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Purification and Characterization of Invertase from *Aspergillus terreus*

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Abstract

Invertase was produced from *Aspergillus terreus* under optimized culture conditions at six days of incubation with pH 7.0 and 25°C, in Czapek Dox media by solid state fermentation (SSF). The enzyme was partially purified by dialysis followed by DEAE-column chromatography. Purification fold and enzyme yield, while stabled at 20-40°C with pH 3.0-5.0. The activation energy for substrate conversion was 1.87Kcal/mol. Thin layer chromatography (TLC) shown that glucose and fructose were the products of sucrose hydrolysis. The partial purified enzyme was immobilized with different metals, while Fe^{+3} gave highest activity with residual activity 76.52%. Storage activity for immobilized enzyme at 4°C after 2 and 4 weeks were 70.94 % and 58.42% respectively.

Key words: Invertase, Purification, Immobilization

1-Introduction

Invertase (EC 3.2.1.26, -D fructofuranosidase) is used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup. 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 (Aranda et al., 2006). Invertase occurs widely in nature like bacteria and fungi such as *Saccharomyces cerevisae* (Herwig et al., 2001), *Candida utilis* (Belcarz et al., 2002), *Aspergillus niger* (Romero et al., 2000.), *Thermomyces lanuginosus* (Romeroz and Maheswari, 1996) and *Penicillium chrisogenum* (Nuero and Reyes, 2002). Invertase has been immobilized with number of supports using physical and chemical methods (Salleh ,1982; Simionescu et al., 1987; Erginer et al., 2000; Nakane et al., 2000; Ahmad et al., 2001 and Amaya et al., 2006). Commercial invertase shows a high degree of hydrolytically activity, besides the fact that its biotechnological application requires an additional stage of immobilization, on an appropriate support. In this work, we will study the purification and characterization of invertase from *Aspergillus terreus* fungus.

2- Materials and Methods

2-1-Enzyme Production under Solid State Fermentation (SSF) (Fermentation Condition)

The media used for enzyme production from *A. terreus* under sold stat fermentation using crushed corn cobs as support comprised of (gm/L): sucrose

30, sodium nitrate 2, magnesium sulphate 0.5, dipotassium hydrogen phosphate 1, potassium chloride 0.5, ferrous sulphate hydrated 0.01. After inoculation ($10^{\wedge 6}$ × 1 pores/gm), the flasks were incubated at 25°C for six days. At the end of fermentation, enzyme extracted by addition 60 ml phosphate buffer, centrifugation at 10,000 rpm for 10 min (4°C) and used as crude enzyme extract.

2-2-Protein Estimation with Lowry's Method

The method of reactions was copper ions with peptide bonds under alkaline conditions to oxidation aromatic protein residues. Lowry's method is best used with protein concentrations of 0.01–1.0 mg/ml. The reactions of Cu^{+} were produced by oxidation of peptide bonds with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin-Ciocalteu Reaction). The reaction mechanism is not well understood, but involves reduction of Folin reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). The concentration of reduced Folin reagent is measured by absorbance at 600 nm. As a result, the total concentration of protein in samples can be calculated from the concentration of Trp. and Tyr. (Lowry et al., 1951).

2-3-Enzyme Assay

Invertase activity was determined as Sumner and Howells (1935). with slight modification by incubating 0.1 ml of enzyme solution with 0.9 ml of sucrose in 0.1 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 (Miller, 1959). One unit of invertase (IU) is defined as the amount of enzyme which liberates 1 moles of glucose/minute/ml under assay conditions.

2-4- Purification and Characterization of Invertase

Crude extract was dialyzed against 0.01M phosphate buffer (pH 6.5) for 24 hours at 4°C. The filtrate was loaded on DEAE-cellulose chromatographic column (8 cm * 2.2 cm) equilibrated with phosphate buffer 0.01M, pH 6.5. The enzyme was eluted with a concentration gradient NaCl (0.01, 0.05, 0.1, 0.15, 0.2, 0.3, 0.5,1 M) in the same buffer, 5.0 ml fractions were collected at a flow rate 60 ml / hour.

2-5-Characterization of Partially Purified Invertase

2-5-1- Optimum pH for Enzyme Stability

Optimum pH was determined by assaying the enzyme activity at different pH ranging from 2 to 8: incubation 1 ml of the partially purified enzyme in 30 °C for 30 mints with 2 ml of 0.2 M buffer then cooling in freeze water. The buffers

was (HCL –KCL) pH 2, Citrate buffer pH (3-5), Phosphate buffer pH (6-8).

2-5-2- Optimum pH for Enzyme Activity

Prepared 0.2 M substrate in different pH ranged (1.5-8) and assaying the enzyme activity.

2-5-3- Optimum Temperature for enzyme stability

Incubation partially purified enzyme in different temperature range 20 -70°C for 30 min then cooling in freeze water, then measuring activation energy for partially purified enzyme as in (Segal,1976).

2-5-4-Optimum Temperature for Enzyme Activity

The optimum temperature was studied by incubation the enzyme at different temperatures 20-70°C, then measuring activation energy for substrate conversion as (Segal, 1976).

2-6- Thin Layer Chromatography (TLC)

Thin layer chromatography was done using TLC plate as(Jejan, 2001).

2-7- Immobilization Enzyme with Transition Metals

Immobilization enzyme as Kennedy and Carbarl (1987) using transition metals following (concentration of 0.65 M dissolved in an acid solution of hydrochloric): ZnCl2, FeCl3, FeCl2, CoCl2 and $CrCL_3$.

3-Results and Discussion

3-1-Invertase Production by Fungi in solid state fermentation

Invertase was produced from *Aspergillus terreus* in solid state culture, the crude enzyme activity was 2.63 U/ml and protein was 0.09 mg/ml.

3-2- Purification of Enzyme

The enzyme was partially purified by two steps: dialysis followed by ion exchange column (DEAE-Cellulose) (Fig. 1). The results of purification are summarized in (Table I). Invertase was purified 8.21-fold on the basis of sucrose-hydrolyzing activity with a yield of 76.04%. The specific activity toward sucrose was 240 U/mg.

(Guimaraes et al., 2007) reported purified invertase 7.1 fold with recovery of 24% by two chromatographic steps DEAE-cellulose and sephacryl S-200 from *Aspergillus ochraceus* , Aafia et al. (2013) showed that an extracellular invertase was purified with ammonium sulfate precipitation and DEAE-Sephadex A-50 by 15 fold with recovery of 38%.

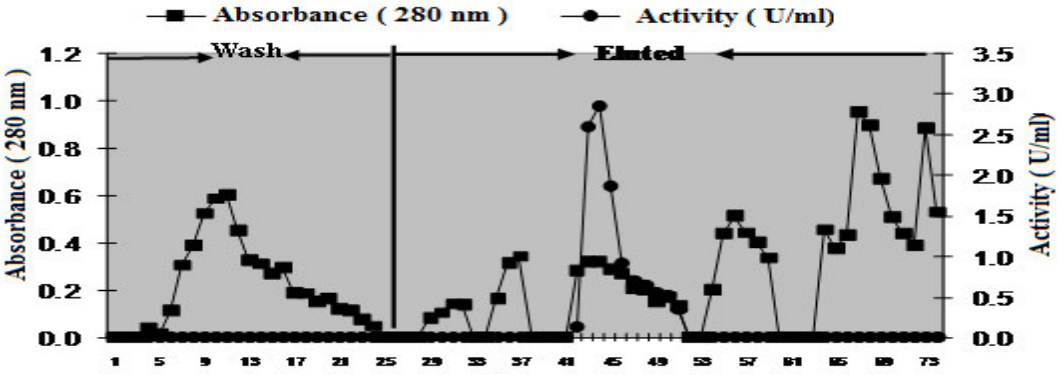


Fig.1 DEAE-cellulose chromatographic column (8 cm * 2.2 cm) equilibrated with phosphate buffer 0.01M, pH 6.5. The enzyme was eluted with a concentration gradient NaCl (0.01, 0.05, 0.1, 0.15, 0.2, 0.3, 0.5,1 M) in the same buffer, 5.0 ml fractions were collected at a flow rate 60 ml / hour.

Table 1. Purification of invertase from *A. terreus*

Steps	Volume (ml)	Activity (U/ml)	Protein con. (mg/ml)	Total Activity(U)	Specific Activity(U/mg)	(%) Yield	Purification fold
Crude Extract	30	2.63	0.090	78.90	29.22	100	1.00
Dialysis	31.5	3.14	0.070	98.91	44.85	125.36	1.53
DEAE-Cellulose Chromatography	50	1.20	0.005	60.00	240.00	76.04	8.21

3-3-Effect of pH and Temperature on Enzyme Activity

The effect of pH and temperature on partially purified enzyme activity was studied under standard conditions. The enzyme showed maximum activity at pH 2.0 (Fig. 2) and temperature 60°C (Fig. 3). Maheshwari et al. (2000) and Hocine et al. (2000) reported maximum activity at pH 4.4 and 5.6 for *A. niger* and 55-60 °C.

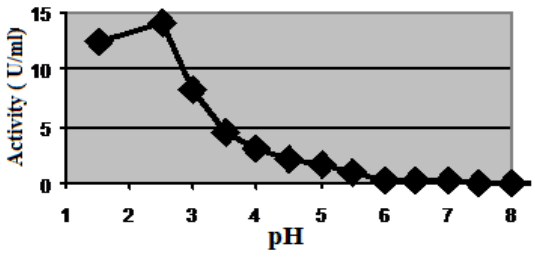


Fig.2 Effect of pH on the activity of purified invertase

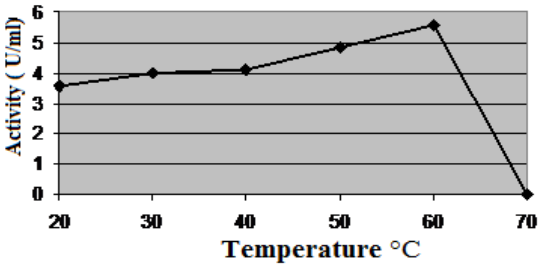


Fig.3 Effect of temperature on the activity of purified invertase

3-4- Effect of pH and Temperature on Enzyme Stability

Enzyme stability at different pH levels were measured by incubating the enzyme solution at 30°C and different pH for 30 min., after cooling in ice bath, the residual activity was measured under standard assay conditions. The enzyme was stable over a range of pH values 3.0 - 5.0 (Fig. 4). Thermo stability was 20 - 40°C (Fig. 5). Hocine et al.(2000) reported enzyme stability at pH 4.5- 11 and

30-50°C.

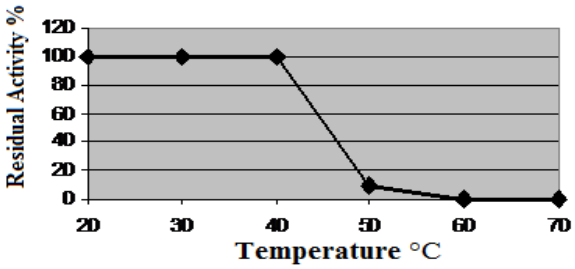


Fig.4 Effect of pH on the stability of purified invertase

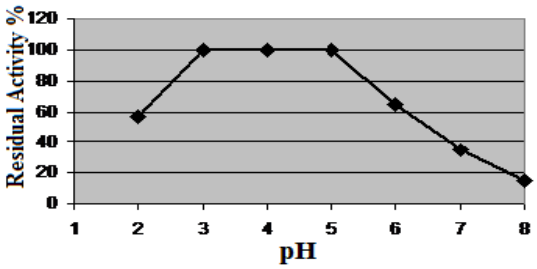


Fig .5 Effect of temperature on the stability of purified invertase

Activation energy with partially purified invertase was 1.87 kilocalories / mol (Fig. 6). Whitaker and Bernhard (1972) found 6-15 kilocalories / mol, which refer to the catalytic efficiency of enzyme under study.

3-5- Thin Layer Chromatography

Thin layer chromatography (TLC) closely in the Rf of glucose and fructose values resulting from the decomposition of sucrose by crude enzyme and partially purified with the Rf of glucose values and fructose indices (Fig. 7), there are three types of obvious spots displayed on the second and third resulting from decomposition of sucrose by crude and partially purified enzyme. Tanriseven and Dogan (2001) use the technique (TLC) to determine the degradation products of sucrose.

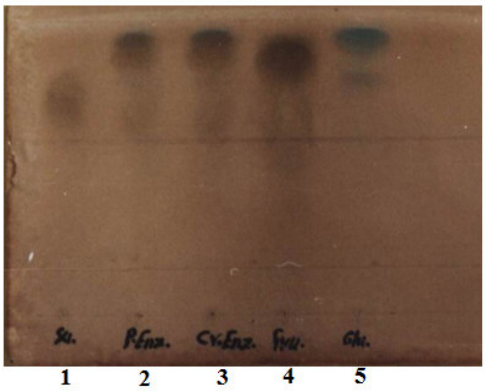


Fig. 7 Thin Layer Chromatography (TLC)
1)Standard sucrose 2) Decomposition of sucrose by partial purified invertase under study 3) Decomposition of sucrose by crude invertase under study 4) Standard fructose 5) Standard glucose

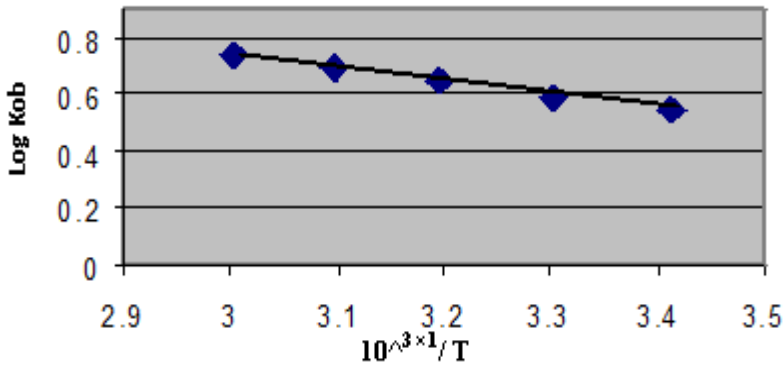


Fig.6 Arrhenius plot for activation energy of purified invertase

3-6- Immobilization Partially Purified Enzyme

Purified invertase was immobilized on several transition metals (Fig. 8). The metal Fe^{+3} and Fe^{+2} was the best residual activity for immobilized enzyme 76.52% and 70.05% respectively, while the percentage of residual immobilized enzyme with Cr^{+3} are 57.04% of the original activity.

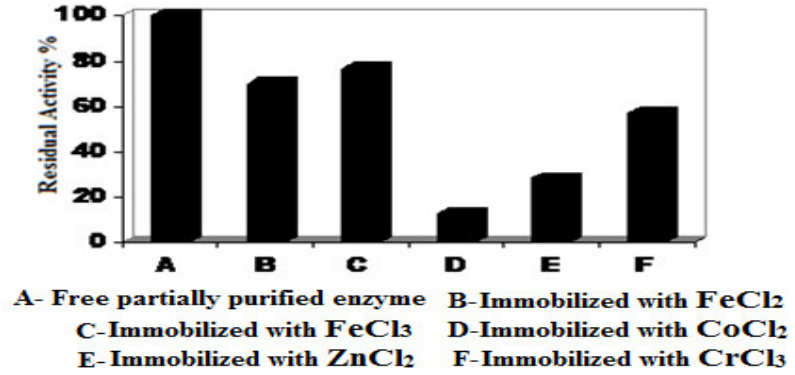
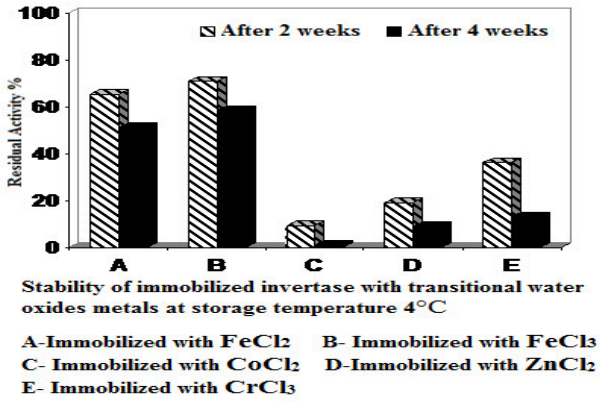


Fig. 8 Immobilization enzyme on several transition metals

These results are encouraging in immobilized enzyme with metals, because the activity of immobilized enzyme within normal ranges 50-80% of the original efficiency (Barker et al. ,1971). The storage stability of immobilized and free enzyme is presented in (Fig. 9)



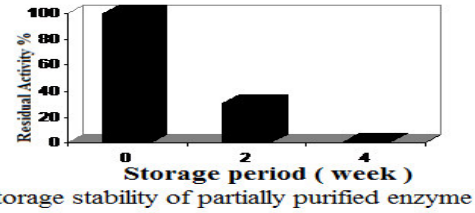


Fig. 9 Storage stability of immobilized and free Partially Purified Enzyme

The residual activity of free invertase was 31.11 % and 0.77 % after 2 and 4 weeks while the residual activity of immobilization invertase with Fe^{+3} was 70.94% and 58.42% after 2 and 4 weeks. The results indicated that the storage stability of immobilized invertase was better than that of free invertase.

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