

Original Article

Optimized Production and Characterization of β -fructofuranosidase from *Penicillium brevicompactum*

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The present study is aimed at the optimization of β -fructofuranosidase from *Penicillium brevicompactum* using orange fruit peel waste as a substrate in Czapek Dox as basal medium by submerged fermentation (SmF). The optimum pH, temperature and incubation period for enzyme production were 5, 30° C and 4th day respectively. Of the carbon source, sucrose was recorded to be the best carbon source for enzyme production yeast extract was ideal nitrogen source for the β -fructofuranosidase production. The enzyme was purified to 5.8 fold with recovery of 3.2% by DEAE-column chromatography and the molecular weight was estimated to be 64 KDa by SDS-PAGE. It has a V_{max} value of 15.8 U/mg and K_m value of 0.23 mg/ml at pH 6. The enzyme activity was found to be stable at 50° C for 30 minutes and it was stimulated by metal ions like Na⁺ and Ca²⁺ and inhibited by Zinc. These results highlight the industrial potentials of the substrates as possible raw materials for β -fructofuranosidase enzyme production from *Penicillium brevicompactum*.

Keywords: β -fructofuranosidase, *Penicillium brevicompactum*, Fruit peel waste, Optimization.

Introduction

β -fructofuranosidase is a special kind of enzyme which catalysis the hydrolysis of terminal non-reducing β -fructofuranoside residue in sucrose. The enzyme has wide range of commercial applications including the production of confectionery with liquid or soft centers, chocolates and fermentation of cane molasses into ethanol. β -fructofuranosidase is also used in pharmaceutical industry as digestive aid tablets, powder milk for infants' foods, as calf feed preparation, assimilation of alcohol in fortified wines and in manufacture of inverted sugars as food for honeybees¹.

β -fructofuranosidase is used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup (HFS). It is one of the most widely used enzymes in food industry where fructose is preferred than sucrose especially in the preparation of jams and candies, because it is sweeter and does not crystallize easily². The enzymatic activity of β -fructofuranosidase has been characterized mainly in plants and microorganisms. Among microorganisms, *Saccharomyces cerevisiae*³, *Candida utilis*⁴, *Aspergillus niger*⁵, *Thermomyces lanuginosus*⁶ and *Penicillium chrisogenum*⁷ has been widely studied.

β -fructofuranosidase exhibits marked stability towards temperature, pH changes and denaturants. Temperature of the reaction mixture determines the rate of sucrose inversion by the active enzyme⁸. The present study is aimed at the optimization of culture conditions for the enhanced production of β -

fructofuranosidase from *Penicillium brevicompactum* using fruit peel waste as cheap substrate has been carried out which has good potential for biotechnological applications.

Materials and methods

Organism and inoculum preparation

Fungal strains were isolated from the soil of sugarcane field Coimbatore, India by dilution plate method. The culture was screened for β -fructofuranosidase enzyme production. The fungal strain *Penicillium brevicompactum* was selected for the production of β -fructofuranosidase and it was prepared from 4 days old slant culture.

Fermentation condition

The medium used for the enzyme production under submerged fermentation was comprised of (g/l): sucrose 20, yeast extract 10, ammonium sulphate 1.0, magnesium sulphate 0.75, potassium dihydrogen phosphate 3.5, and pH 5.0. Cultivation was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. Instead of sucrose, orange peel waste was substituted in the present study. After inoculation (10⁶ spores/ml), the flasks were incubated at 30° C for seven days in an incubator shaker at 125 rpm. At the end of fermentation, the supernatant was harvested by centrifugation at 10,000 rpm for 10 min (4° C) and was used as crude enzyme extract. The substrate (orange peel) was fed with varying sizes of inoculum of the organism. The

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inoculum size was varied from 1.0 to 5.0% and the enzyme activity was measured every 24 hours.

Enzyme assay

β -fructofuranosidase activity was determined using the method of Sumner and Howells⁹ with slight modification by incubating 0.1 ml of enzyme solution with 0.9 ml of sucrose in 0.03 M acetate buffer (pH 5.0). To stop the reaction, 1 ml of dinitrosalicylic acid reagent was added and heated for 5 min in a boiling water bath. Finally the absorbance was read at 540 nm in spectrophotometer¹⁰. One unit of β -fructofuranosidase (IU) is defined as the amount of enzyme which liberates 1 μ moles of glucose/minute/ml under the assay condition.

The optimization of the culture condition and the effects of various factors like inoculum size, carbon sources, nitrogen sources, pH and temperature on the production of β -fructofuranosidase were studied.

Purification and characterization of β -fructofuranosidase

Crude extract (100 ml) was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 100mM Tris phosphate buffer (pH 7.5) for 24 hours at 4^o C. The filtrate was loaded onto a DEAE-cellulose chromatographic column (25 cm * 2.6 cm) equilibrated with Tris-HCl buffer, 100mM, and pH 7.5. The enzyme was eluted with a linear salt concentration gradient (NaCl, 0-0.4 M) in the same buffer and 3.0 ml fractions were collected at a flow rate of 20 ml per hour.

SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by the method of Lowry¹¹. The kinetic parameter of the purified β -fructofuranosidase enzyme was determined and the optimum pH and temperature on the activity of the enzyme were also assayed. All experiments were conducted with triplicates and their mean values are represented.

Results

The optimization of fermentation medium was done by measuring the enzyme activity by varying the single parameter such as pH, temperature, inoculum size, carbon, nitrogen and substrate concentration of the medium while keeping the remaining parameter unaltered. The highest level of extracellular β -fructofuranosidase was obtained in Czapek Dox medium using fruit peel waste under orbital agitation (125 rpm) at 30^oC for seven days.

The selected substrates (Orange, Pineapple, and Pomegranate) were analyzed for carbon and nitrogen content in CHNS analyzer and it was found that C: N ratio was higher in the case of orange peel when compared to other two substrates pomegranate and pineapple (Fig. 1). Different concentrations (1-5%) of the selected

substrates were studied and 4% of the substrates produced better results of enzyme production (Fig. 2). Hence further experiments were carried out with orange peel as the substrate.

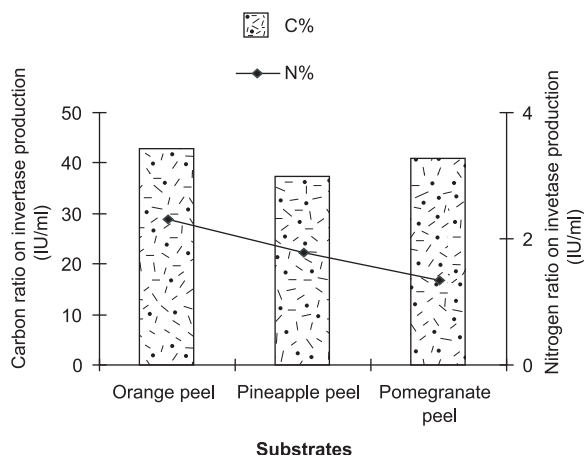


Figure 1. Carbon and Nitrogen content of the fruit peel waste as substrate using CHNS analyzer.

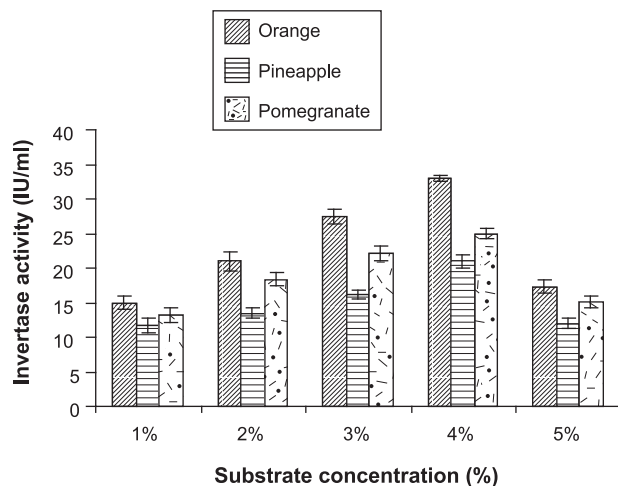


Figure 2. Effect of different concentration (1-5%) of fruit peel waste as substrate on β -fructofuranosidase production by *P. brevicompactum*. Results are mean of three independent determinations. Bars correspond to standard deviation.

The enzyme activity was measured at regular intervals of 24 hrs at pH 3-8 and with varying temperature 20-60^oC. The maximum enzyme production occurred at 3% of inoculum size was 25.0 IU/ml at pH 5 and kept at 30^oC when orange peel was used as substrate (Table 1).

The effect of different carbon (fructose, glucose, lactose, raffinose and sucrose) and nitrogen sources (peptone, urea and yeast) were tested by incorporating 1% of the above set sources into fermentation medium. Production was more pronounced by the addition of sucrose and yeast extract as the nutritional factors (Table 1).

Table 1. Effects of various factors on α -fructofuranosidase production from *P. brevicompactum*

Factors	β -fructofuranosidase production (IU/ml) Incubation days (hours)					
	24	48	72	96	120	144
pH						
3	4.8	8.5	11.1	12.5	10.3	7.6
4	6.2	9.1	13.4	16.8	12.9	9.2
5	10.5	14.6	19.2	23.8	19.7	14.3
6	5.6	7.2	12.6	19.5	15.2	10.1
7	3.2	5.7	7.2	10.4	8.3	5.8
8	2.9	4.2	6.0	7.9	5.2	3.2
Temperature (°C)						
20	10.2	14.6	16.2	18.6	17.0	12.2
30	15.2	23.3	26.8	28.8	20.6	14.0
40	10.9	17.6	19.9	21.6	17.3	11.1
50	9.9	14.9	16.1	17.8	15.0	13.3
60	11.9	12.6	14.0	15.3	12.3	7.3
Inoculum size (%)						
1	5.8	9.5	10.5	15.0	13.4	7.5
2	8.9	11.7	15.6	20.0	12.5	8.4
3	9.6	12.9	18.2	25.0	17.6	11.2
4	7.4	9.1	12.3	18.4	11.8	5.4
5	4.9	7.0	10.8	14.1	9.2	4.2
Carbon Source (g/100mL)						
Fructose	14.3	16.2	19.3	23.9	19.8	13.3
Glucose	9.6	11.5	15.5	18.7	14.2	7.9
Lactose	11.3	14.2	16.3	20.3	11.3	6.7
Rafinose	6.1	9.5	11.4	14.6	7.4	5.3
Sucrose	9.3	11.2	20.1	25.6	20.3	10.6
Nitrogen Source (g/100mL)						
Nutrient broth	10.6	14.6	16.6	19.0	17.7	11.6
Peptone	14.6	21.3	23.9	25.0	23.1	19.6
Urea	19.9	26.6	30.3	33.0	30.4	25.3
Yeast Extract	18.6	26.6	29.9	33.7	31.2	24.3

Standard conditions used: pH 5.0, temperature 30°C, age of inoculum 96 hrs, size of inoculum 3%, 10^6 spores (v/v), agitation rate 125 rpm, sucrose 3% (w/v) and yeast extract.

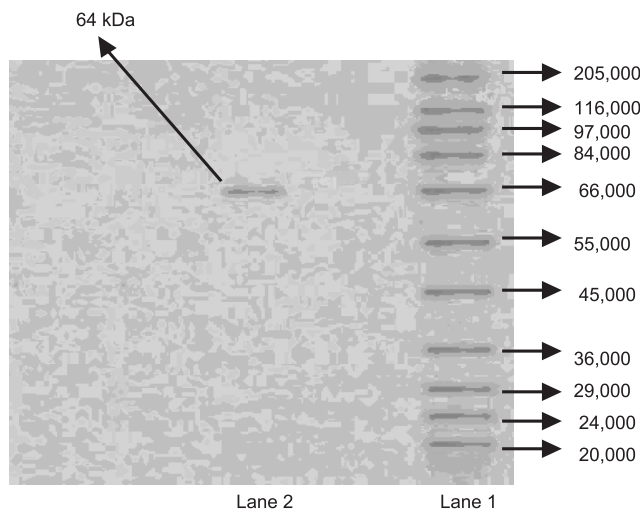
Purification

The purification of α -fructofuranosidase from *Penicillium brevicompactum* has been shown in Table 2. The specific activity of the final purified preparation was 211.0 U representing a total purification factor of 7.85.

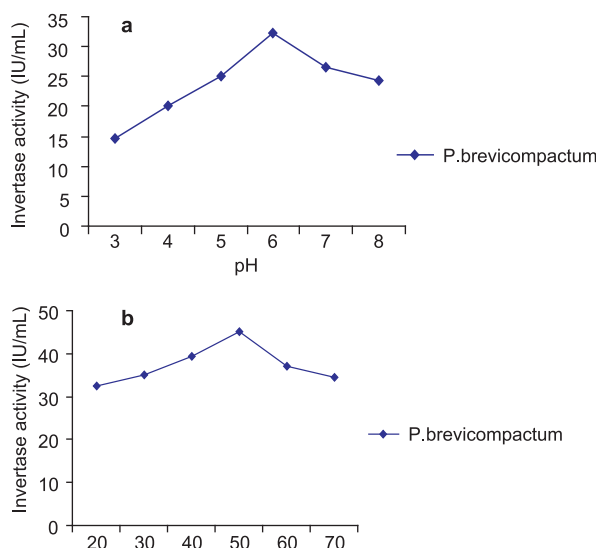
The elution profiles from DEAE Sephadex A-50 chromatographic column, from which a homogeneous enzyme was eluted with a linear gradient of (0-0.4M) NaCl, showed a single peak with a symmetrical distribution of activity. The SDS-PAGE of the enzyme revealed a single protein band, whose estimated molecular weight was 64 KDa (Fig. 3).

Table 2. Purification and recovery of β -fructofuranosidase from *P. brevicompactum*

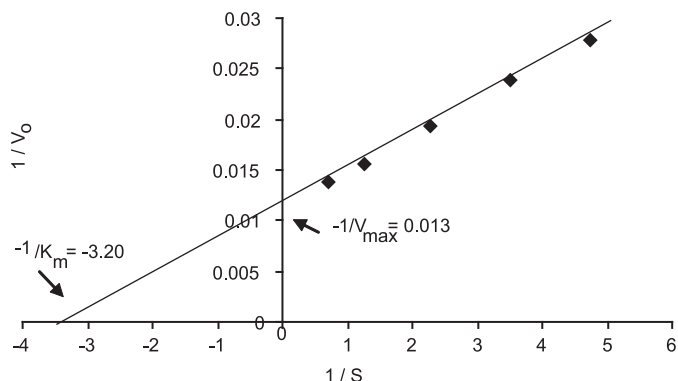
Steps	β -fructofuranosidase Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	9970	369.45	27	1	100
70% Ammonium sulphate precipitation	7012.50	157.58	44.50	1.65	70.3
Dialysis	511.2	9.80	52.1	1.93	5.12
DEAE Cellulose Column Chromatography	424.25	2.01	211.0	7.85	4.25

**Figure 3.** SDS-Polyacrylamide gel electrophoresis of purified α -fructofuranosidase from *P. brevicompactum*. Lane 1: Molecular weight marker; Lane 2: Molecular weight of α -fructofuranosidase

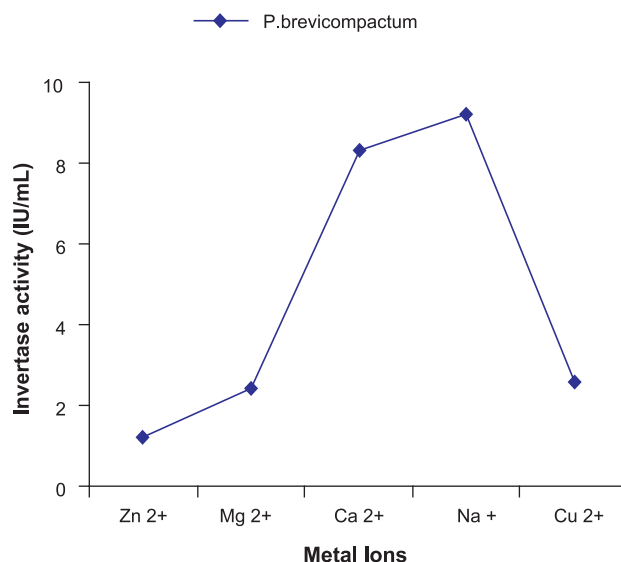
The activity of the enzyme α -fructofuranosidase from *Penicillium brevicompactum* was stable exhibited a relative broad pH 5-7 with an optimum pH of 6.0 (Fig. 4a) and it was found to exhibit maximum activity at 50 °C with a slight change of stability at 70°C (Fig. 4b).

**Figure 4a and 4b.** Stability and optimum activities of the enzyme α -fructofuranosidase at different pH and temperature from *P. brevicompactum*.

The kinetic parameters for purified extracellular β -fructofuranosidase activity were determined using sucrose, in the concentration range of 0.2 – 1.0 mM. The values of K_m and V_{max} were calculated by Lineweaver Burk plot (Fig. 5).

**Figure 5.** Enzyme kinetics of *P. brevicompactum*. A Lineweaver-Burk plot of initial velocity against sucrose concentration. The values are means of three experiments done in duplicate.

It can be seen from the Fig. 6, that the metal ions Na^+ and Ca^{2+} supported the maximum enzyme activity whereas Zn^{2+} was found to be inhibitor of the enzyme β -fructofuranosidase.

**Figure 6.** The effect of metal ions (0.05M) on activity of β -fructofuranosidase by *P. brevicompactum*.

Discussion

Increasing the potential of β -fructofuranosidase application prompt screening for newer β -fructofuranosidase producing microorganisms, hence the present production and characterization of β -fructofuranosidase by *P. brevicompactum* was studied in shake flask culture technique by inoculating 10^6 spores/ml of fermentation medium containing the fruit peel waste as substrate.

Optimization of medium composition is one of the essential steps to maintain a balance between the various medium components to minimize the amount of unutilized components at the end of fermentation and have cost-effective metabolite yield^{12, 13}. Medium components such as nitrogen and carbon sources, physiological factors such as pH, temperature, incubation time and biological factors such as genetic nature of the organism influences the metabolic and chemical behavior of the microbial strain and subsequent metabolite production pattern^{12, 13}.

Quantity of inoculum had a definite effect on β -fructofuranosidase titers. Increase in quantity of the inoculum increased β -fructofuranosidase titers 25% of inoculum gave the highest titre in 72 hours at pH 5¹³. The maximum enzyme production occurred at 4% of inoculum size was 25.8 IU/ml at pH 5 at 30°C at 96 hours of incubation. Similar report was observed by Guimaraes¹⁴ who reported the highest level of enzyme production at 40°C at 96 hours, where as Shafiq¹⁵ reported an optimum temperature of 25°C for β -fructofuranosidase production by *Saccharomyces cerevisiae* GCB-K5. A high level of β -fructofuranosidase was produced by *Penicillium brevicompactum* when sucrose was used as a carbon source and yeast extract was used as the nitrogen source. Maximum enzyme activity was found at sucrose concentration of 30.0 g/L by *Saccharomyces species* GCA-II¹⁶. Different organic nitrogen sources and their concentrations have a major effect on the ability of yeast to synthesize β -fructofuranosidase¹⁷.

Our result was in consonance with the work of Guimaraes¹⁴ who purified the enzyme to 7.1 fold with a recovery of 24%, by two chromatographic steps in DEAE-cellulose and sephacryl s-200, in *Aspergillus ochraceus*.

The relative activity of the enzyme was retained between pH 5 and 7 whereas Rubio¹⁸ reported 4.5 as optimum for *Rhodotorula glutinis* while its half-life was 30 minutes when assayed between 20-70°C. The stability was higher than that exhibited by the α -fructofuranosidase from *Azotobacter chroococcum*, whose half-life at 60°C was 3 minutes¹⁴. Stability decreased to 50% when temperature increased to 70°C.

A Lineweaver-Burk plot of the enzyme affinity for sucrose gave a straight line plot from which the K_m as 0.32 mg/ml and V_{max} was

74.64 U/mg. The values were similar to that obtained with the β -fructofuranosidase from *Rhodotorula glutinis*¹⁸.

The activity of the enzyme β -fructofuranosidase was accelerated by the addition of metal ions like Na^+ and Ca^{2+} . Similar observation was seen from *Rhodotorula glutinis*, which was activated by Na^+ and Mg^{2+} ¹⁴. This result suggests that the metal ions protect the enzyme against thermal denaturation at high temperatures¹⁴.

β -fructofuranosidase production by *Penicillium brevicompactum* under optimized cultural condition where studied and the enzyme was purified to 7.85 fold. The behavior of β -fructofuranosidase activity at different temperature, pH, substrate concentration were analyzed and it showed good stability at pH 6.0 and temperature 50°C. Moreover agro wastes are used as substrate for enzyme production which substantially lowers the cost of production qualifying it for application in sucrose hydrolysis and fructose syrup production.

From the present study, we could see that parameters like pH, temperature, substrate concentration, carbon and nitrogen source had different effect in the enzyme production. It can be concluded that the orange peel waste can be more effectively used as a substrate for the production of enzyme under optimized culture conditions. The yield of the enzyme was greatly enhanced when the fungi was grown in shaken flask condition supplemented with sucrose and yeast extract.

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