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Optimization of Production of Fructooligosaccride using *Aureobasidium pullullans*

Awani Kumar Singh * and S.K. Tank
Department of Biosciences Veer Narmad South Gujrat university , Gujrat , India
*Corresponding Author: Awani Kumar Singh

Abstract

This present study 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 intracellulraly by fungi. 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, Fructosyltransferase, HPLC, Probiotics and sugar substitute

Introduction

Fructooligosaccharides (FOS) are oligosaccharides of fructose containing a single glucose moiety; they are produced by the action of fructosyltransferase (FTase,) from many plants and microorganisms. The FOS formed contains fructosyl units bounded at the β-2, 1 position of sucrose; they are mainly composed by 1-kestose, nystose and 1-β- fructofuranosylnystose (Sangeetha *et al*. 2005). Among FOS, the ones with low polymeric grade show better therapeutic properties than those with a high polymeric degree. 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 (Biedrzycka *et al*. 2004). FOS present 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 (Modler *et al.* 1994). FOS are industrially produced from sucrose by microbial enzymes with transfructosylating activity. Most of these enzymes have been found in fungi such as *Aspergillus* sp., *Aureobasidium* sp., *Arthrobacter* sp. and *Fusarium* (Sangeetha*et al*. (2005). Fructooligosaccharides are prebiotic substances found in several vegetables or natural foods. FOS are found in small amounts in vegetables such as onion, garlic, Jerusalem artichokes, asparagus, bananas, rye, wheat and tomatoes, They are calorie-free and non cariogenic sweeteners, stimulate the growth of *Bifidobacteria*, (Modler*et al.* 1994) and have been claimed to contribute towards the prevention of colon cancer and to reduce cholesterol, phospholipids and triglyceride levels in serum. Fructooligosaccharides (FOS) also sometimes called oligofructose or oligofructan is a class of oligosaccharides used as an artificial or alternative sweetener. (Stamp *et al*. 1990) FOS exhibits sweetness levels between 30 and 50 percent of sugar in commercially-prepared syrups. Its use emerged in the 1980s in response to consumer demand for healthier and calorie-reduced foods.

Two different classes of fructooligosaccharide (FOS) mixtures are produced commercially, based on inulin degradation or transfructosylation processes. 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. This process also occurs to some extent in nature, and these oligosaccharides can be found in a large number of plants, especially in Jerusalem artichoke and chicory. 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 *Aspergillusniger*on saccharose. The resulting mixture has the general formula of GF$_n$ with n ranging from 1 to 5. Contrary to the inulin derived FOS, the binding is not only β(1-2), but other linkages do occur, though in limited numbers. Because of the configuration of their glycosidic bonds fructooligosaccharides resist hydrolysis by salivary and intestinal digestive enzymes. In the colon they are fermented by anaerobic bacteria. In other words, they have a lower caloric value, whilst contributing to the dietary fiber fraction of the diet. Fructooligosaccharides are more soluble than inulins and are therefore sometimes used as an additive to yoghurt and other (dairy) products.

FOS serves as a substrate for microflora in the large intestine, increasing the overall gastrointestinal tract (GI Tract) health. 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. The intestinal microflora in the lower gut can ferment FOS, which results in a reduced pH. Calcium is

more soluble in acid, and, therefore, more of it comes out of food and is available to move from the gut into the bloodstream (Van den Heuvel *et al.* 1999). FOS can be considered a small dietary fiber with (like all fibers) low caloric value. The fermentation of FOS results in the production of gases and acids. The latter provide some energy to the body (Roberfroid *et al.* 1993). In the recent times the research work for the production of fructooligosaccharides using microorganisms especially fungi is much in demand industrially. Present work is intended towards production of FOS from *Aureobasidium pullulans* and effect of different physical and chemical parameters on FOS production.

Materials and Methods

Chemicals

All the chemicals used were of analytical grade, Fructooligosacchrides standard 1-kestose (GF2) 1-nystose (GF3) and 1-fructofuranosylnystose (GF4) were from Wako Pure chemical industries, ltd. (Osaka, Japan)

Micro organism and Culture Conditions

The fungal strain *Aureobasidium pullulans (ATCC 20524)* was procured from culture collection of Food and Drug Administration, Vadodara.

Inoculum Development of culture *Aureobasidium pullulans*

Removed the culture vial from the deep freezer dept at -20°C, and kept at 4°C for two hours. Heat the tip of the vial in flame and pour a few drop of water on the hot tip in order to crack cap. One ml 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 dehydrations

Preparation Yeast extract malt extract agar (YEME) Slant of *Aureobasidium Pullulans*

Sterilized YEME agar containing Sucrose (0.4gm), yeast – extract (0.4 gm), malt – extract (1gm), and agar (2 gm) were dissolved in 100 ml of distilled water with pH 6.5 were used to prepare slants. The revived culture of *Aureobasidium pullulans* was streaked on these YEME slants with the help of sterile loop. After streaking these YEME slants were incubated at 25°C. After 72 – hours of incubation good puffy growth were observed on the YEME slants of *Aureobasidium pullulans (ATCC 20524).*

Identification of Culture of *Aureobasidium pullulans* by Microscopic Morphology

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 Fructosyltransferase Enzyme

The shake flask medium 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* (YEME slants) 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.

Pack cell volume (PCV)

50ml broth was taken in centrifuge tube and kept in cooling centrifuge at 2000 rpm for 15 minute. After centrifugation the supernatant was measured in a measuring cylinder. PCV was calculated using following formula:

Packed Cell Volume = (50 ml broth – supernatant) x 2 = approx 4 %

Packed Cell Mass (PCM)

Weight of empty centrifuge tube was taken and then 50 ml broth was taken in a measuring cylinder. 25 ml broth was transferred each into two centrifuge tubes (weighted of empty centrifuge tube) with cell mass.

Packed Cell Mass (PCM) = weight of the tube cell mass - weight of empty centrifuge tube) X 2 = approx 4.1 %

Culture Broth media separation

At the end of respective periods of culture it was centrifuged (4^0C, 6000 rpm) using a refrigerated centrifuge (Remi cooling centrifuge C-30, Mumbai, India), the supernatant and sediment (pellet formed) the cells were separated in culture shake flask broth for cell Immobilization.

Cell immobilization of *Aureobasidium pullulans*

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 -15^{o}C for 24 hours to ensure freeze dehydration process. This helped to study enzyme kinetics by using different techniques (Tal *et al.* 1999).

Enzyme assay (Fructosyltransferase activity)

The enzyme activity was determined by measuring the release of glucose into culture medium, it was helpful to measure the turn over 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*. 2006) 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.

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. The samples are 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.

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. The samples are taken at 4-hr, 8-hr 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.

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

Fructooligosacchrides production was carried out using sucrose (as substrate) at 3 different concentration (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. The samples are taken at 4-hr, 8-hr 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.

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. The samples are taken at 4-hr, 8-hr and 12-hr of the reaction time separately and filtered through HYFLOW with 0.45 µm membrane.

Effect of different incubation periods At 8-hrs, 12-hrs, 16-hrs, 20-hrs, 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. The samples are taken at 4hr, 8hr, 12hr, 16hr, 20hr and 24hr of the reaction time separately and filtered through HYFLOW with 0.45 µm membranes. The levels of different sugars were analyzed using HPLC.

HPLC Analysis:

The Fructooligosaccharides were analyzed by high performance liquid chromatography (HPLC). Chromatographic Conditions:- Column: - ShodexAsahipak 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}) by the Madlova et al (1999),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

Result and discussion

The culture revival result healthy of A. pullulans after inoculam prepration, moreover optimization of FOS conversion has also shown great significance at each parameter. Temperature validation results showed that 1-kestose increased, Gradually at 45^0 C, 50^0 C, 55^0 C and

60^0 C from 13.85 to 35.91% which was on 55^0 C (Graph 1.),significantly 31.79 % was maximum Kestose conversion at 120 RPM (Graph 2.), where as 25.23 maximum at pH 5.5 and 28.43 on 24 hrs (Graph 3.) of reaction incubation. Where as over all FOS conversion was quit high at pH 6.0. Similarly, nystose showed similar variation and increased progressively at 12.43% at 55^0 C, similarly high on same above parameter points (13.16%, 10.27 and 17.47) after over night reaction. Over all FOS conversion was optimized at 35.91 at 55.C, 49.39 at pH 6.0, 31.79 at 120 RPM and 48.40 after 24 hrs of incubation(Graph 4 and 5).

Reaction time interval		4hrs sample	8hrs sample	12hrs sample
45^0 C	Fructose%	0.33	0.60	0.46
	Glucose%	6.50	7.99	9.89
	Sucrose%	35.58	29.72	24.84
	Kestose%	13.85	16.67	20.28
	Nystose%	2.71	3.37	4.93
	GF4%	0	0	0
	FOS%	16.02	20.04	25.21
50^0 C	Fructose%	0.30	0.21	0.44
	Glucose%	7.41	9.46	11.86
	Sucrose%	35.65	26.96	21.26
	Kestose%	15.01	18.57	22.37
	Nystose%	3.21	4.45	5.78
	GF4%	0	0	0
	FOS%	18.22	23.02	28.15
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%	24.29	33.66	35.91
60^0 C	Fructose%	0.14	0.22	0.56
	Glucose%	5.05	7.10	9.87
	Sucrose%	48.51	41.98	26.90
	Kestose%	9.90	16.27	21.48
	Nystose%	1.43	2.69	4.27
	GF4%	0	0	1.21
	FOS%	11.33	18.96	26.96

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
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%	26.05	35.01	36.91
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%	33.15	41.82	49.39
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%	25.80	30.69	33.80
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%	21.58	29.02	33.51
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%	23.50	27.90	33.32

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
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%	29.20	32.06	32.63
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%	24.92	30.31	37.44
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%	24.84	34.41	32.49

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

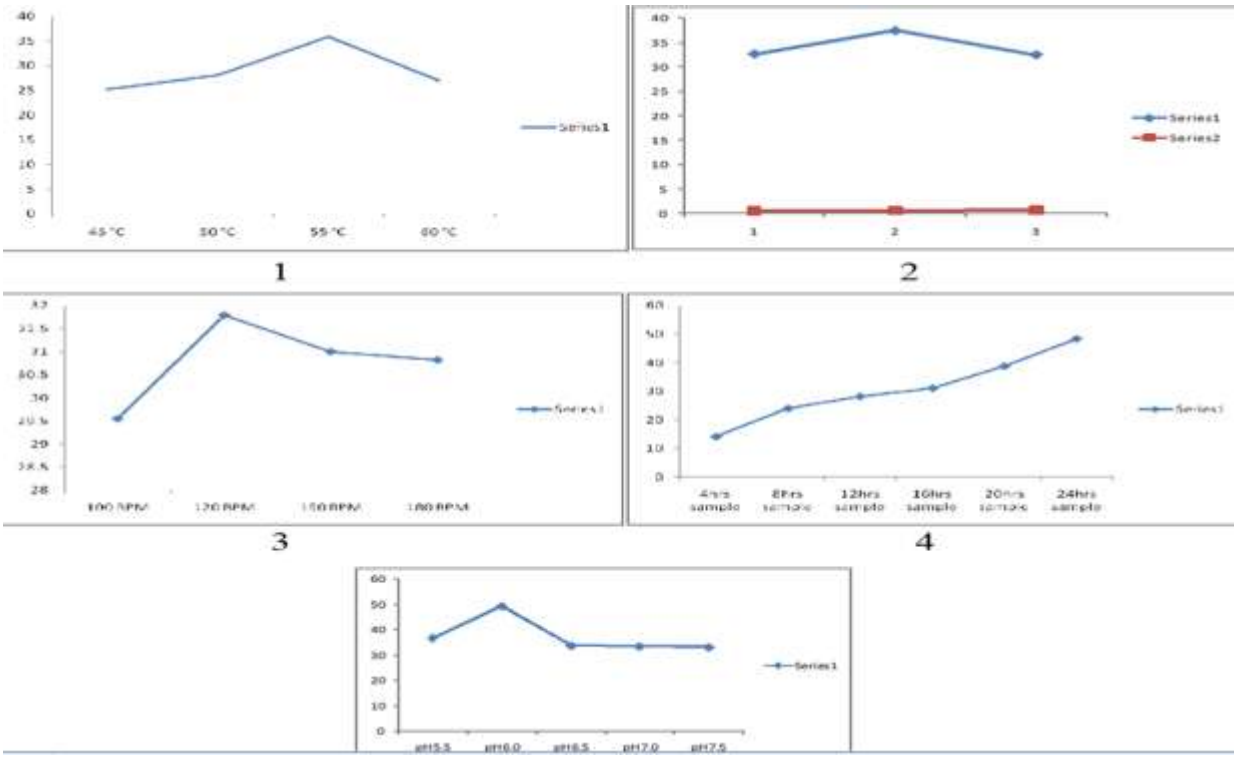
Reaction time interval		4hrs sample	8hrs sample	12hrs sample
100 rpm	Fructose%	0.28	0.57	0.64
	Glucose%	10.37	12.40	14.16
	Sucrose%	25.64	17.70	13.39
	Kestose%	16.70	18.42	19.28
	Nystose%	5.50	7.92	10.27
	GF4%	0	0	0
	FOS%	22.20	26.34	29.55
120 rpm	Fructose%	0.38	0.55	0.88
	Glucose%	11.10	13.89	16.17
	Sucrose%	22.20	15.20	8.98
	Kestose%	17.87	19.22	18.63
	Nystose%	6.49	9.49	13.16

	GF4%	0	0	0
	FOS%	24.36	28.31	31.79
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	28.31	31.00
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%	26.97	29.28	30.82

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

Reaction time interval	Fructose %	Glucose %	Sucrose %	Kestose %	Nystose %	GF4 %	FOS %
4hrs sample	0.55	7.59	39.97	10.14	4.09	0.00	14.23
8hrs sample	0.21	9.61	33.98	19.63	4.43	0.00	24.06
12hrs sample	0.25	11.34	27.66	22.74	5.53	0.00	28.27
16hrs sample	0.60	12.57	23.31	24.27	6.84	0.00	31.16
20hrs sample	0.46	14.57	15.92	24.69	9.10	4.91	38.79
24hrs sample	1.22	21.92	11.97	28.43	17.47	2.5	48.40

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



Graph 1, Showing maximum conversion on 55 °C, Graph 2, Showing optimization of carbon source, indicating 55 % ideal for maximum

conversion, Graph 3, Showing 120 RPM was optimized for maximum conversion, Graph 4, Showing incubation period for sucrose conversion based on incubation period and Graph 5,Showing maximum conversion on pH 6.0.

Conclusion

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. The increasing industrial need to develop different processes to produce this alternate sugar in low cost can be fulfilled by present work.

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