	Kestose %	Nystose %	GF4 %	FOS %
4hrs sample	0.53	7.62	39.07	10.14	4.09	0.00	14.66
8hrs sample	0.22	10.01	32.87	19.63	4.53	0.00	24.35
12hrs sample	0.26	11.56	26.49	22.74	5.53	0.00	29.53
16hrs sample	0.58	12.86	22.12	24.27	6.74	0.01	33.22
20hrs sample	0.66	15.03	15.89	24.69	9.10	4.91	39.82
24hrs sample	1.22	22.08	10.99	28.43	16.56	2.5	50.04



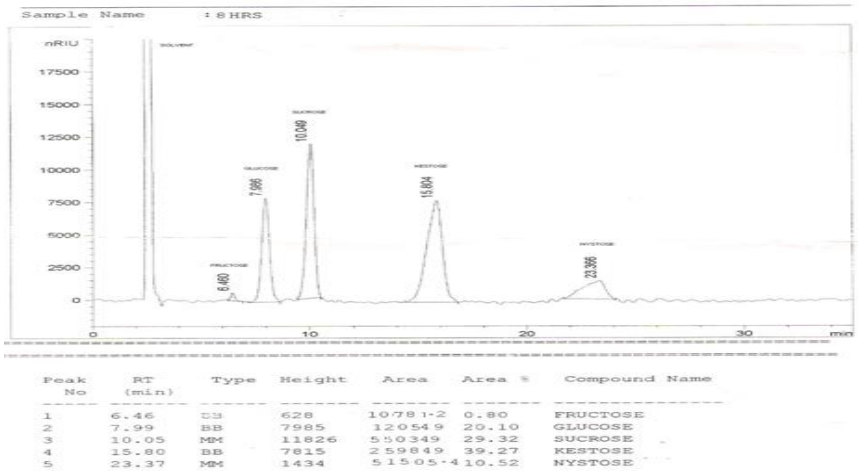
Peak No	RT (min)	Type	Height	Area	Area %	Compound Name
1	6.31	BB	1573	89546	2.49	FRUCTOSE
2	7.52	BB	11351	75377	23.93	GLUCOSE
3	9.18	BB	10577	92583	24.75	SUCROSE
4	13.44	BB	9028	77575	34.44	KESTOSE
5	18.67	BB	2531	79221	13.11	NYNSTOSE
6	26.37	MM	349	22251	1.28	GF-4

Fig. 2: Typical HPLC chromatogram of standard solution FOS



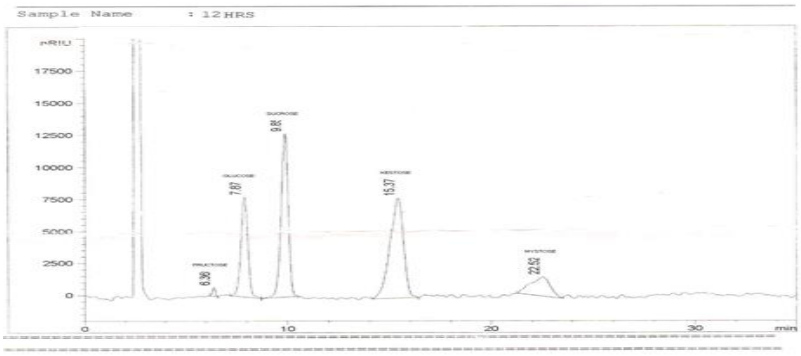
Peak No	RT (min)	Type	Height	Area	Area %	Compound Name
1	6.44	BB	480	5938	0.57	FRUCTOSE
2	7.99	BB	6959	991629	16.86	GLUCOSE
3	10.06	BB	12957	658969	34.57	SUCROSE
4	15.84	BB	7100	214923	36.37	KESTOSE
5	23.38	MM	1434	42979	11.64	NYSTOSE

Fig. 3: Typical HPLC chromatogram of 4 hrs sample



Peak No	RT (min)	Type	Height	Area	Area %	Compound Name
1	6.46	BB	628	10781-2	0.80	FRUCTOSE
2	7.99	BB	7985	120549	20.10	GLUCOSE
3	10.05	MM	11826	550349	29.32	SUCROSE
4	15.80	BB	7815	259849	39.27	KESTOSE
5	23.37	MM	1434	51505-4	10.52	NYSTOSE

Fig. 4: Typical HPLC chromatogram of 8hrs sample



Peak No	RT (min)	Type	Height	Area	Area %	Compound Name
1	6.37	BB	697	8401	0.76	FRUCTOSE
2	7.87	BB	7813	149237	19.12	GLUCOSE
3	9.86	BB	12747	460031	32.17	SUCROSE
4	15.38	BB	7824	314734	37.52	KESTOSE
5	22.53	MM	1519	78235-3	10.43	NYSTOSE

Fig. 5: Typical HPLC chromatogram of 12hrs sample

CONCLUSION

It is becoming increasingly clear that there is a strong relationship between the food we eat and our health. Scientific knowledge of the beneficial role of various food ingredients (nutrients) for the prevention of specific diseases is rapidly accumulating. Functional foods, designer foods, pharma foods and nutraceuticals are synonyms for foods that can prevent and treat diseases. Generally, a functional food can be defined as 'any food that has a positive impact on an individual's health, physical performance or state of mind in addition to its nutritional content'. They include dietary fiber, oligosaccharides, sugar alcohols, peptides and proteins, prebiotics and probiotics, phytochemicals and antioxidants and polyunsaturated fatty acids.

Fructooligosaccharides help the absorption of calcium and magnesium; it is useful for diabetics products and is used as prebiotics to stimulate the growth of *bifidobacteria* in the human colon. Although many microorganisms have been reported to be producing FOS by different scientists, not much record is available or accessible for optimized production and scale up. In the industrial production of fructooligosaccharides, the cells with the FTase activity are produced by aerobic cultivation of fungi such as *Aureobasidium pullulans***,** *Aspergillus orizae* or *Aspergillus japonicus*. They are applied for the biocatalytic process in immobilized form. The survey for FTase producers has been the focus of several studies in this field. The increasing industrial need to develop different processes to produce this alternate sugar in low cost using fungus *Aureobasidium pullulans* can be fulfilled by present work.

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