ISSN 1996-0700

Asian Journal of Biotechnology

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

http://knowledgiascientific.com

Asian Journal of Biotechnology 2 (2): 86-98, 2010
ISSN 1996-0700

Highly Thermostable β-fructofuranosidase from *Aspergillus niger* PSSF21 and its Application in the Synthesis of Fructooligosacharides from Agro Industrial Residue

[1]P. Praveen Reddy, [2]G.S.N. Reddy and [1]M.B. Sulochana
[1]Departement of Research and Studies in Biotechnology,
Gulbarga University, Gulbarga-585106, India
[2]Center for Cellular and Molecular Biology,
Uppal-Hyderabad-580032, India

Abstract: The aim of this study is to establish optimal conditions for the maximum production of β-Fructofuranosidase by *Aspergillus niger* PSSF21 using cheaper sources. The experiment is to evaluate the effect of initial pH, temperature, different carbon and nitrogen sources of the medium on maximal enzyme production. The maximum enzyme activity (30.84±0.447 U mL^{-1}) was achieved at 96 h of cultivation at pH 3.5 and 30°C in a basal medium containing Molasses (2%) as the carbon and energy source supplemented with 0.5% Soya bean meal. Partial characterization of β-Fructofuranosidase produced from *Aspergillus niger* PSSF21 results showed high optimal pH and temperature at 3.5 and 60°C, respectively. The pH and thermo stability of enzyme was good in absence of any stabilizers (50% of residual activity was maintained up to 20 h) at optimal pH 3.5 to 4.5 and temperature 55 to 65°C, respectively. The 6-kestose monohydrate and nystose were identified by LCMS as the major products of fructoligosaccharides using molasses as carbon source. Hence, it is a promising strain for food industry to utilize the agro waste for the production of β-Fructofuranosidase having specific properties to produce fructo oligosaccharides.

Key words: *Aspergillus niger* PSSF21, β-fructofuranosidase, liquid chromatogram mass spectroscopy, thermostable β-fructofuranosidase, fructo oligosaccharides

INTRODUCTION

In response to an increasing demand from the consumer for healthier and calorie-controlled foods, a number of so-called alternative sweeteners are present. One such functional ingredient is fructoligosaccharides (FOS). FOS are synthesized by β-fructofuranosidases (FFase; EC.3.2.1.26) having transfructosylating activity and able to convert the disaccharide sucrose to FOS which gained commercial importance in food processing industry (Mussatto and Manicilha, 2007; Sangeetha *et al*., 2005). These FOS are made of 1-3 fructose units bonded to one molecule of sucrose and they are classified as 1-kestose (GF2), nystose (GF3) and 1Ffructofuranosyl nystose (GF4) (Gibson and Roberfroid, 1995). FOS exert several physiological effects, like decrease in levels of serum cholesterol, phospholipids, triglyceride, including calorie free, non cariogenic, improvement of

Corresponding Author: Dr. M.B. Sulochana, Departement of Biotechnology, Gulbarga University, Gulbarga-585106, India Tel: 08472 247635

gastrointestinal conditions and an increase in mineral absorption by stimulating the growth of bifid bacteria in the large intestine (Tokunaga, 2004; Biedrzycka and Bielecka, 2004; Yun, 1996). Considerable knowledge had been reported about production of Ffase from synthetic sources and non synthetic sources through different microbial sources e.g., *S. cerevisiae* ATCC36858 (Atiyeh and Duvnjak, 2001), *C. utilis* (Ali and Ashiq, 2009), *Sporotrichum thermophile* (Katapodis *et al*., 2004), *Aspergillus* sp. 27H (Cuervo *et al*., 2004), *Aspergillus ochraceus* (Guimarães *et al*., 2007), Xanthophyllomyces (Dolores *et al*., 2009). But little can be found about the production of thermostable FFase possessing high transfructosylation property from agro industrial residues. Owing to the increasing biotechnological importance of thermostable β-fructofuranosidases. In the present study, a isolated strain from our laboratory *Aspergillus niger* PSSF21 which is a good producer of β-fructofuranosidase was undertaken for following studies. Optimization of β-fructofuranosidase produced from *Aspergillus niger* PSSF22 using cheaper sources; partial characterization of β-fructofuranosidase and Detection of Hydrolysis products by Liquid Chromatogram Mass Spectroscopy (LCMS).

MATERIALS AND METHODS

Materials

Chemicals used in the experiments are 3, 5-dinitro salicylic acid, sucrose, Inulin, $MgSO_4$, yeast extract, ammonium salts etc., were analytical grade reagents.

Microorganism and Cultural Conditions

The fungus *Aspergillus niger* PSSF21 capable of producing extracellular β-fructofuranosidase was isolated in our laboratory and it is deposited in National Collection of Industrial Microorganisms (NCIM), Pune with accession number 1342. It was maintained on PDA (potato dextrose agar) slants and stored at 4°C for long term studies. The active culture was produced through submerged cultivation of the *Aspergillus niger* PSSF21 in a culture growth medium containing $NaNO_3$-0.5%, K_2HPO_4-0.5%, $MgSO_4.7H_2O$-0.05% and glucose-1% pH 5 in a 250 mL Erlenmeyer flask and cultured at 30°C and 160 rev min (Steel meet Novatech, Pune, India) for 24 h.

Fermentation and Growth Profile of *Aspergillus niger* PSSF21 Strain

The *Aspergillus niger* PSSF21 was grown aerobically at 30°C in growth medium pH 5.0 in a 250 mL Erlenmeyer flask containing 50 mL liquid medium. After 24 h incubation, 160 rev min^{-1}, a 5% (v/v) inoculum was transferred to a 250 mL Erlenmeyer flask containing 50 mL basal salt medium containing sucrose (1%) as sole carbon source. The culture condition in incubator shaker was maintained at 30°C rotating at 160 rev min^{-1}. The flasks were removed at regular intervals for every 24 h, the contents were analyzed for biomass, intra and extra cellular β-fructofuranosidase activity up to ten days.

Enzyme Extraction

The mycelia was collected by filtration using Whatman No. 1 filter paper and washed three times using 50 mM sodium acetate buffer, pH 3.5. The wet mycelia was ground with approx. 2 times of glass beads and minimal amount of buffer. The homogenized mycelia was centrifuged at 10,000x g for 10 min and the supernatant was used as an intracellular enzyme. The culture filtrate from molasses medium was used as an extracellular enzyme.

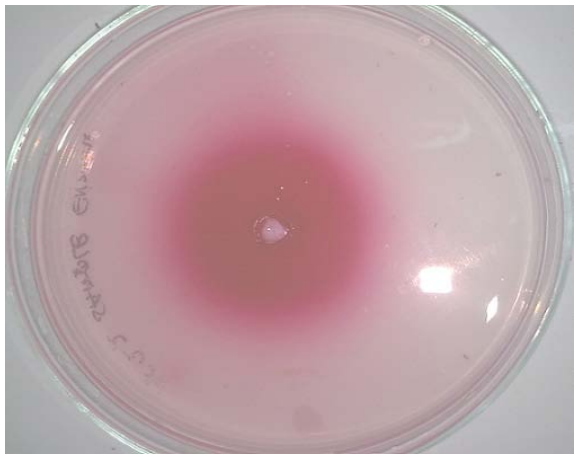

Fig. 1: Hydrolytic action of *Aspergillus niger* PSSF21 on Sucrose by Radial diffusion method

β-Fructofuranosidase Assay

Radial Diffusion Method

Wells are made aseptically by using gel puncture in the Petri plate containing basal culture media. Two hundred microliter of enzyme was loaded in to well and kept for incubation at room temperature for overnight. TTC staining was carried out by spraying the agar plate with TTC reagent (0.1% triphenyl tetrazolium chloride in 0.5 M NaOH) and kept for 20 min in the dark. After washing in the 0.1 M acetate buffer (pH-5), the extra cellular production of β-fructofuranosidase was confirmed by the appearance of the red zone around the well (Fig. 1). The reaction mixture (0.2 mL crude extract, 0.3 mL 50 mm sodium acetate buffer pH 3.5 and 0.5 mL 5% sucrose solution) was carried out at 60°C for 30 min and terminated with DNS addition followed by boiling at 100°C for 5 min (Bernfeld *et al*., 1955). The reducing sugar was subsequently analyzed by using 3, 5-dinitrosalicylic acid (Miller, 1959) One unit of β-fructofuranosidase was defined as 1 μmole of glucose or fructose liberated per minute under specified condition.

Protein Estimation

Protein concentrations were determined with the method of Lowry *et al*. (1951) using Bovine serum albumin as a standard.

Pretreatment of Lignocellulosic Materials

To remove the lignin content from the lignocellulosic materials, the pretreatment of agro residues (rice bran, wheat bran, banana waste, sugarcane bagasse, chicory and sawdust) was carried out according to the method Silverstein *et al*. (2007) except molasses. Lignocellulosic materials are finely ground with grinder for 15 min each and sieved through 20 mesh size. The ground substrates were pretreated with 2% NaOH at 121°C for 2 h followed by washing several times with distilled water until neutral pH condition attained and then dried in an oven at 500°C to obtain a constant weight. These substrates are sieved and kept in desiccator for further use.

Effect of pH and Temperature on Growth and Enzyme Production

The effect of pH and temperature on the enzyme production was studied. This was carried out by cultivating the organism at different pH values using adequate buffers (3.0-6.0

sodium acetate, 7.0-8.0 sodium phosphate and 9.0-11.0 glycine-NaOH) at 50 mM concentration and different temperatures (10-60°C). The β-fructofuranosidase activity and biomass in these studies was measured at optimum growth period.

Effect of Carbon and Nitrogen Sources on the Growth and β-Fructofuranosidase Production

The fungal strain was grown in the basal salt medium containing 1% (w/v) of different carbon sources to study their effect on the production of the enzyme. The various carbon sources tested were maltose xylose, dextrose, galactose, fructose, sucrose, lactose, starch and inulin; various concentrations of lignocellulosic materials were rice bran, wheat bran, banana waste, sugarcane bagasse, chicory, molasses and sawdust. The nitrogen sources tested were peptone, tryptone, gelatin, beef extract, casein, ammonium chloride, sodium nitrate, ammonium sulphate, ammonium nitrate and urea.

Time Course of β-Fructofuranosidase Production

The time course of β-fructofuranosidase production was carried out before and after media formulation. After inoculation, β-fructofuranosidase activity in the supernatant was followed from the first to eight day.

Determination of Temperature and pH Optima for β-Fructofuranosidase Activity

The optimum temperature was determined by assaying the enzyme activity at various temperatures (30-100°C) for 30 min in 0.2 M sodium acetate buffer at pH 3.5. The optimum pH was determined by measuring the activity at 60°C over the pH range of 3.0-9.0 using the following buffers: sodium acetate (pH 3.0-6.0), sodium phosphate (pH 7.0-8.0) and glycine-NaoH (pH 9.0 to 11.0).

Determination of pH and Thermal Stability on β-Fructofuranosidase Activity

To investigate pH stability of the crude enzyme, the enzyme preparation was incubated at 60°C at different pH values (3.5-4.5) in the absence of substrate and enzyme stabilizers. Aliquots were withdrawn at periodic intervals and residual activity in each sample was calculated by doing the assay against enzyme control sample at optimum pH and temperature as explained above. Thermal stability of β-fructofuranosidase was studied by incubating the enzyme in 50 mm sodium acetate buffer (pH 3.5) at different temperatures (60-75°C) in the absence of substrate and enzyme stabilizers at regular time intervals up to 24 h. Residual activity was determined as mentioned above.

Action of β-Fructofuranosidase from *Aspergillus niger* PSSF21 on Molasses

A 10 mL of reaction mixture containing 5 mL of 2% (w/v) Molasses, 3.0 mL of 50 mM of sodium acetate buffer pH 3.5, 2 mL of crude enzyme was incubated for 10 h at 60°C. The aliquots (0.5 mL) were used to collect hydrolysis products at regular time intervals. The 0.1 mL of each was used to determine the reducing sugar by Miller *et al.* (1959) method. The remaining sample (0.4 mL) was used to perform on mass spectrometer connected to an HPLC system fitted with column C-18 at 25°C with 90: 10 methanol: water as the mobile phase. Electro-Spray-Ionization (ESI) of the sample was carried out in the positive-ionization mode at 51.0 p.s.i. nebulizer pressure, 0.2 mL min^{-1} dry gas flow rate at 360°C dry gas temperature. The detector used was an ion-trap analyzer.

Statistical Analysis

The results were subjected to statistical Analysis of Variance (ANOVA), using a statistical analysis system (Graphad Instat). The significant differences between means were determined Duncan's Multiple Range Test (DMRT), at $p<0.05$.

RESULTS AND DISCUSSION

Fermentation and Growth Profile of *Aspergillus niger* PSSF21

Proper incubation time was very important and critical for maximal enzyme production. Figure 2 shows the rate of β-fructofuranosidase production by *Aspergillus niger* PSSF21. It is evident from the Fig. 2 maximum β-fructofuranosidase production was observed at 96 h of incubation time. Similar observations was found by Chang (1996), Katapodis and Christakopoulos (2004) in *Aspergillus japonicas* and *Thermoascus aurantiacus* at 96 h, respectively. Further increase in the incubation period did not enhance the enzyme activity due to a decrease in nutrient availability in the medium, or carbon catabolite repression. Similar literature reported by Gomez *et al.* (2000).

Effect of pH and Temperature

Maximum fructofuranosidase production was obtained when initial pH of the fermentation medium was 3.5. Further increase in pH (4.0-9.0), slowly declines the production of enzyme (Fig. 3). Where as maximum fructofuranosidase production from in *Aspergillus niger* and *Aspergillus niger* ATCC 20611 were optimally found at pH 4.5 (Rubio *et al*., 1997) and pH-5.0 (Sirisansaneeyakul *et al*., 2000) respectively. Growth of fungal cultures are strongly influenced by the temperature. It is evident from the Fig. 3 maximum growth and enzyme production was found at 30°C. Similar observation reported by Rajoka and Yasmeen (2005) in *Aspergillus niger* strain. Good amount of fructofuranosidase production occurred at 30 to 40°C suggesting it is moderate thermophilic in nature (Fig. 4). Further increase in temperature declines the production of enzyme.

Effect of Carbon Source

The influence of environmental factors on product formation in batch culture is determined by biomass concentration, the specific production rate, product yield from the substrate, the duration of synthetic activity and rate of decomposition of the product (Chen *et al*., 1996). In the present study, different carbon sources of synthetic and non-synthetic nature (Table 1) were employed to study their effect on growth and production

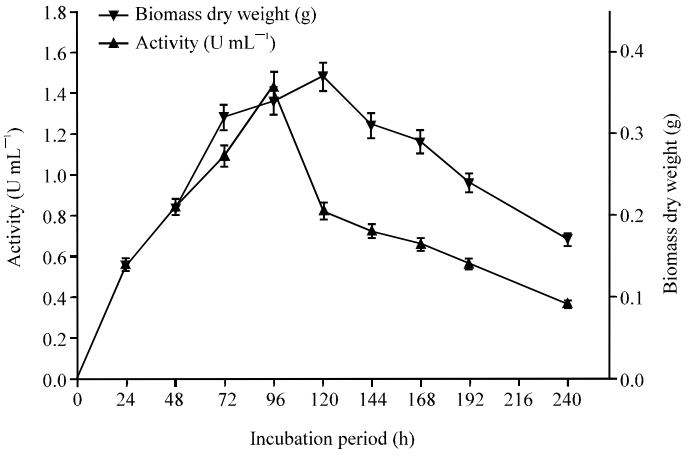

Fig. 2: Effect of incubation period on growth β-fructofuranosidase activity in *Aspergilus niger* PSSF21

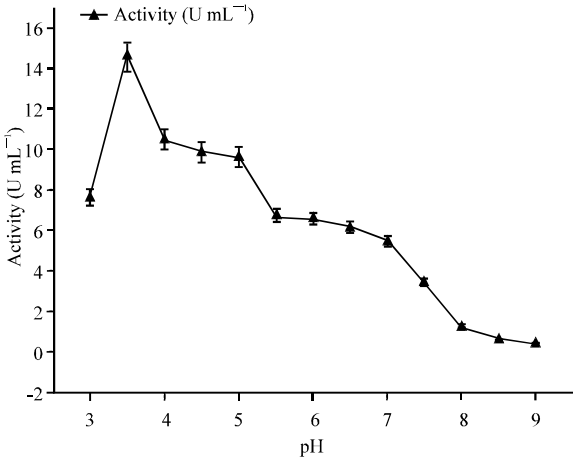

Fig. 3: Effect of pH on the production of β-fructofuranosidase in *Aspergilus niger* PSSF21

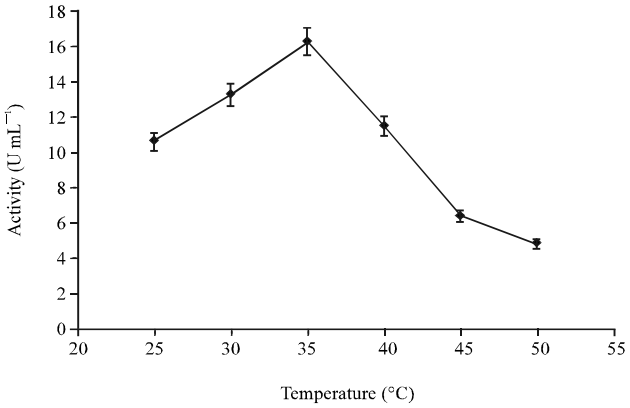

Fig. 4: Effect of temperature on the production of β-fructofuranosidase in *Aspergilus niger* PSSF21

Table 1: Effect of different carbon sources (2%) on production of β-fructofuranosidase

Carbon source (2% w/v)	β-Ffase activity (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (IU mg^{-1} protein)
Inulin	10.16±0.08	11.880±0.020	0.861±0.037
Maltose	07.30±0.09	10.890±0.060	0.627±0.008
Starch	06.50±0.08	10.840±0.020	0.582±0.015
Lactose	03.57±0.09	06.641±0.012	0.543±0.007
Dextrose	05.61±0.12	09.854±0.009	0.560±0.008
Glucose	05.63±0.07	10.274±0.020	0.543±0.006
Galactose	00.82±0.06	05.450±0.030	0.152±0.004
Sucrose	16.36±0.13	12.640±0.040	1.287±0.006
Fructose	6.99±0.13	10.850±0.050	0.652±0.007
Chicory	3.779±0.06	08.770±0.069	0.450±0.030
Wheat bran	07.56±0.19	11.460±0.006	0.655±0.040
Saw dust	04.38±0.14	9.064±0.060	0.476±0.006
Molasses	19.81±0.21	14.320±0.060	1.379±0.006
Banana leaf	17.39±0.09	13.567±0.004	1.265±0.017
Bagasse	14.51±0.44	12.130±0.104	1.221±0.037
Ricebran	05.24±0.21	09.830±0.030	0.572±0.046
Banana peel	12.30±0.14	11.910±0.040	1.102±0.061

of extracellular β-Ffase from *Aspergillus niger* PSSF21. It makes clear from Table 1 production of fase was greatest in Sugarcane molasses followed by banana leaf (2%). The maximum production of extracellular β-fructofuranosidase activity from *Aspergillus niger* PSSF21 was 19.81±0.21 and 17.39±0.090 U mL^{-1}, respectively. In contrast Rajoka and Yasmeen (2005) found wheat bran (2%) as best carbon source for the production of fructofuranosidase. Among synthetic substrates sucrose and inulin was found to be the best carbon source with higher fructofuranosidase activity 16.36±0.13 and 10.16±0.08 U mL^{-1} where as fructose (3.779±0.063) and glucose (03.57±0.09 mL^{-1}) showed lower production of enzyme. But the rate of biomass formation was lower with sucrose (0.182 g mL^{-1}) and inulin (0.169 g mL^{-1}) compare to fructose (0.328 g mL^{-1}) and glucose (0.428 g mL^{-1}). Normally, Monomeric carbon sources which feed quickly into early steps of metabolic pathways cause catabolic repression, due to formation of the CreA protein (Bohm and Boos, 2004). Present findings are similar in good agreement with the work reported by other workers (De Groot *et al*., 2003). Many findings are cited in literature that sucrose was the best carbon source for the production of β-fructofuranosidases (Hayashi *et al*., 1992). The use of commercial sucrose as a substrate is uneconomical for industrial production of Ffase, therefore several lignocellulosic materials (at 2% carbohydrate level) were included in these studies. Banana was found to be second most preferred agrowaste residue followed by molasses which enhance the enzyme activity by 2.5 times fold compare with Sucrose. Reason is found to be the formation of Cellobiose and xylobiose as a primary products of lignocellulose (LC) hydrolysis, they are believed to be strong inducers of the hydrolyses as glucose and xylose are liberated slowly (Hrmova *et al*., 1991) and may not support the formation of CreA (De Vries *et al*., 1999) resulting in inhibition of catabolite repression.

Effect of Nitrogen Sources

Application of appropriate nitrogen source is very important for optimal production of enzymes. From Table 2 it gives proof that organic nitrogen sources induces more β-fructofuranosidase production than inorganic sources. These data are in agreement with those reported in literature, where maximum β-fructofuranosidase activities are observed in different fungal strains (*Aspergillus japonicus*, *Aspergillus foetidus*) growing on organic nitrogen sources (Hayashi *et al*., 1992; Wang and Rakshit, 1999). Among organic nitrogen sources soyabean meal showed maximum β-fructofuranosidase activity (30.84±0.447 U mL^{-1}) followed by yeast extract and peptone. In contrast Bhatti *et al*. (2006) reported peptone as best nitrogen source showing maximum β-fructofuranosidase activity (9.90 U mL^{-1}) in *Fusarium solani*. Present results shows three fold higher in enzyme levels compare to previous literature mentioned above. Inorganic nitrogen sources (Table 2) significantly not supported the enzyme activity, Urea showed lowest β-FFase activity (4.72±0.183 U mL^{-1}) because higher concentrations of urea induce denaturation of cells by increasing in cell pore size there by reducing enzyme production. Similar observation was found by (Pitombo *et al*., 1994; Ahsen Baig *et al*., 2003). Ammonium nitrate showed maximum activity (22.11±0.22 U mL^{-1}) among inorganic nitrogen sources. Present findings are similar with Katapodis *et al*. (2004) were ammonium nitrate (3.2±0.8 U mL^{-1}) found as best inorganic nitrogen source.

Time Course of β-Fructofuranosidase Production

The time course of β-Fructfuranosidase production was carried out before and after media formulation. The results presented in Fig. 5 interestingly found β-Fructfuranosidase was maximally produced at the 96 h of fermentation, in both formulated and non-formulated media. The enzyme activities were 27.34±0.064 and 9.459±0.072 U mL^{-1}, respectively. There is a decrease in the biomass (data not shown) and enzyme production was observed in non formulated media as compare to formulated one.

Table 2: Effect of different nitrogen sources on production β-fructofuranosidase (using 2% Molasses)

Nitrogen source (0.5%)	β-Ffase activity (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (IU mg^{-1} protein)
Sodium nitrate	18.27±0.320	18.40±0.144	1.026±0.060
Peptone	23.00±0.480	22.05±0.720	1.113±0.020
Tryptone	22.11±0.220	15.56±0.101	1.454±0.010
Ammonium nitrate	24.43±0.360	17.67±0.158	1.371±0.010
Soya bean	30.84±0.447	18.64±0.570	1.654±0.030
Ammonium sulphate	18.64±0.185	14.73±0.110	1.260±0.009
Yeast extract	24.39±0.300	26.30±0.560	0.956±0.022
Urea	4.72±0.183	6.32±0.310	0.753±0.009

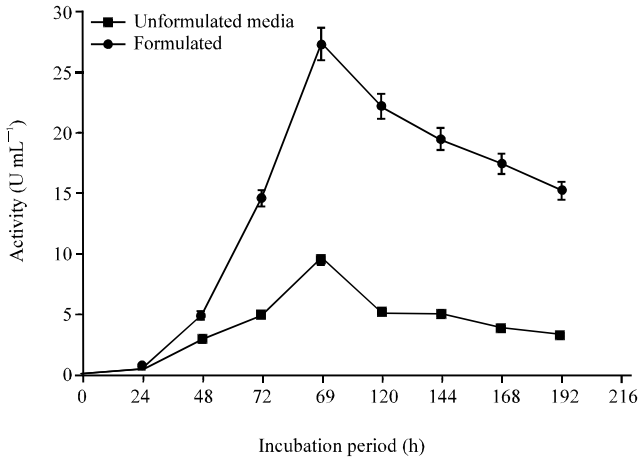

Fig. 5: Time course of β-fructofuranosidase production by *Aspergilus niger* PSSF21 in submerged fermentation un-formulated media and formulated media

Determination of pH and Temperature Optima

β-Fructofuranosidase from *Aspergillus niger* PSSF21 was optimally active at pH 3.5 (Fig. 6) where as more than 40% of maximum activity was retained from 3.5 to 6.5 pH. In contrast Rubio *et al.* (1997) reported maximum activity was found at pH-4.5 in *Aspergillus niger*. The optimum temperature for the enzyme activity was 60°C but the enzyme activity decreased only 15% at 75°C. From the Fig. 7 it makes clear the enzyme produced by *Aspergillus niger* PSSF21 was highly thermo tolerant than results reported earlier in *Aspergillus niger, Fusarium oxysporum* (Jung *et al*., 1989; Kaur *et al*., 1992) were optimally active below 60°C.

Determination of Thermal and pH Stability on Enzyme Activities

Thermal stability of β-fructofuranosidase was studied by incubating the enzyme in 50 mM sodium acetate buffer (pH 3.5) at different temperatures (60-75°C) in the absence of substrate. Samples were withdrawn at periodic intervals and cooled in an ice bath prior to assay. β-fructfuranosidase produced from *Aspergillus niger* PSSF21 showed good thermostability at 60-75°C in absence of stabilizers and maintained more than 50% of residual activity up to 20 h (Fig. 8).The observed temperature stability was higher by the results reported for β-fructofuranosidases from most microbial sources. The enzyme was showed good stability at the optimum pH range (3.5 to 4.5 pH) and more than 80% of residual activity was retained at 4 h (Fig. 9).

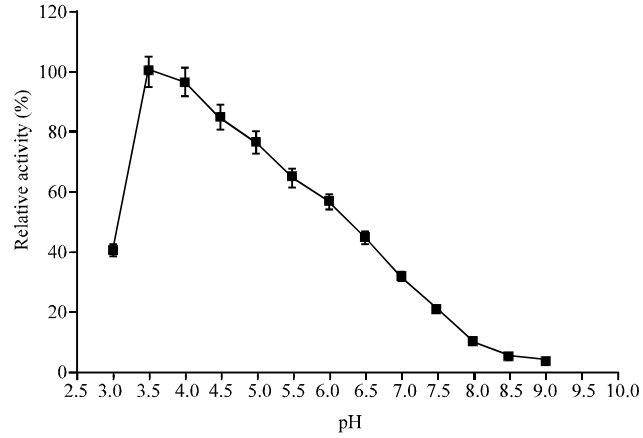

Fig. 6: Effect of pH on β-fructofuranosidase activity in *Aspergilus niger* PSSF21

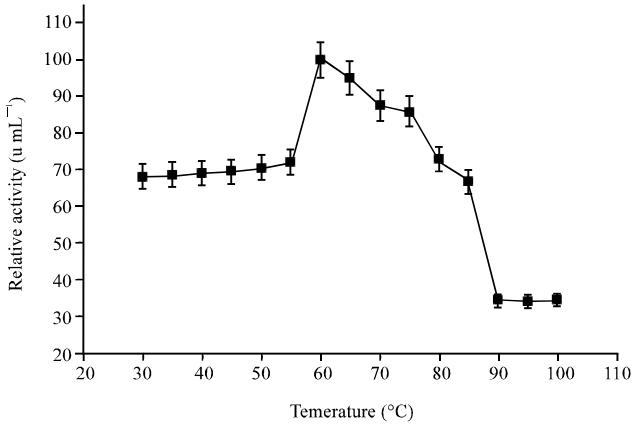

Fig. 7: Effect of temperature on β-fructofuranosidase activity in *Aspergilus niger* PSSF21

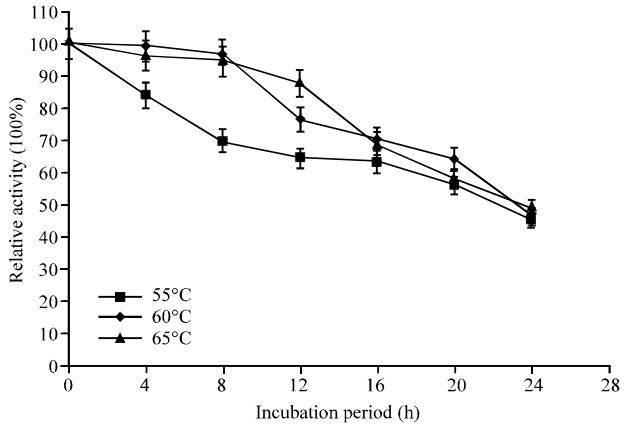

Fig. 8: Thermostability of β-fructofuranosidase at different optimal temperatures from *Aspergilus niger* PSSF21

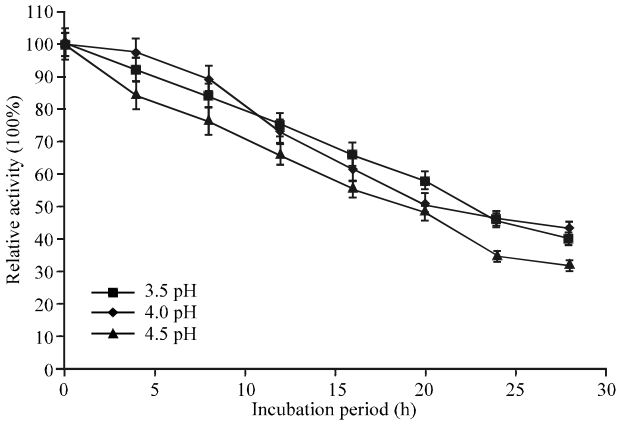

Fig. 9: pH stability β-fructofuranosidase at different optimal pH from *Aspergilus niger* PSSF21

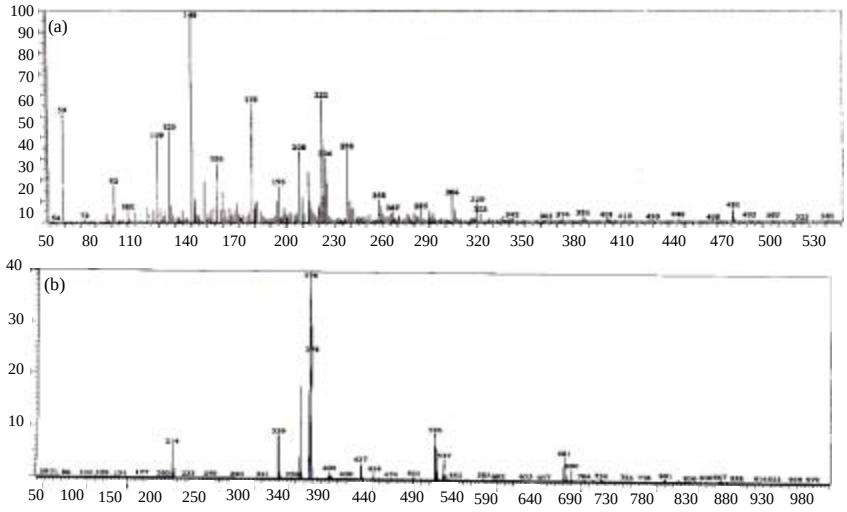

Fig. 10: (a, b) LCMS profile of the products formed by the action of β-fructofuranosidase from *Aspergilus niger* PSSF 21

Action of β-Fructofuranosidase from *Aspergillus niger* PSSF21 on Molasses

The product of the time course of hydrolysis (pH-3.5) of molasses by the crude β-fructofuranosidase was analyzed by Liquid chromatogram Mass Spectroscopy (LCMS). The LCMS procedure employed for separation of fructose oligomer enables one to characterize the mode of action of the enzyme and particularly, it is having hydrolytic activity or transfructosylation activity or both. There have been many reports on the analysis of FOS by HPLC, GC-MS, NMR, etc. (Sachio *et al*., 1991). In this study, attempts have been made to explore the application of LC-MS for the direct analysis of FOS. The LCMS results were obtained in the positive-ionization mode, using a sodium salt contained in the mobile phase to facilitate ionization. Hence, all the masses are presented as sodium adducts. At the early stages of reaction (60 min) β-fructofuranosidase hydrolyzed molasses to yield fructose and glucose as main product (Fig. 10a). The signals at m/z 178 correspond to glucose or fructose

and minor signal was observed at m/z 342 correspond to sucrose. After 4 h of incubation the major end products were Fructooligosacharides. Figure 10b shows the signals at m/z 526 correspond to 6-kestose monohydrate, signal m/z 681 corresponds to nystose. The actual masses of 6-kestose monohydrate and nystose were calculated as 522 and 666.88, respectively.

CONCLUSION

To the best of our knowledge FFase producing by *Aspergillus niger* PSSF21 showed good pH and thermo stability among all the strains reported in *Aspergillus* species and have transfructosylation activity in synthesis of fructoligosaccharides. Considering this results further work should be carried out for the production of FOS in large scale and determination of its concentration through HPLC analysis. Moreover, *Aspergillus niger* does not produce toxins; their metabolic products enjoy Generally Recognized as Safe status (GRAS) and this can be used in the food industry.

ACKNOWLEDGMENTS

One of the authors, Praveen Reddy. Patil wishes to thank the Gulbarga University, Gulbarga, India for its financial support in the form of a University-Junior Research Fellowship during this work. We are also Grateful to Dr. G. S. N Reddy, Center for Cellular and Molecular Biology, Hyderabad for his constant support and encouragement.

REFERENCES

Ahsen Baig, M., S. Kiran, M. Shazia, A. Sikander and Ikram-ul-Haq, 2003. Effect of urea as an inducer of β-fructofuranosidase in saccharomyces fermentation. Pak. J. Nutr., 2: 106-108.

Ali, S. and M. Ashiq, 2009. Enhanced production of an extracellular β-D-fructofuranosidase fructohydrolase from a 2-deoxy-D-glucose stabilized mutant of *Candida utilis*. J. Applied Biochem. Biotechnol., 159: 453-463.

Atiyeh, H. and Z. Duvnjak, 2001. Study of the production of fructose and ethanol from sucrose media by *Saccharomyces cerevisiae*. Applied Microbiol. Biotechnol., 57: 407-411.

Bernfeld, P., S.P. Colowick and N.O. Kaplan, 1955. Amylases α and β, pp. Method in Enzymol. Vol. I. Academic Press Inc., New York, pp: 149-158.

Bhatti, H.N., A. Muhammad, A. Abbas, R. Nawaz and M.A. Sheikh, 2006. Studies on kinetics and thermostability of a novel acid invertase from *Fusarium solani*. J. Agric. Food Chem., 54: 4617-4623.

Biedrzycka, E. and M. Bielecka, 2004. Prebiotic effectiveness of fructans of different degrees of polymerization. Trends Food Sci. Technol., 15: 170-175.

Bohm, A. and W. Boos, 2004. Gene regulation in prokaryotes by sub cellular relocalization of transcription factors. Curr. Opin. Microbiol., 7: 151-156.

Chang, W., 1996. Fructofuranosldase production by *Aspergillus japonicus* in shaking batch cultures as affected by initial sucrose concentration. Biotechnol. Lett., 18: 69-72.

Chen, J.S., J. Saxton, F.W. Hemming and J.F. Peberdy, 1996. Purification and characterization of high and low molecular weight forms of invertases secreted by *Aspergillus nidulans*. Biochem. Biophys. Acta, 1296: 207-218.

Cuervo, R., B. Guilarte, A. Juarez and J. Martýnez, 2004. Production of fructoligosaccharides by β-fructofuranosidase from *Aspergillus* sp. 27H. J. Chem. Technol. Biotechnol., 79: 268-272.

De Groot, M.J., P.J. van de Vondervoort, R.P. de Vries, P.A. Vankuyk, G.J. Ruijter and J. Visser, 2003. Isolation and characterization of two specific regulatory *Aspergillus niger* mutants show antagonistic regulation of arabinan and xylan metabolism. J. Microbiol., 149: 1183-1191.

De Vries, R.P., J. Visser and L.H. de Graaff, 1999. CreA modulates the XlnR-induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. Res. Microbiol., 150: 281-285.

Dolores, L., I. Macias, L. Fernández-Arrojo, F.J. Plou, A. Jiménez and M. Fernández-Lobato, 2009. Molecular and biochemical characterization of a β-fructofuranosidase from *Xanthophyllomyces*. Applied Environ. Microbiol., 75: 1065-1073.

Gibson, G.R. and M.D. Roberfroid, 1995. Dietary modulation of the colonic microbiota: Introducing the concept of prebiotics. J. Nutr., 125: 1401-1412.

Gomez, S.J.R., C. Augur and G. Viniegra-Gozalez, 2000. Invertase production by *Aspergillus niger* in submerged and solid-state fermentation. Biotechnol. Lett., 22: 1255-1258.

Guimarães, L.H.S., H.F. Terenzi, M.L.T.M. Polizeli and J.A. Jorge, 2007. Production and characterization of a thermostable extra cellular ß-d-fructofuranosidase produced by *Aspergillus ochraceus* with agroindustrial residues as carbon sources. Enzyme Microbial Technol., 42: 52-57.

Hayashi, A., K. Matsuzaki, Y. Takasaki, H. Ueno and K. Imada, 1992. Production of β-fructofuranosidase by *Aspergillus japonicus*. World J. Microbiol. Biotechnol., 8: 155-159.

Hrmova, M., E. Petrakova and P. Biely, 1991. Induction of cellulose and xylan degrading enzyme systems in *Aspergillus terreus* by homo and hetero-disaccharides composed of glucose and xylose. Gen. Microbiol., 137: 541-547.

Jung, K.H., J.W. Yun, K.R. Kang, J.Y. Lim and J.H. Lee, 1989. Mathematical model for enzymatic production of fructo-oligosaccharides from sucrose. Enzyme Microbial Technol., 11: 491-494.

Katapodis, P. and P. Christakopoulos, 2004. Induction and partial characterization of intracellular â-fructofuranosidase from *Thermoascus aurantiacus* and its application in the synthesis of 6-kestose. World J. Microbiol. Biotechnol., 20: 667-672.

Katapodis, P., E. Kalogeris, D. Kekos, B. J. Macris and P. Christakopoulos, 2004. Biosynthesis of fructo-oligosaccharides by *Sporotrichum thermophile* during submerged batch cultivation in high sucrose media. Applied Microbiol. Biotechnol., 63: 378-382.

Kaur, N., M. Kaur, A.K. Gupta and R. Singh, 1992. Properties of β-fructosidase (Invertase and Inulinase) of *Fusarium oxysporum* grown on an aqueous extract of *Cichorium intybus* roots. J. Chem. Technol. Biotechnol., 53: 279-284.

Lowry, D.H., N.J. Rosenbrough, A.L. Far and R.J. Randal, 1951. Protein measurement with folin phenol reagent. J. Biol. Chem., 193: 265-275.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Mussatto, S.I. and I.M. Mancilha, 2007. Non-digestible oligosaccharides: A review. Carbohydrate Polymers, 68: 587-597.

Pitombo, R.N.M., C. Spring, R.F. Passos, M. Tonato and M. Vitole, 1994. Effect of moisture content on invertase activity of freeze-dried *Saccharomyces cerevisiae*. Cytobiology, 31: 383-392.

Rajoka, M.I. and A. Yasmeen, 2005. Improved productivity of â-fructofuranosidase by a derepressed mutant of *Aspergillus niger* from conventional and non-conventional substrates. World J. Microbiol. Biotechnol., 21: 471-478.

Rubio, M.C., A.C. Maldonado and A.R. Navarro, 1997. Effect of culture conditions on invertase production in a strain of *Aspergillus niger*. M.A.N. Microbiol. Aliments Nutr., 15: 17-22.

Sachio, H., J. Kinoshita, M. Nonoguchi, Y. Takasaki and K. Imada, 1991. Continuous production of 1-kestose by β-fructofuranosidase immobilized on shirasu porous glass. Biotechnol. Lett., 13: 395-398.

Sangeetha, P.T., M.N. Ramesh and S.G. Prapulla, 2005. Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trends J. Food Sci. Technol., 16: 442-457.

Silverstein, R.A., Y. Chen, R.R. Sharma-Shivappa, M.D. Boyette and J. Osborne, 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour. Technol., 98: 3000-3011.

Sirisansaneeyakul, S., S. Jitbanjongkit, N. Prasomsart and P. Luangpituksa, 2000. Production of β-fructofuranosldase from *Aspergillus niger* ATCC20611 kasestart. J. Nat. Sci., 34: 378-386.

Tokunaga, T., 2004. Novel physiological function of fructooligosaccharides. Biofactors, 21: 89-94.

Wang, X.D. and S.K. Rakshit, 1999. Improved extracellular transferase enzyme production by *Aspergillus foetidus* for synthesis of oligosaccharides. Bioprocess Biosystems Eng., 20: 429-434.

Yun, J.W., 1996. Fructooligosaccharides-occurrence, preparation and application. Enzyme Microbial Technol., 19: 107-117.