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Optimization and characterization of invertase by *Aspergillus fumigatus* using fruit peel waste as substrate

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ABSTRACT

The filamentous fungi *A. fumigatus* produced high levels of invertase when cultured for 96 hr at 30^0C and pH 5 in Czapek Dox supplemented with fruit peel waste as substrate. Enhanced production occurred on addition of sucrose and yeast extract as nutritional factors. The crude extract was purified to 4.73 fold with recovery of 4.91% by DEAE-column chromatography and the molecular weight was estimated to be 67 KDa by SDS-PAGE. The enzyme activity was stimulated by Na^+ and Ca^{2+} and inhibited by Zinc. The enzyme hydrolyzed sucrose exhibiting V_{max} value of 27.53 U/mg and K_m value of 0.28 mg/ml. The activity of the invertase was found to be stable at 50^0C for 30 minutes at pH 6.

Keywords: Invertase, *A. fumigatus*, Fruit peel waste, Optimization.

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INTRODUCTION

Invertase 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 easily [1]. The enzymatic activity of invertase has been characterized mainly in plants and microorganisms. Among microorganisms, *Saccharomyces cerevisae*[2], *Candida utilis* [3], *Aspergillus niger* [4], *Thermomyces lanuginosus* [5] and *Penicillium chrisogenum* [6)]has been widely studied.

Invertase exhibits marked stability towards temperature, pH changes and denaturants. Temperature of the reaction mixture determines the rate of sucrose inversion by the active enzyme [7].

The present study trend is the utilization of waste material for production of byproducts which boosts up high economic returns in many industries. The aim of the study is optimization of culture condition for the enhanced production of invertase from *A. fumigatus* using fruit peel waste as substrate has been carried out which has good potential for biotechnological applications.

MATERIALS AND METHODS

Organism and inoculum preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by dilution plate method. Culture was screened for invertase enzyme production and fungal strain *A. fumigatus* selected for the production of invertase was prepared from 4 days old slant culture.

Fermentation condition

The medium used for enzyme production under submerged fermentation comprised of (gm/L): sucrose 20, yeast extract 10, ammonium sulphate 1.0, magnesium sulphate 0.75, potassium dihydrogen phosphate 3.5, pH 5.0. Cultivation was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. After inoculation (10^6 spores/ml), the flasks were incubated at 30 °C for seven days in a incubator shaker at 125rpm. At the end of fermentation, the supernatant was harvested by centrifugation at 10,000 rpm for 10 min (4 ^{o}C) and was used as crude enzyme extract. The sucrose in the media was substituted with fruit peel waste as substrate. The mycelial mass was collected by filtration and its dry weight was determined.

Processing of the substrate

The fruit peel waste (Orange, Pineapple and Pomegranate) were obtained from the fruit market Coimbatore, washed and then sliced. The sliced pieces were spread on the trays and then sieved which was used as substrate and was stored in the polyethylene bags at room temperature. They were autoclaved at 15 lbs for 20 minutes before use.

Enzyme assay

Invertase activity was determined using the method of Sumner and Howells [8] 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 spectrophotometer [9]. One unit of invertase (IU) is defined as the amount of enzyme which liberates 1µ moles of glucose/minute/ml under the assay condition.

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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 invertase were studied.

Purification and characterization of invertase

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 100mM Tris phosphate buffer (pH 7.5) for 24 hours at 4^0 C. the filtrate was loaded onto a DEAE-cellulose chromatographic column (25 cm * 2.6 cm) equilibrated with Tris-Hcl buffer, 100mM, 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 Lowry *et al*., [10]. The kinetic parameter of the purified invertase enzyme was determined and the optimum pH and temperature on the activity of the enzyme was also assayed. All experiments were conducted with triplicates and their mean values represented.

RESULTS AND DISCUSSION

Production of invertase by fungi in shaken flask culture

Invertase production by *A. fumigatus* was studied in shaken flask culture technique by inoculating 10^6 spores/ml of fermentation medium containing the fruit peel waste as substrate. The C: N ratio in CHNS analyzer was estimated (Fig. 1) which shows the carbon content in orange and pomegranate was similar and comparatively more than pineapple peel whereas in the case of nitrogen, orange peel showed high value than other two substrates.

To determine the optimum incubation period for invertase enzyme production, fermentation flasks were incubated for different time duration. (1 – 7 days). Enzyme activity was analyzed at every 24 hrs time intervals. Maximal titers of enzyme were reached between 72 and 96 hrs with the fungal tested (Table 1) after which the rate declined; this might be on the basis of consumption of nutrients. Similar trend was noticed [11] for *penecillium chrysogenum* in SmF. The optimum production of invertase by *Saccharomyces cerevisiae* was found to be 48 hours [12].

Inoculum level for optimum production of invertase by *A. fumigatus* was worked out (Table 1). The maximum enzyme production occurred at 3% of inoculum size was 25.8 IU/ml when orange peel was used as substrate whereas it was less when pineapple and pomegranate was used as substrate. Quantity of inoculum had a definite effect on invertase titers. Increase in quantity of the inoculum increased invertase titers 25% of inoculum gave the highest titre in 72 hours [11].

The behaviour of enzyme invertase from fungal strain using peel waste as substrate was examined under different conditions of temperature {20-60 ^{0}C} and at varying pH {3-8} The enzyme was found to be active when reaction mixture was kept at 30^0 C (Table 1) but at high temperature the enzyme activity was not significant, because of high temperature denaturation of enzyme active site [13]. Peak enzyme production was observed at pH 5 for all the selected substrates but enzyme production varied (Table 1). Maximum production was observed for pomegranate peel when compared with other two substrates followed by marked decline in enzyme activity on increasing the pH. This shows that enzyme is not stable towards alkaline conditions so the sucrose inversion efficiency is also affected in direct way [14].

Different carbon sources such as glucose, fructose, sucrose, lactose and raffinose at 1% concentration were selected for the invertase production. For all the carbon sources tested, sucrose gave the best result (Table 1). The results was supported by the findings of Cairns *et al*., [15] who reported that invertase production in some

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other fungi was induced by sucrose. Glucose and fructose are not involved in the induction of the synthesis of β - D- fructofuranosidase in *A. niger* [16].

Table - 1: Effect of various factors on the optimization of invertase production in shaken flask by *A. fumigatus* Standard conditions used: age of inoculum, 96 hrs; size of inoculum: 3%, 10^6 spores (v/v); agitation rate 125 rpm, sucrose 3% (w/v), pH 5.0; and temperature 30^0 C.

Conditions	Extra cellular invertase production (IU/ml)				
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
Size of inoculum (3%)	8.8	16.4	19.6	25.8	17.3
Carbon source (Sucrose)	11.5	16.7	20.3	23.8	15.0
Nitrogen source (Yeast)	8.2	12.3	16.4	20.0	14.2
Optimum pH (5)	9.2	15.7	19.3	25.0	20.1
Optimum temperature(30^0C)	14.6	25.9	33.5	42.1	36.5

The effect of different nitrogen sources were tested by incorporating 1% nitrogen sources like nutrient broth, peptone, urea and yeast extract into the fermentation medium. Production was more pronounced by the addition of yeast extract. Different organic nitrogen sources and their concentrations have a major effect on the ability of yeast to synthesize fructofuranosidase [17]. Our result differs from the observation of Shafiq *et al*., [18] who reported that among all the nitrogen sources peptone gave maximum production of invertase activity using *saccharomyces cerevisae* under the temperature of 30^0 C and pH 6.0 and agitation rate 200 rpm.

Purification

The purification of invertase from *A. fumigatus* is showed in Table 2. The specific activity of the final purified preparation was 139.2 U/mg protein, representing a total purification factor of 4.73. Our result was in consonance with the work of Guimaraes et al., [19] 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*.

Table. 2: Purification and recovery of invertase from *A. fumigatus*

Steps	Invertase Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	7650	260.0	29.42	1	100
70% Ammonium sulphate precipitation	5370	142.0	37.8	1.28	70.1
Dialysis	484	7.4	65.7	2.23	6.32
DEAE Cellulose column chromatography	376	2.7	139.2	4.73	4.91

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The elution profiles from DEAE Sephdex 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 (Fig. 2). The SDS-PAGE of the enzyme revealed a single protein band, whose estimated molecular weight was 67 KDa (Fig. 3).

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

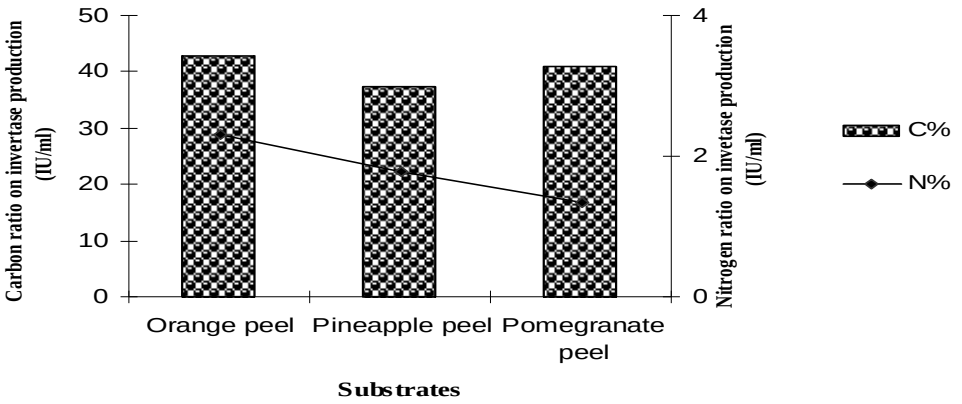

Figure - 2: Shows that elution profile of partially purified fractions on DEAE-Cellulose column chromatography. Elution was carried out with a linear gradient from zero to 0.4M NaCl with Tris HCl (pH 6.0)

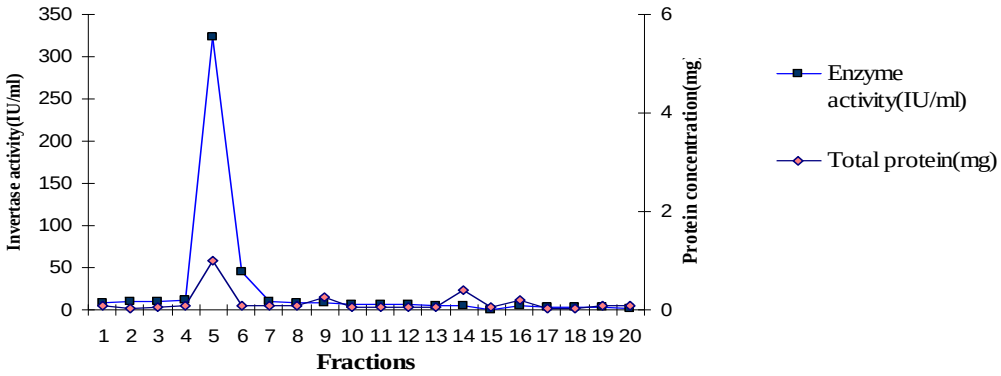

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Figure -3: SDS-Polyacrylamide gel electrophoresis of purified invertase from *A. fumigatus*. Lane 1: Molecular weight marker; Lane 2: Molecular weight of invertase.

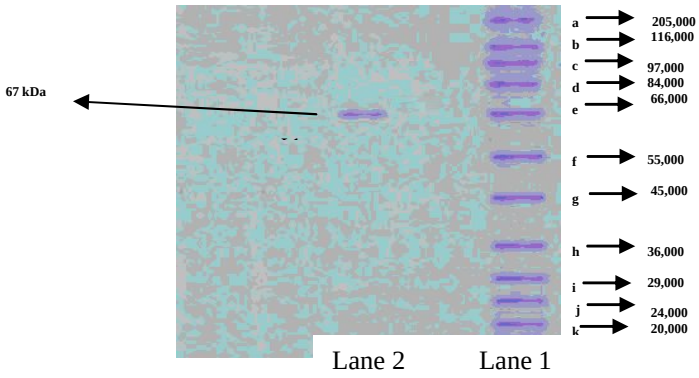

Figure - 4: The effect of optimum pH (6.0) on the activity of the enzyme invertase from *A. fumigatus.*

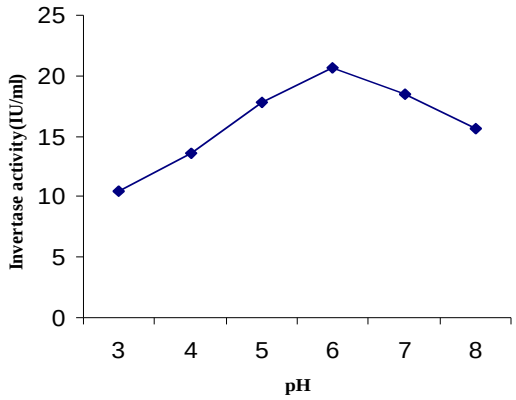

Figure - 5: The effect of temperature and stability (50 ^{0}C) on the activity of the enzyme invertase from *A. fumigatus*.

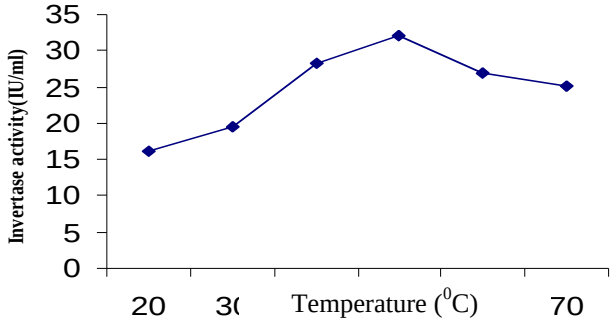

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Figure - 6: A Lineweaver-Burk plot of initial velocity against sucrose concentration. The values are means of three experiments done in duplicate

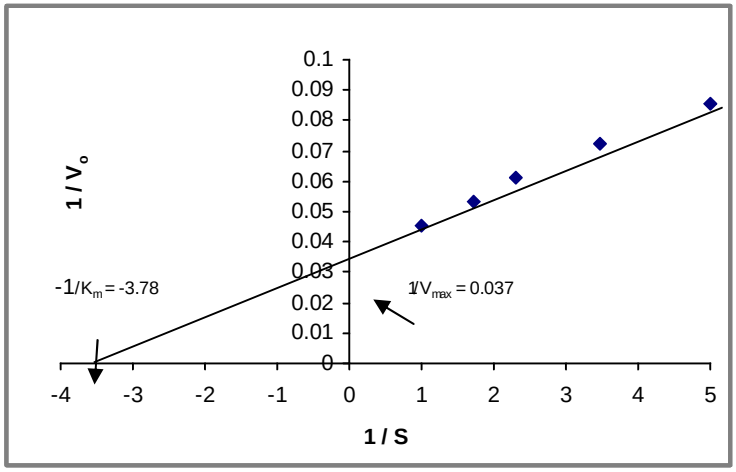


Figure -7: Eadie-Hofstee plot of initial velocity against sucrose concentration. The values are means of three experiments done in duplicate.

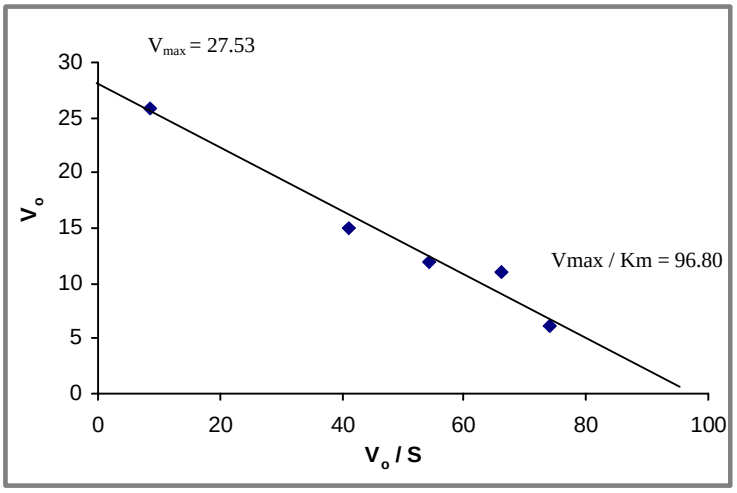
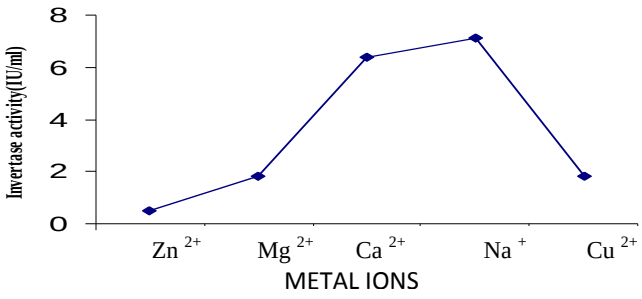


Figure – 8: The effect of metal ions (0.05M) on activity of invertase by *A. fumigatus*



The enzyme exhibited a relative broad pH 5-7 with an optimum pH of 6.0 (Fig. 4). The relative activity was retained between pH 5 and 7 whereas Rubio *et al.,* [20] reported 4.5 as optimum for *Rhodortorula glutinis* The activity of the enzyme invertase from *A. fumigatus* was stable at 50 ^{0}C (Fig. 5) while its half-life was 30 minutes when assayed between 20- 70 ^{0}C. The stability was higher than that exhibited by the invertase from *Azotobacter chroococcum*, whose half-life at 60 ^{0}C was 3 minutes [21]. Stability decreased to 50% when temperature increased to 70 ^{0}C. Results suggested that in these conditions bacterial contamination decreased.

The kinetic parameters for purified extracellular invertase activity were determined using sucrose, in the concentration range of 0.2 – 1.0 mM. The values of Km and Vmax were calculated by Linewaver Burk plot and E-H plot (Fig.6 & 7).

A Lineweaver-Burk plot of the enzyme affinity for sucrose gave a straight line plot from which the Km as 0.28 mg/ml and Vmax was 27.53 U/mg. The values were similar to that obtained with the invertase from *Rhodortorula glutinis* [20].

It can be seen from the Fig. 8, that the metal ions Na^+ and Ca^{2+} supported the maximum enzyme activity whereas Zn^{2+} was found to be inhibitor of the enzyme invertase. Similar observation was seen from *Rhodortorula glutinis*, which was activated by Na^+ and Mg^{2+} [19]. This result suggests that the metal ions protect the enzyme against thermal denaturation at high temperatures.

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. The yield of the enzyme was greatly enhanced when the fungi was grown in shaken flask condition supplemented with sucrose. The enhancement was explained as being due to the gradual liberation of the sugar from the ester by the action of a slowly acting esterase.

Invertase production by *A. fumigatus* under optimized cultural condition where studied and the enzyme was purified to 4.73 fold. The behaviour of invertase activity at different temperature, pH, substrate concentration were analysed and it showed good stability at pH 6.0 and temperature 50 ^{0}C moreover agrowaste are used as substrate for enzyme production which substantially lower the cost of production qualifying it for application in sucrose hydrolysis and fructose syrup production.

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