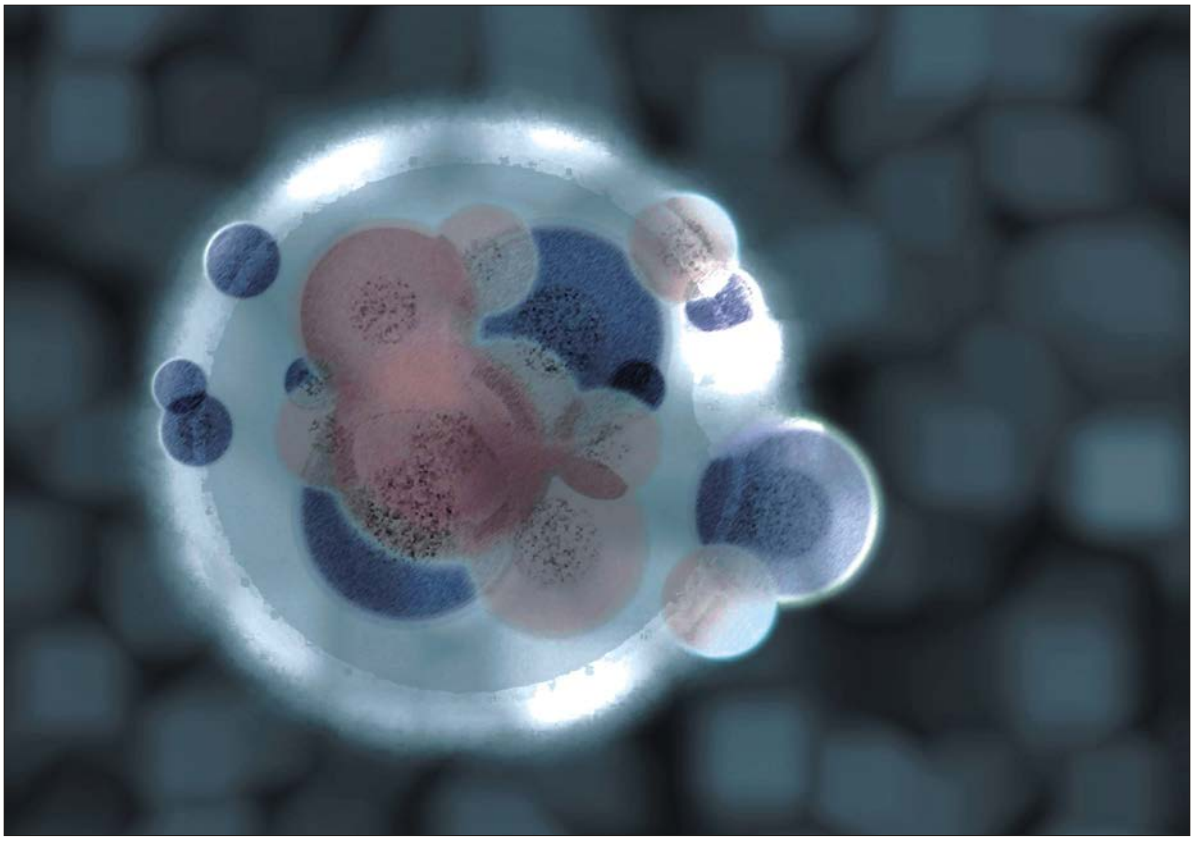

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Screening of β-Fructofuranosidase Producers with High Transfructosylation Activity and its 3^2 Experimental Run Studies on Reaction Rate of Enzyme

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Abstract: β-fructofuranosidases are a group of hydrolytic and transfructosylating enzymes that play an important role in food processing industry to synthesize Fructo Oligosaccharides (FOS). The present study aimed to screening of filamentous fungi strains and growing them in batch cultures to compare their abilities in production of β-Fructofuranosidase (β-FFase) using sole carbon source as sucrose. Radial diffusion method was used to evaluate the potent β-fructofuranosidase producers by using crude enzyme. Among screened strains, *Aspergillus niger* PSSF21 (2.20±0.100 cm), *Chrysonilia sitophila* PSSF84 (2.00±0.026 cm) and *Aspergillus flavus* PSSF22 (1.64±0.040 cm) showed significant hydrolysis zone in the plate. In addition, the β fructofuranosidase of the above strains also showed high fructosyltransferase activity to hydrolytic ratio (*Aspergillus niger* PSSF21-6.335 U mg^{-1}, *Chrysonilia sitophila* PSSF84 3.487 U mg^{-1}, *Aspergillus flavus* PSSF22-3.256 U mg^{-1}). The effects of pH and temperature on the sucrose- β-FFase reaction rate were studied using a 3^2 experimental design matrix. Acidic pH influences positively on the fructosyl transferase activity of three isolated strains, where as effect of temperature showed remarkable variations on high fructosyltransferase activity-to-hydrolytic activity ratio.

Key words: Fructosyltransferase, radial diffusion method, sucrose, At/Ah, hydrolytic activity

INTRODUCTION

From the last decades functional foods have been described as physiologically active food components, which provide benefits beyond basic nutrition and may prevent disease or promote health. A functional ingredient received much attention in the scientific literature is Fructooligosaccharides (FOS). The FOS is easily understood as inulin-type oligosaccharides of D-fructose, attached by β-(2-1) linkages that carry a D-glucosyl residue at the end of the chain. FOS constitutes a series of homologous oligosaccharides derived from sucrose usually represented in the form of GFn. The GFn units of interest are used as food ingredients (Spiegel *et al*., 1994) in various nutritional foods. In addition it has important physiological properties, as they are low sweetness, calorie free, act as bifidogenic agent, non cariogenic, reduce cholesterol, phospholipids and triglyceride levels in blood serum, respectively (Yun, 1996; Tokunaga *et al*., 1986). From the literature survey, FOS producing enzymes are classified as β-Fructofuranosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26; β-FFase) which catalyzes the hydrolysis of sucrose as well as the transfructosylation from sucrose (Kim *et al*., 1998). Hydrolytic action of β-Fructofuranosidase results in production of invert sugar which is completely healthy sweetener for the preparation of confectionery products like Jams, candies etc. (Klein *et al*., 1989; Vandamme and Derycke, 1983) and transfructosylation action results in synthesis of FOS which has several applications as mentioned above. Fructosyltransferase (EC 2.4.1.9) is another enzyme possessing only transfructosylating activity which is useful for the production of fructooligosaccharides (Chien *et al*., 2001). Several research papers reported on FOS production using β-FTases by different strains at high sucrose concentrations (*Pencillium citrinium* FERMP 15994 (Hayashi *et al*., 2000); *Aspergillus japonicus* TIT90076 (Hayashi *et al*., 1992); *Pencillum citrinum* KCTC 18080P (Lim *et al*., 2006). To the best of our knowledge only few reports available on screening of β-Ffase possessing FTase activity (Oskar *et al*., 2005; Dominguez *et al*., 2006; Jun *et al*., 2008). As we all familiar too screening of new strains is complicated and tedious work due to large number of evaluations. Considering above points in this study we reported radial diffusion which is cheap and economically viable technique (mentioned below), to screen potent producers of β-fructofuranosidase. The manuscript also describes the transfructosylation property of β-fructofuranosidase produced by different strains and its effect of physical parameters on the reaction rate of enzyme.

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MATERIALS AND METHODS

Materials: Triphenyl Tetrazolium Chloride (TTC), 3,5 Dinitro salicylic acid and Sucrose. All other chemicals used were of analytical grade.

Culture media: The basal culture medium for β-fructofuranosidase production was composed of NH_4SO_4-0.5 g, KH_2PO_4-3 g, $NaNO_3$-1.5 g, $MgSO_4.7H_2O$-0.02 g, Sucrose-2 g and agar -20 g L^{-1}, pH was adjusted to 7.

Isolation and preliminary screening: Different soil samples were collected from agricultural fields, garden soil and compost soils in and around Northern parts of Karnataka (India) in the year 2008. To isolate β-fructofuranosidase producing fungal strains soil samples were serially diluted and spread on basal culture medium mentioned above and incubated at 30°C for 96 h. Petri Plates were constantly observed for the appearance of colonies with greater diameter and cultures were purified by repeated transfer of cultures and maintained on nutrient agar.

Secondary screening of β-fructofuranosidase producing microbes: The isolated organisms from the preliminary screening were selected and fermented in basal culture media at pH 5.0 excluding agar in 250 mL Erlenmeyer flasks. After incubation on a rotary shaker (30°C, 160 rpm) for 7 days, the culture broth was centrifuged (10000 rpm for 10 min) and the supernatant was collected for enzyme assay.

β-fructofuranosidase assay: Radial diffusion method: It was performed by using TTC reagent (0.1% Triphenyl tetrazolium chloride in 0.5 M NaOH) which easily soluble in water. In the presence of reducing sugars TTC reduce in to triphenyformazon which is a red, water insoluble compound. The quantity of formazan formed is directly proportional to the quantity of reducing sugars present. Wells are made aseptically by using cock borer in the Petri plate containing basal culture media (mentioned above). Two hundred microliter of crude enzyme was loaded in to well and kept for incubation at room temperature for overnight. Staining was carried out by spraying TTC reagent to the agar plate and kept incubation 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.

Quantitative assay: Hydrolytic and Transfructosylating activities: A 2 mL reaction mixture containing 0.6 mL of 50 mM sodium acetate buffer pH 5.0, 1.0 mL of 5% (w/v) sucrose solution and pre-incubated at 30°C for 10 min. A 0.4 mL of appropriately diluted enzyme was added and the incubation continued for further 30 min at 60°C. The reaction was terminated by inactivating the enzyme keeping in boiling water bath at 100°C for 10 min. After cooling, equal volumes of the reaction mixture was used for determining the reducing sugar and glucose by DNS method (Miller, 1959) and Glucose oxidase-Peroxidase (Wood and Bhat 1988), respectively. The colour developed in both cases was read at 540 nm and converted into reducing sugar and glucose using glucose standard curve prepared under identical conditions. The hydrolytic activity and transfructosylation activity was determined by Nguyen *et al.* (1999) method.

Determination of protein concentration: Proteins were measured by the Lowry *et al.* (1951) method using Bovine Serum Albumin (BSA) as the standard protein.

Biomass concentration: Cell mass concentration was determined by dry cell weight per volume (g L^{-1}). The cell mass obtained by filtration of the fermentation broth was washed with distilled water and dried at 120°C for 4 h.

Statistical analysis: All the experiments were repeated at least three times and the results were reproducible. The data points represented the mean values within ±1% of the individual values.

Identification of fungal strains: Analysis of morphology a very small sample of a colony was removed from the agar plate, mixed with a drop of water and smeared on a glass slide (Wei, 1979). Then observations were made with a microscope. For further conformation the strains were stored in PDA (potato dextrose agar) slants at 4°C send to Agarkhar research institute, Pune (India).

Temperature and pH effects on the reaction rate: The temperature and pH effects on the sucrose-fructofuranosidase reaction rate were studied using a 3^2 experimental design. The proposed experimental design matrix is presented in Table 3. All the experiments were performed by preparing the reaction mixture as mentioned above. The enzyme reaction was carried out for 30 min at pH and temperature to be tested. The reaction rate results of fructose transference (At) and of hydrolysis (Ah) were analyzed mathematically and statistically using Instat Graphpad Software.

RESULTS AND DISCUSSION

Thirty strains that can grow on basal culture medium containing sucrose as sole carbon source were isolated in

Table 1: Measurements of zone of inhibition produced by β-fructofuranosidase producers by Radial diffusion method

Strain	Zone of inhibition (cm)
PSSF21*	2.20±0.100
PSSF14[a]	1.50±0.090
PSSF16[a]	1.38±0.020
PSSF08[a]	1.84±0.058
PSSF22*	1.59±0.081
PSSF84*	2.00±0.026
PSSF13[a]	1.32±0.036
PSSF18[a]	1.64±0.040
PSSF15[a]	0.76±0.040
PSSF06[a]	1.57±0.030
PSSF07[a]	1.38±0.025
PSSF03[a]	1.33±0.032
PSSF09[a]	1.04±0.030
PSSF02[a]	0.98±0.041
PSSF01[a]	0.52±0.0153
PSSF17[a]	0.32±0.025

*Identified strains: PSSF21, *Aspergillus niger*; PSSF22, *Aspergillus flavus*; PSSF84, *Chrysonilia sitophila*. [a]Non-identified strains

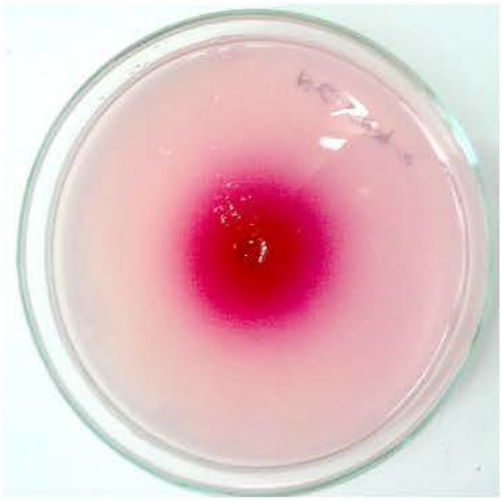

Fig. 1: Hydrolysis of sucrose by *Aspergillus niger* PSSF21 by radial diffusion method

a pure state. By considering large number of evaluations (growth, size etc.) 16 strains are subjected for secondary screening as mentioned above. Radial diffusion method was found significant to determine the hydrolytic activity of crude enzyme. Activities of β-fructofuranosidase collected from secondary screening were examined to calculate the zone diameter. As shown in Table 1. Zone of hydrolysis produced by strains PSSF21, PSSF84, PSSF08 was found significant comparing with the other isolates producing β-fructofuranosidase (Fig. 1). Based upon morphology and Biochemical characteristics the strains were identified as PSSF21-*Aspergillus niger*, PSSF84- *Chrysonilia sitophila* and PSSF22-*Aspergillus flavus*. Among screened ones PSSF01, PSSF15 and PSSF17 were least producers of β-fructofuranosidase.

In order to determine the relationship between transfructosylation (At) and hydrolytic activity (Ah) the mycelium (ICE) and mycelia broth (ECE) were assayed. The average values of the biomass, enzyme activities of both intracellular (ICE), extra cellular (ECE) and specific activity (SA) were presented in Table 2. The strains PSSF09, PSSF03 PSSF14 reached biomass concentration higher than 7 g L^{-1}. Where as the result with the strains PSSF16, PSSF13, PSSF18, PSSF06 PSSF07, *C.sitophila* PSSF84, *A. flavus* PSSF22 and *A. niger* PSSF21 showed lower biomass concentration (<7 g L^{-1}). Correlation between the biomass concentration and enzyme activity was not found. These results could be caused by the use of different microorganisms that synthesize β-fructofuranosidase with different specific properties and activities, our reports are well in agreement with Hidemasa *et al.* (1988). In contrast, Fernandez *et al.* (2007) observed no correlation between cell growth and final pH in shaken flasks.

On the other hand nine strains reached the highest values of extra cellular (ECE) At values which are mentioned in Table 2. Among them *A. niger* PSSF21 (21.61±0.055 U mL^{-1}), *C. sitophila* PSSF84 (19.50±0.355 U mL^{-1}), PSSF06 (19.43±0.217 U mL^{-1}) and *A. flavus*PSSF22 (17.47±0.469 U mL^{-1}) were possessing good transfructosylation activity . But it is known that At/Ah values are the most important parameters to be considered when the objective is screening of strains to produce β-fructofuranosidase with transfructosylation activity (Hidemasa *et al*., 1988). It is evident from Table 2 significant differences can be observed between the At/Ah ratio values of extra cellular (ECE), intracellular enzyme activities (ECE) and specific activity (S.A). Present observations found similar with previous cited literature (Fernandez *et al*., 2007). Among twelve screened strains, three of them were selected which shows higher At/Ah values as shown in Table 2. The extra cellular At/Ah ratio of these strains are followed in the order *A. niger* PSSF21 (11.940 U mL^{-1}) >*C. sitophila* PSSF84 (8.024 U mL^{-1}) >*A. flavus*PSSF22 (5.041 U mL^{-1}) were intracellular At/Ah ratio values are in the order *C. sitophila* PSSF84 (4.042 U mL^{-1}) >*A. flavus* PSSF22 (3.616 U mL^{-1}) >*A. niger* PSSF21 (3.038 U mL^{-1}). In contrast PSSF08 strain showed relatively poor At/Ah value (0.657 U mL^{-1}) and high hydrolytic activity (12.84±0.4801 U mL^{-1}) compare to other strains previously listed (Table 2). Lower sucrose concentration might be unfavorable climate to dominate transferase activity in PSSF08 which results in poor At/Ah ratio.

A 3^2 experimental design was used, to study the effect of pH and temperature on reaction rate of enzyme for three better strains. These strains are selected due

Table 2: Biomass, extra cellular, intracellular fructosyltransferase (A_t) and hydrolytic activities (A_h) reached in shake flask experiments

		ECE (U mL^{-1})			ICE (U mL^{-1})			ECE-S.A (U mg^{-1})			ICE-S.A (U mg^{-1})		
Strain	Biomass	At	Ah	At/Ah	At	Ah	At/Ah	At	Ah	At/Ah	At	Ah	At/Ah
PSSF21*	6.41±0.036	21.61±0.055	1.81±0.070	11.940	14.830±0.446	4.88±0.070	3.038	9.395±0.032	1.483±0.047	6.335	3.830±0.026	3.480±0.048	1.100
PSSF14^{a}	7.10±0.075	15.40±0.091	12.45±0.387	1.2510	5.900±0.051	3.31±0.064	1.782	3.130±0.065	2.139±0.063	1.464	5.900±0.036	5.516±0.063	0.297
PSSF16^{a}	6.45±0.034	16.87±0.072	10.64±0.480	1.5850	6.300±0.076	2.78±0.092	2.266	3.923±0.085	2.121±0.047	1.849	6.300±0.027	3.432±0.084	0.369
PSSF08^{a}	6.17±0.085	08.44±0.061	12.84±0.4801	0.6570	3.400±0.020	4.89±0.052	0.696	1.411±0.046	2.136±0.028	0.660	3.400±0.096	6.791±0.096	0.132
PSSF22*	5.86±0.113	17.47±0.469	3.47±0.041	5.0410	8.570±0.045	2.37±0.055	3.616	4.852±0.035	1.470±0.082	3.256	8.570±0.120	3.160±0.024	0.651
PSSF84*	6.18±0.122	19.50±0.355	2.43±0.05	8.0240	11.400±0.151	2.82±0.036	4.042	6.724±0.031	1.928±0.027	3.487	11.400±0.031	4.208±0.036	0.697
PSSF13^{a}	4.48±0.147	16.56±0.151	9.68±0.268	1.7100	5.596±0.385	2.56±0.049	2.185	3.600±0.028	1.898±0.016	1.896	5.596±0.041	2.813±0.041	0.379
PSSF18^{a}	5.86±0.097	15.34±0.206	5.19±0.154	2.9530	7.300±0.075	2.13±0.050	3.420	2.950±0.036	1.221±0.057	2.416	7.300±0.023	3.435±0.025	0.483
PSSF06^{a}	6.75±0.097	19.43±0.217	7.43±0.050	2.6150	6.340±0.040	2.056±0.08	3.083	5.887±0.034	2.835±0.072	2.620	6.300±0.036	2.562±0.052	0.524
PSSF07^{a}	5.92±0.096	14.58±0.270	8.21±0.016	1.7740	10.870±0.072	7.86±0.062	1.380	2.589±0.028	1.130±0.036	2.291	10.870±0.041	5.614±0.104	0.458
PSSF03^{a}	8.09±0.020	09.52±0.096	12.07±0.0757	0.7890	3.836±0.055	4.60±0.036	0.830	1.602±0.074	1.360±0.089	1.178	3.830±0.026	4.181±0.084	0.235
PSSF09^{a}	9.61±0.083	07.18±0.026	4.74±0.093	1.5150	2.83±0.0667	3.86±0.026	0.733	1.204±0.052	0.788±0.025	1.528	2.836±0.063	3.299±0.069	0.305

*Identified strains: PSSF21, *Aspergillus niger*, PSSF22, *Aspergillus flavus*, PSSF84, *Chrysonilia sitophila*. ^{a}Non- Identified strains isolated from soil Extra cellular Enzyme = ECE, Intra cellular enzyme = ICE, At = Transfructosylation activity, Ah = Hydrolytic activity, S.A = Specific activity

Table 3: Effect of physical parameters used to study the β-fructofuranosidase reaction rate by 3^2 experimental run

	Variables	
Experimental run	pH	Temp (°C)
1	5	40
2	6	40
3	8	40
4	5	50
5	6	50
6	8	50
7	5	60
8	6	60
9	8	60

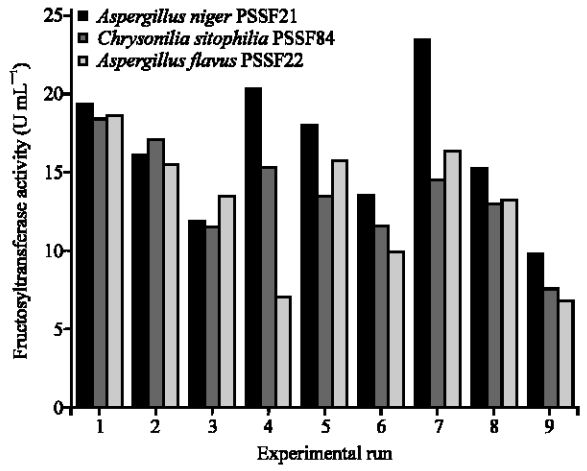

Fig. 2: Effects of temperature and pH on fructosyltransferase activity from *A. niger* PSSF21, *C. sitophila* PSSF84 and *A. flavus* PSSF22

to their At/Ah values. Experiments were performed under nine conditions using the extra cellular β-fructofuranosidase as described in Table 3. It is evident from Fig. 2. *Aspergillus niger* PSSF21 achieved higher values of At (23.5±0.75 U mL^{-1}) under experimental conditions of run 7 which indicates good sign of FOS synthesis. Present results are in well agreement with Madlová *et al.* (1999). Where as the reaction conditions of PSSF84 and PSSF22 strains exhibited higher values of At (18.46±0.537 U mL^{-1}, 18.32±0.285 U mL^{-1}) under conditions of run 1, respectively.

For these microorganisms, the repeated ANOVA was performed (data not shown). The analysis of the obtained results shows that decrease in pH values influences positively on the At value for three strains (*A. niger* PSSSF21, *C. sitophila* PSSF84, *A. flavus* PSSF21), where as temperature effect shown remarkable variation. *C. sitophila* PSS84 and *A. flavus* PSSF22 strains showed lower contribution to the At values as the temperature increases and showed positive contribution to Ah values. Concerning the strain *A. niger* PSSF21 increase in the temperature showed higher At values (mentioned above), besides variation in the pH from 5 to 8 led to decrease in the Ah values and it results remarkable increase in the At/Ah ratio (18.3±0.057 U mL^{-1}) comparing to other two strains (PSSF84 and PSSF22). In contrast Fernandez *et al.* (2007) reported *A. oryzae* IPT-301 showed meaningful increase in At/Ah ratio when the variation of pH from 5.5 to 8.0.

CONCLUSION

In view of great demand for FOS production, microbial β-fructofuranosaidase having transfructosylation property has attracted special attention. Researchers showing great interest to survey on new β-fructofuranosidase having unique properties which have ability to synthesis FOS. In this paper, three fungi strains *Aspergillus niger* PSSF21, *Chrysonilia sitophila* PSSF84 and *Aspergillus flavus* PSSF22 have greater potential for FOS production. Considering the results, inspite of non optimization of culture conditions, these microorganisms showed higher values of At/Ah ratio compared to the earlier strains reported. Further studies will be carried out on these strains to meet the demands for FOS production.

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