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Study on Fructosyltransferase enzyme from *Aspergillus* sp. in Fructooligosaccharides production

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Abstract

The fructosyltransferase intra- and extra- cellular enzyme preparations obtained from Aspergillus sp. was used to produce fructooligosaccharides by enzymatic conversion of sucrose. The crude enzyme preparation of Aspergillus sp. exhibited fructosyltransferase activity of 5.0 U.mg^{-1} at 60 per cent sucrose concentration. The specific activity was recorded at its highest rate at the substrate concentration of 40 per cent (9.05 U.min^{-1}.mg^{-1} of protein). The high performance liquid chromatography analysis of the end products of fructosyl enzyme activity on sucrose had shown the formation of, 1-kestose and nystose along with glucose, fructose and unhydrolyzed sucrose. The enzyme activity was stable at a temperature of 52°C and at a pH of 4.5. The activity of enzyme was enhanced by addition of 1 mM $FeSO_4$ (7.1 U.min^{-1}.mg^{-1} of protein) and also by addition of Fe^{2+} and its combination with Ca^{2+} (9.2 U.min^{-1}.mg^{-1} of protein). Susceptibility to detergents was observed. The selectivity of conversion of sucrose to fructooligosaccharides obtained by the enzyme was approximately 70 per cent under optimized conditions. The partially purified fructosyltransferase preparation from Aspergillus sp. is found applicable for industrial production of fructooligosaccharides.

Keywords: 1-Kestose, Nystose, Fructosyltransferase, HPLC, Parameter optimization.

Introduction

The design of food products that confer a health benefit is relatively a new trend and recognizes the growing acceptance of the role of diet in disease prevention, treatment and well-being. Consumers believe that foods contribute directly to their health. So an increasingly active population, the pursuit of healthier lifestyles and the desire to live longer has risen to a new category of food products referred to as "Nutraceuticals". The term "Nutraceuticals" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen Defelice and was originally defined as a wide range of food and food components with a claimed medical or health benefits[1]. Nutraceuticals can come from plant, marine, animal and microbial sources; specifically the food products include whole foods, food additive, probiotics, vitamins and mineral[2]. A lot of attention is being paid to dietary carbohydrates, especially on the various kinds of oligosaccharides[3]. In the current context of functional foods, it generates a global market in which oligosaccharides could play a major role as functional ingredients compared to dietary fibers, sugar alcohols, peptides, probiotics, polyunsaturated fatty acids and antioxidants[4].

The generic term "oligosaccharides" is customarily used for saccharides having the degree of polymerization of 2-10 and are found either free or in combined forms in all living organisms as polymeric carbohydrates. Structurally, oligosaccharides are composed of 2-10 monosaccharide residues linked by glycosidic bonds that are readily hydrolysed to their constituent monoaccharides either by acids or by specific enzymes[5]. Oligosaccharides are functional food ingredients, they have a great potential to improve the quality of many foods[6]. In this way, a compound named as fructooligosaccharides (FOS) coming under the category of sucrose related oligosaccharides has emerged as one of the important product in the functional food market. They are naturally occurring sugars that has beneficial effects as food ingredient. FOS act as prebiotic which represent selective nutrient for beneficial microorganisms and they have the potential to increase the effectiveness of probiotic products. Furctooligosaccharides consist mainly of 1-kestose, nystose and fructofuranosyl nystose.

Fructosyltransferase (FTase) (E.C. 2.4.1.9) catalyze the formation of FOS from sucrose, which have broad applications in the food and pharmaceutical industries. Several microorganisms have been reported to posses FTase activity and produce FOS from sucrose. The characterization of the enzyme was undertaken as a necessary step towards understanding the properties of the FTase enzyme. The activity of the FTase enzyme obtained from the strain *Aureobasidium pullulans* (CFR 77) was assayed under pre-determined conditions by incubating the FTase enzyme with sucrose (60%w/v). The activity of the enzyme was determined based on the amount of enzyme required to liberate 1 micromole of glucose under the specified conditions [7]. The fructosyltransferase enzyme from *Rhodotorula* sp. showed a rather sigmoid cooperative type behavior similar to that expressed by the Hill's model with a V_{max} value of 236.1 µmol.ml^{-1} [8]. A behaviour associated with the ping-pong mechanism for fructosyltransferase produced by *Aspergillus* sp. N74 leading to the highest sucrose bioconversion at 24 h of

reaction (84 per cent w/v) was reported[9]. Batch fructooligosaccharides production was done and obtained the yield of 69 per cent (43 per cent kestose and 26 per cent nystose). Fructooligosaccharides production of 51.8 per cent kestose and 74.6 per cent nystose in the batch culture mode and 102.4 $g.l^{-1}$ for 1-kestose and 59 $g.l^{-1}$ for nystose by two step batch culture mode using fructosyltransferase from *Aureobasidium* sp. ATCC 20524 was reported[10].

The fructooligosaccharides are called as, 'non digestable food ingredients' that beneficially affects the host health by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon. The low calorie sweetner like fructooligosaccharides has an important role in control of diabetes. They also prevent the colonization of human gut by pathogenic microorganisms because it encourages the growth of beneficial bacteria. Dietary treatment with FOS significantly potentiated the effects of sub therapeutic doses of cytotoxic drugs commonly utilized in human cancer treatment. On optimizing the conditions for higher enzyme activity, we achieve maximum FOS production and also maximum conversion of sucrose substrate into product. The substrate concentration, at which there is maximum enzyme activity and minimum substrate inhibition, is optimized.

Material and Methods

All chemicals, reagents and enzyme were of analytical grade. HPLC grade chemicals obtained from Sigma were used for chromatographic studies. Borosil grade glasswares were used in all the experiments.

The *Aspergillus* sp. obtained from fermented jaggery was developed for fructooligosaccharides production in the previous studies. The crude protein extract of *Aspergillus* sp. exhibited fructosyltransferase activity, producing fructooligosaccharides from sucrose. The culture was stored and propagated in potato dextrose agar to be used as the inoculum for further experiments. The fungal isolate *Aspergillus* sp. was developed by transferring 1 ml of spore suspension in to 100 ml Erlenmeyer flask containing 50 ml of pre-inoculum medium (sucrose: 1per cent; yeast extract: 0.2 per cent, pH 5.50).

Development of fungal inoculum in fermentation medium: The 36 h old pre-inoculum at 20 per cent (v/v) was transferred to 100 ml of fermentation medium (Sucrose - 20 $g.l^{-1}$,Yeast extract – 5 $g.l^{-1}$, Sodium nitrate – 1 $g.l^{-1}$, Potassium hydrogen orthophosphate – 0.5 $g.l^{-1}$, Dipotassium hydrogen orthophosphate – 0.25 $g.l^{-1}$, Sodium chloride – 0.25 $g.l^{-1}$, Magnesium sulphate – 0.05 $g.l^{-1}$, Ammonium chloride – 0.05 $g.l^{-1}$, pH – 5.5) prepared in 250 ml Erlenmeyer flasks [11]. The culture flasks and uninoculated fermentation medium were incubated at 30 ± 1^0C in environment incubator shaker (New Brunswick Scientific, USA) at 250 rpm. An uninoculated fermentation medium served as control.

Preparation of crude protein extract by ammonium sulphate precipitation: At the end of 48 h of incubation, the culture broths were centrifuged under 6000 g at 4^0C for 30 min in refrigerated centrifuge (KUBOTA 6800) to separate the biomass from the culture broth. The clear cell free extract thus obtained was used for further assay. The cell free extract and the mycelial / cell extract were pooled and saturated with 30, 40, 50, 60 and 70 per cent ammonium sulphate (AR grade). The precipitate obtained from each fractionation was dissolved in a minimal amount of phosphate buffer (0.2 M, pH 7.0) and used as crude enzyme source for fructosyltransferase assay.

Fructosyltransferase enzyme assay: The crude protein extract obtained was used as an enzyme source and fructosyltransferase activity was assayed under the predetermined conditions as suggested[11].

The substrate sucrose solution was prepared at various concentrations, 20–80 per cent (w/v) (58mM–234mM) in 0.1 M citrate buffer (pH 5.0). The reaction mixture consisted of 250 µl of substrate and 250 µl of enzyme source. The reaction mixture was placed at 55^0C in shaking water bath (JULABO SW20) at 100 rpm for 1 h. After the period of incubation the reaction mixture was heated at 90^0C for 15 min to arrest the reaction. The amount of glucose released at the end of the reaction was determined by dinitrosalicylic acid assay[12]. In case of fructosyltransferase enzyme activity one unit was defined as the amount of enzyme required to liberate 1 µ mole of glucose per minute.

Detection of fructooligosaccharides by high performance liquid chromatography: High performance liquid chromatography was used to quantify the fructooligosaccharides synthesized by fructosyltransferase activity in the reaction products at various substrate concentrations[13].

The fructooligosaccharides present in the samples was analyzed by polar bonded phase high performance liquid chromatography (Shimadzu, Japan). A sample volume of 20 µl was injected into the amino column with a mobile phase of acetonitrile and water in 60:40 as binary gradient at a flow rate of 1.0 $ml.min^{-1}$. The sample was run for 10 min. The saccharides were identified by comparing retention times with those of known standards viz., glucose, fructose, sucrose, kestose and nystose (HPLC grade, Sigma).

Analysis of yield of fructooligosaccharides: The yield of fructooligosaccharides, which was the measure of enzyme activity, was calculated[14]. The selectivity of conversion from sucrose to fructooligosaccharides (FOS) was calculated from the following formula,

$$S\ (FOS) = \frac{2c\ (GF_2) + 3c\ (GF_3)}{2c\ (GF_2) + 3c\ (GF_3) + c(F)}$$

where c (F) is the molar concentration of fructose.

Determination of optimal temperature and pH for enzyme activity: This experiment was conducted to find out the temperature and pH for maximum enzyme activity. The fructosyltransferase activity on sucrose was determined by incubating the enzyme (15 $U.ml^{-1}$) at temperatures of 53°C, 54°C, 55°C, 56°C and 57°C in medium consisting of sucrose (200 – 800 $g.l^{-1}$) in 0.1 M citrate buffer (pH 5.0) and enzyme preparation in 0.2 M phosphate buffer (pH 7.0). The fructosyltransferase activity was studied at pH values from 4.0-8.0. The 0.1 M citrate buffer was used to prepare sucrose with pH values of 4.0, 5.0 and 6.0 whereas substrates of pH 7.0 and 8.0 were prepared using 0.2 M phosphate buffer. The enzyme solution was maintained at 55°C in each buffer. The fructosyltransferase activity was determined by the fructosyltransferase enzyme assay procedure.

Effect of metal ions on fructosyltransferase activity: The metal ions were added as NaCl, $CaCl_2$, $CuSO_4$ and $FeSO_4$ at concentration of 1 mM to the enzyme and incubated for 60 min. Upon incubation the hydrolytic activity was measured by the standard assay with sucrose at a concentration of 1.76 M.

Effect of detergents on fructosyltransferase activity: The detergent susceptibility was assessed by incubating the crude fructosyltransferase enzyme with polyethylene glycol (5 per cent w/v), tween 20 (5 per cent w/v) and tween 80 (5 per cent w/v) in 0.2 M sodium acetate buffer (pH 5.5). Upon incubation an aliquot was withdrawn, chilled on ice and the fructosyltransferase activity from 1.76 M sucrose concentration was measured by the enzyme assay.

Statistical analysis: The experimental set up in all the investigations was duplicated and three samples were taken for analysis. Statistical analysis was done in completely randomized block design [15].

Results and Discussion

Results: The isolate *Aspergillus* sp. when grown for 48 h in 100 ml of fermentation medium yielded a mycelial biomass of 0.4 g dry weight. In the ammonium sulphate protein extract, the quantity of protein estimated was in the range of 3 to 5.0 mg. The maximum fructosyltransferase activity exhibited in the crude enzyme extract was 9.05 $U.mg^{-1}$ in the reaction conditions of 250 µl of substrate sucrose at 40 per cent (w/v) concentration in 0.1 M citrate buffer (pH 5.0) and 250 µl of enzyme source (table 1).

Table-1
Activity of fructosyltransferase from *Aspergillus* sp. at various sucrose concentrations

S. No.	Sucrose concentration (mM)	Total activity (U. min^{-1})	Specific activity (U. min^{-1}. mg^{-1} of protein)
1	58 (20)	65.90	7.01
2	88 (30)	74.17	7.89
3	116 (40)	85.10	9.05
4	146 (50)	71.95	7.65
5	176 (60)	61.05	6.49
6	204 (70)	54.60	5.81
7	234 (80)	48.50	5.16
	Mean	65.64	6.98
	CD (0.05)	3.50	0.37

*Values in paranthesis indicate percent substrate (sucrose) concentration, U – amount of enzyme required to liberate 1 µ mole of glucose

Table-2
Fructosyltransferase activity and fructooligosaccharides production at various sucrose concentrations by *Aspergillus* sp.

S. No.	Sucrose concentration (mM)	Total activity (U. min^{-1})	Specific activity (U. min^{-1}. mg^{-1} of protein)	FOS concentration ($g.l^{-1}$)
1	58 (20)*	60.70	6.89	75
2	88 (30)	72.07	7.45	90
3	116 (40)	84.10	8.90	110
4	146 (50)	70.95	7.15	85
5	176 (60)	60.05	6.19	67
6	204 (70)	53.60	5.61	45
7	234 (80)	47.50	5.06	31
	Mean	63.64	6.98	73.43
	CD (0.05)	3.50	0.37	2.72

*Values in paranthesis indicate percent substrate (sucrose) concentration, U – amount of enzyme required to liberate 1 µ mole of glucose

The total activity and specific activity recorded in different sucrose concentrations were given in table-1. The reaction products of crude fructosyltranferase enzyme from *Aspergillus* sp.AFJ3 with sucrose concentrations, 58 mM – 234 mM (200 $g.l^{-1}$ – 800 $g.l^{-1}$) were analyzed by high performance liquid chromatography for the detection of sugars (figure-1) formed from sucrose due to fructosyltransferase enzyme activity. The sugars formed from sucrose concentrations 58 mM – 234 mM (20-80 per cent) presented in table-2 shows fructooligosaccharides production at all sucrose levels. The rest of carbohydrates in the mixture were fructose, glucose and remaining sucrose. The chromatogram also revealed the presence of sugars with high polymerization.

The enzyme activity at various sucrose levels (58 mM – 234 mM) and temperature (53ºC, 54ºC, 55ºC, 56ºC and 57ºC) on frutosyltransferase enzyme activity was determined and the results are presented in figure-2. The fructosyltransferase activity was maximum (8.50 $U.min^{-1}.mg^{-1}$ of protein) at 55ºC when incubated with 116 mM of sucrose. The activity of fructosyltransferase was minimum (4.40 $U.min^{-1}.mg^{-1}$ of protein) at 53ºC when incubated with 234 mM of sucrose.

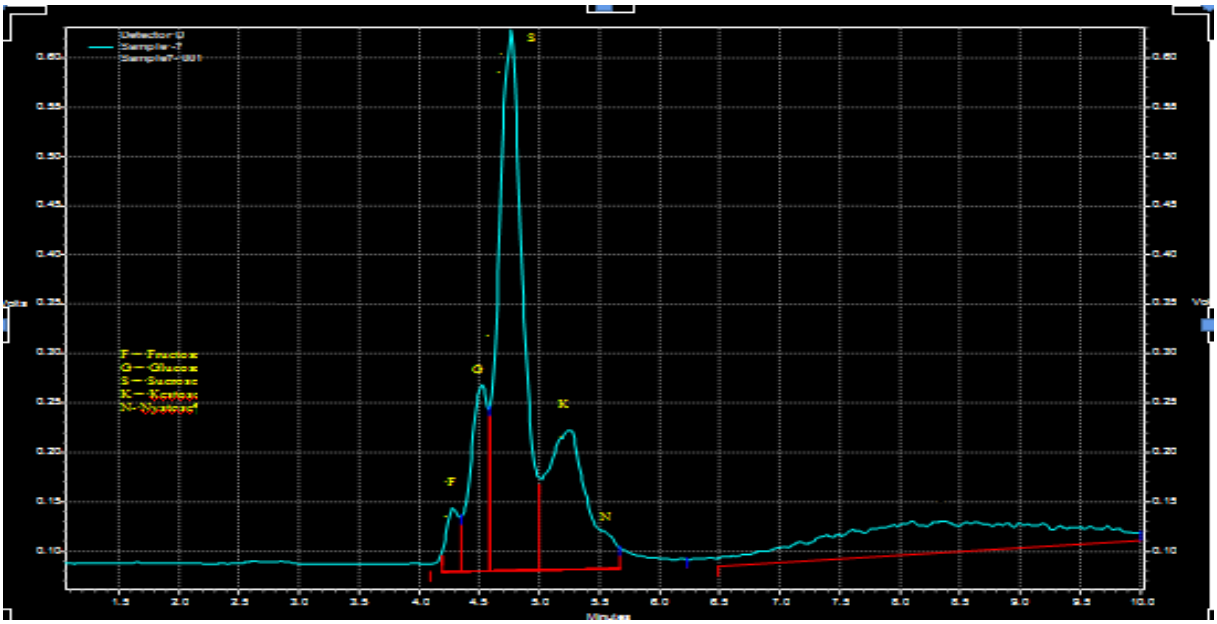
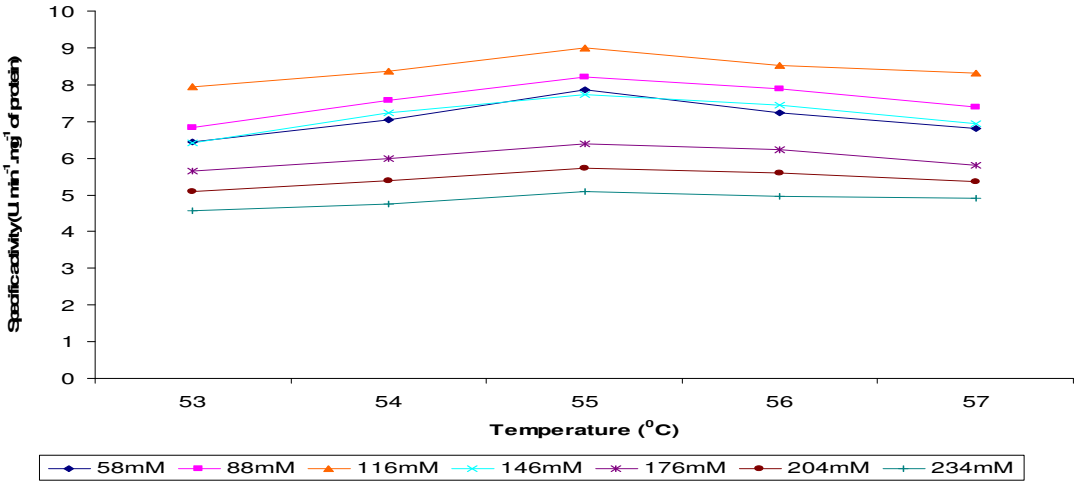


Figure-1

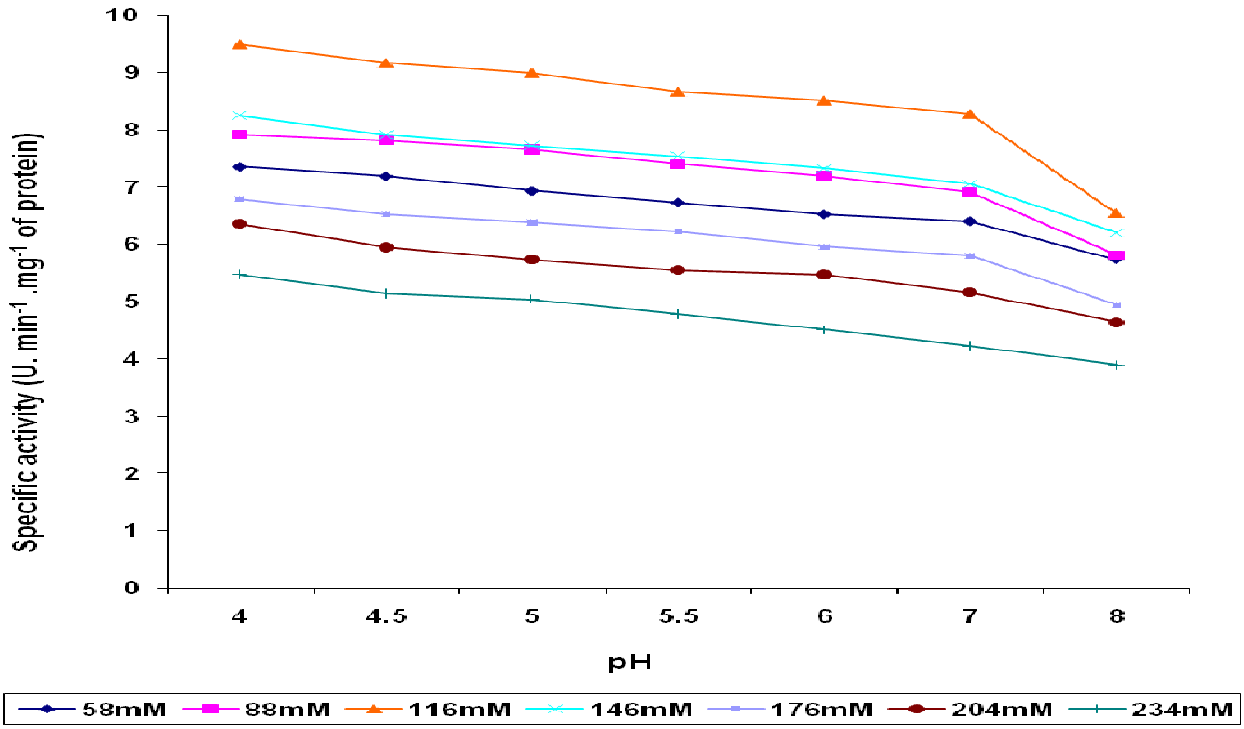
Chromatogram of sugars formed by fructosyltransferase activity of *Aspergillus* sp., from sucrose by high performance liquid chromatography



U – amount of enzyme required to liberate 1 µ mole of glucose

Figure-2

Effect of temperature on fructosyltransferase activity of *Aspergillus* sp. AFJ3 at various sucrose concentrations



U – amount of enzyme required to liberate 1 µ mole of glucose

Figure-3

Relationship of fructosyltransferase activity and pH at various sucrose concentrations

The influence of temperature and pH on the fructosyltranferase activity was studied for substrate sucrose levels at pH of 4.0, 5.0, 6.0, 7.0 and 8.0. The enzyme was stable at temperature of 52°C and in the pH of 4.5. According to the results shown in figure-3, the maximum enzyme activity of 9.30 $U.min^{-1}.mg^{-1}$ of protein was recorded at pH 4.0 when incubated at sucrose concentration of 116 mM.

The crude fructosyltransferase enzyme displayed activity using sucrose as substrate (176 mM) even in the absence of metal ions (table 3). Of those tested, only Fe^{2+} added in the form of $FeSO_4$ increased the fructosyltransferase activity by 13.5 per cent (7.1 $U.min^{-1}.mg^{-1}$ of protein). Most of the ions tested produced an inhibitory effect. The combination of metal ions Fe^{2+} and Ca^{2+} added in the form of $FeSO_4$ and $CaCl_2$ respectively, had a positive effect on fructosyltransferase activity by 28.6 per cent (9.2 $U.min^{-1}.mg^{-1}$of protein). The effect of detergents on fructosyltransferase activity is shown in (table 4). None of the detergents tested exhibited positive effect.

Discussion: Fructooligosaccharides have enormous functional properties that make them commercially interesting for applications in pharmaceutical and food industries. In the human digestive tract, fructooligosaccharides are almost exclusively fermented by bifidobacteria and lactobacilli which have beneficial health effects. Sucrose can be converted in to fructooligosaccharides by using a range of transfructosylating enzymes originating from plants, bacteria and fungi. Fructooligosaccharides synthesis has been reported for the commercially important fungus *Aspergillus* sp. as a result of the specific fructosyltransferase enzyme activity [16].

The fungi of the genera *Aspergillus, Fusarium, Aureobasidium* and *Penicillium* have been described as good producers of fructooligosaccharides with potential for industrial processes [17]. Fructooligosaccharides producing fungal strain, *Aspergillus* sp. N74 was isolated from sugarcane crop[18]. The use of sugar by-products as substrates for growing fructooligosaccharides producing fungi also have been reported [19].

The chromatographic profile of the reaction product from *Aspergillus* sp. AFJ3 revealed the presence of sucrose, fructose, glucose and fructooligosaccharides namely, kestose and nystose. This shows the presence of transfructosylation activity in enzymatic product. Presence of sugar polymers were also detected in the enzymatic product chromatographic profile. The maximum fructooligosaccharides production was found at 40 per cent initial sucrose concentration. The fructooligosaccharides produced were identified as kestose and nystose. Presence of glucose, fructose and residual sucrose were also found. It is understood that the lower fructooligosaccharides yield above 40 per cent sucrose concentration may be due to poor hydrolysis of substrate at high sucrose concentrations.

Table-3
Effect of metal ions and EDTA on fructosyltransferase activity

S. No.	Metal ion/ Chelator	Total activity (U. min^{-1})	Specific activity (U. min^{-1}. mg^{-1} of protein)
1	NaCl	30.15	3.21
3	$CaCl_2$	54.71	5.82
7	$CuSO_4$	26.85	2.86
8	$FeSO_4$	74.20	7.1
12	$FeSO_4$ + $CaCl_2$	88.60	9.2
13	Control	61.05	6.49
	Mean	41.55	4.39
	CD (0.05)	1.56	0.19

U – amount of enzyme required to liberate 1 µ mole of glucose

Table-4
Effect of detergents on fructosyltransferase activity

S. No.	Detergent	Total activity (U. min^{-1})	Specific activity (U. min^{-1}. mg^{-1} of protein)
1	Polyethylene glycol	27.55	1.12
2	Tween 20	25.55	2.10
3	Tween 80	29.40	2.12
7	Control	61.05	6.49
	Mean	29.10	3.12
	CD (0.05)	1.04	0.15

U – amount of enzyme required to liberate 1 µ mole of glucose

The fructosyltransferase activity of crude enzyme from *Aspergillus* sp. AFJ3 was maximum (8.99 U. min^{-1}.mg^{-1} of protein) at 55°C and minimum at 53°C. A large number of reports have described the temperature ranges of microbial fructosyltransferase as 50-60°C. The results were found to be similar with that of the enzyme from *Aspergillus niger* which exhibited an optimal activity at 50-60°C [7]. Temperatures greater than 65°C inactivated the fructosyltransferase enzyme from *Aspergillus aculeatus*[20]. The crude fructosyltransferase from *Aspergillus* sp. AFJ3 exhibited maximum enzyme activity at pH 4.0 irrespective of the substrate sucrose concentration. Production of fructooligosaccharides from sucrose by the enzyme preparation was optimal at pH 5.0 - 6.0. Similarly fructosyltransferase from other fungi are most active in the pH range of 5.0 - 6 .0 [7, 21, 22]. The activity of fructosyltransferase from *Aspergillus aculeatus* was elicited 1.4-1.9 fold by ions like Mn^{2+}, K^{+} and Co^{2+} whereas the activity showed inhibition at low concentration of Hg^{2+} and Zn^{2+}. The addition of ethylene diamine tetraacetic acid (EDTA) did not result in a decrease in activity, indicating that the fructosyltransferase activity was independent of the divalent ions [20].

Conclusion

In the present study, the fructosyltransferase specific activity exhibited in the crude protein extract of *Aspergillus* sp. from sucrose was 5.0 U.mg^{-1} of protein. The chromatographic profile of the cell free extract from *Aspergillus* sp. revealed the presence of sucrose, fructose, glucose and fructooligosaccharides namely, kestose and nystose. Presence of sucrose, fructose, glucose, kestose and nystose were also detected in the enzymatic product. This shows the presence of transfructosylation activity in both cell free extract and enzymatic product. The study of optimal temperature and pH of fructosyltransferase activity were needed to understand the suitability of the enzyme in industrial applications. The study of how one metal ion could influence the reaction rate is important to understand the structure-function relationship in enzymes. Further elaborate studies are required in optimizing the culture conditions and reaction conditions for maximum production of fructooligosaccharides. Studies are required to find the suitability of the purified enzyme from *Aspergillus* sp. in food and nutraceutical industries.

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