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EXTRACTION, PARTIAL PURIFICATION AND KINETICS CHARACTERIZATION OFβ- FRUCTOFURANOSIDASE (INVERTASE) FROM AGARICUS BISPORUS

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ABSTRACT
A simple preparative procedure was adopted for Extraction and Partial purification of Invertase enzyme from White edible mushroom (Agaricus bisporus). A homogenised extract of the mushroom was first saturated by Ammonium sulphate (30% - 80%) and followed by the partial purification by Dialysis method. The partially purified mushroom samples were subjected to Lowry's method for Protein Estimation and Enzyme Activity. The apparent Vmax and Km values were determined for different concentrations of sucrose as substrate respectively. The Maximum activity of Invertase from the partially purified Mushroom sample at optimum temperature and pH were determined respectively. The kinetic properties of Invertase were determined by Michealis – Menten Kinetics and using the inverse Km and Vmax values, the double reciprocal, Lineweaver burk Kinetics was studied.
Keywords: Enzymes, Iinvertase, Biochemical characterization, extraction, purification, Enzyme activity and Enzyme kinetics

INTRODUCTION
Metabolism is a dual process that has of chemical and physical change which goes on in a cyclic manner in the living organism. The phenomenon of catalysis makes possible biochemical reactions necessary for all life processes. Catalysis is the process that simply means accelerating a chemical reaction by an potential enzyme that eventually does not undergo any permanent chemical change. Enzymes are complex globular proteins ,acting as a bio-catalyst that exclusively performs metabolic reactions in an organism's body. The term 'enzyme' was coined by Kuhne in 1878 from the Greek word, "enzumas", which means to the leavening of dough by microbe yeast. Enzymes catalytic nature is responsible for the functioning. Because of their specific nature enzymes can differentiate between chemicals with similar structures and can catalyze reactions over a wide range of temperatures (0º C -110º C) and in the pH range 2 -14. Invertase enzyme is widely distributed among the biosphere. They arefound in wild growing plants, skin of grapes and other fruits (Romero-Gomez et al. 2000). Plants like Pea (Pisum sativum), Japanese Pear fruit (Pyrus pyrifolia), Oat (Avena sativa) with excess invertase composition are used, but generally microorganisms like A.niger, S.cerevisiae, and Candida utilis, are considered ideal for their study(Uma et al .2010) The presence of multiple isoform of Invertase in nature have functionally beneficial 6 role to the plants (Kim et al. 2011). For example, intracellular fructofuranosidase has a weight of 135K Daltons, while extracellular fructofuranosidase has a weight of 270 K Daltons (Nakano H et al, 2000) It has been reported that in depressed cells most of the invertase is external where as in fully repressed state all the intracellular fructofuranosidase (Vu et al, 2008)

MATERIALS AND METHODS

MUSHROOM COLLECTION
The Fresh edible mushrooms Agaricus bisporus was purchased from nearby super market and was stored in Refrigerator.

CRUDE EXTRACTION:
100 g of fresh mushroom was homogenized using Mortar & pestle with 150 ml of 0.1 M Sodium phosphate buffer (pH 5.8) (Anjana Sharma et al.2016). The suspension was thoroughly stirred for 30 minutes at room temperature and was filtered by Whatman No.1 filter paper and centrifuged at 4000 rpm for 30 min. The pellet was discarded and the resulting supernatant was collected (Soundhari 2017) and it was designated as crude enzyme (Ayat adnan Abbas, 2015) was further used for protein estimation, screening of enzyme activity, ammonium sulphate precipitation and dialysis analysis. 3.2.1 Sodium Phosphate Buffer preparation: In a beaker aliquots of 1 M Stock solutions were pipetted according to the desired pH of 5.8. Approximately about 145 ml of distilled water was added to 250 ml conical flask. pH of the solution was measured using pH meter and adjusted by adding NaOH in case to raise the pH , and by adding Phosphoric acid to lower the pH .Now add distilled water to bring the total volume to 150 ml.

AMMONIUM SULPHATE PRECIPITATION
The collected supernatant from the crude was transferred to the conical flasks containing a stir bar and were placed in a magnetic stirrer. While the samples were stirring, the weighed ammonium sulphate powder for different concentrations (30%- 80%) was added slowly to each flask respectively. The volume of ammonium sulphate weighed was equal to the volume of supernatant. The flasks were allowed to be stirred for 30 minutes. After the total amount of ammonium sulphate was added the conical flasks were kept in 4ºC for overnight freezing (kamaldhin et al. 2004) The next day, transferred the

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samples of different concentrations from 30% - 80% to the conical tubes labeled and centrifuged the precipitate at 3000 rpm for about 30 minutes and the supernatant was carefully removed and discarded using a pipette from each tube and the pellets was collected. The pellets were suspended with 0.1M cold Sodium phosphatebuffer in all conical tubes and used for protein estimation and enzyme activity screening procedure.

DIALYSIS

Dialysis membrane (>10KDa) was cut into required size and soaked in distilled water to remove glycerol. Dialysis Membrane was sterilized by boiling in 2% NaHCO3 followed by rinsing in distilled water. The membrane was stored in Methanol at 4ºC. The membrane was again washed with distilled water. One end of the dialysed membrane was closed and two third of the bag was filled with the sample using a pipette and this end was knotted and checked for any leakage The Bagwas kept in a beaker filled with 50 M Sodium acetate buffer (pH6). The buffer was changed for every 3 hrs with the same pH buffer (kamaldhin et al. 2004) the same was repeated for thrice and the bag was dialysed overnight for 4ºC.The next day the buffer was discarded and the sample from the bag was carefully collected and stored for further analysis like protein estimation and enzyme assay.

LOWRY'S METHOD

Protein was measured according to Lowry et al. (1951) method using bovine serumalbumin (BSA) as a standard. 0.2, 0.4, 0.6,
0.8 and 1 ml of the working standard was pipette into a series of test tubes. 0.5 ml and 1 ml of the sample extract was pipette in two other test tubes Made up the volume to 1 ml in all the test tubes. The tube with 1 ml of water was considered as blank. 5 ml of reagent C was added to all the tube including the blank and were mixed well and left undisturbed for about 10 minutes. Then, 0.5 ml of reagent D was added and mixed well and incubated at room temperature in the dark for 30 minutes. Blue colour was developed and readings were taken at 620. A standard graph was drawn and the amount of protein in the sample was calculated and the amount of protein was expressed in µg/ml.

Calculation : Concentration of the Protein (mg %) = {OD (std) ÷ OD (test)} X {Aliquot (test) ÷ Conc (std) X 100}

ENZYME ASSAY FOR INVERTASE

Into each of a series of four 30 ml test tubes the following solutions were added consecutively in required volume 1.4 ml of water, 0.5 ml of acetate buffer and 0.1 ml of diluted enzyme and equilibrated the tubes in a 30° water bath. 1 ml of 0.3 M sucrose solution was added to 3 of the 4 tubes (Sumner and Howells .1935). The fourth tube was taken as an enzyme blank, 2 ml of DNS solution was added beforeadding 1.0 ml of 0.3M sucrose solution. Blank as 0.1ml of water in place of diluted enzyme was used in the last test tube. The test tubes were allowed for incubation time of 15 minutes and the absorbance @ 540 nm was measured in colorimeter. The Graph was plotted as concentration (µg/ml) Vs OD @ 540nm from which the Enzyme Activity was calculated from the formula below.

$$\text{Enzyme activity :} \quad \frac{(\mu \text{ mol of invertase released X total volume (ml) X dilution factor)(volume of enzyme X time of assay X Volume used in cuvette)}}{}$$

BIOCHEMICAL CHARACTERIZATION

Determination of optimum pH and Effect of pH on enzyme activity

A Set of test tubes were marked as ET &EB were taken. 2.5 ml of buffer (pH from 3 - 10) was added , followed by 2.5 ml of substrate was added and 1 ml of activatorto all the test tubes .The test tubes were pre incubated at 37º C for 10-15 minutes.0.5ml of enzyme was added to the test tubes marked as ET and incubated it at 37ºC for 15

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min (Hina Ashraf *et al .2015*) Then the reaction was arrested by addition of 0.5 ml of NaOH and 0.5 ml of enzyme was added to the test tubes marked as EB. 0.5 ml of DNS solution was added to all the test tubes. The colour developed was read at 540 nm. The graph was plotted pH vs OD @ 540nm.

Determination of optimum Temperature and Effect of temperature on enzyme activity

A Set of test tubes were marked as ET & EB were taken. 2.5 ml of buffer (pH 5.8) was added, followed by 2.5 ml of substrate was added and 1 ml of activator to all the test tubes (Russell Pressey *et al.*1966).The test tubes were pre incubated at 37º C for 10-15 minutes. 0.5 ml of enzyme was added to the test tubes marked as ET and incubated it at different temperatures from 20 ºC - 80 ºC for 15 min (Hina Ashraf *et al .2015*) Then the reaction was arrested by addition of 0.5 ml of NaOHand 0.5 ml of enzyme was added to the test tubes marked as EB. 0.5ml of DNS solution was added to all the test tubes and incubated for 30 min (Abeer N Shehata*et al,* 2016). The colour developed was read at 540nm. The graph was plottedTemperature vs OD @ 540 nm.

Determination of Effect of substrate concentration on enzyme activity

A Set of test tubes were marked as ET &EB were taken. 2.5 ml of buffer (pH 5.8) was added, followed by 2.5 mlof substrate was added and 1 ml of activator to all the test tubes. The test tubes were pre incubated at 37º C for 10-15 minutes. 0.5 mlof enzyme was added to the test tubes marked as ET and incubated it at different temperaturesfrom 20 ºC - 80 ºC for 15 min. Then the reaction was arrested by addition of 0.5 ml of NaOH and 0.5 ml of enzyme was added to the test tubes marked as EB. 0.5 ml of DNS solution was added to all the test tubes. The colour developed was read at 540 nm (Kofi Kwegyir Essel *et al*.2014). The graph was plotted concentration Vs OD @ 540 nm.

RESULTS AND DISCUSSION

CRUDE EXTRACTION: The 100g of fresh Mushroom was washed and sliced, were homogenised in 150 g of sodiumphosphate buffer (pH 5.8) followed by filtration & centrifugation.

Representative image of filtration and centrifugation of crude mushroomextract

The filtrate was then subjected to centrifugation for about 15 minutes at 5000 rpm.The supernatant was collected and the pellet was discarded. The crudesupernatant was subjected to protein determination by Lowry's method and found to have 81.5 mg/ml and then invertase enzyme assay was calculated which was found to have (2.35 $x10^{-3}$ IU/ml) of enzyme activity.

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Table1: Determination of protein and enzyme activity from crude extract

TOTAL PROTEIN (mg/ml)	TOTAL ENZYME ACTIVITY(U)	SPECIFIC ENZYME ACTIVITY(IU/ml)
81.5	$2.35x\ 10^{-3}$	0.028

AMMONIUM SULPHATE PRECIPITATION

The crude extract from the Agaricus Mushroom obtained by Homogenisation was subjected to partial purification -Ammonium sulphate precipitation method. The samples were saturated at different concentrations from 30%, 40%, 50%, 60%,70% and 80 % saturation with thorough stirring and was stored @4°C.The 80% ammoniumsulphate saturated sample showed maximum enzyme activity (3.025 $x10^{-3}$ IU/ml) andthe minimum enzyme activity (1.225 x 10^{-3} IU/ml) was found to be associated with 40% saturated sample. The total protein from different saturated samples werecalculated from lowry's method where the protein quantity gradually decreased after saturation when compared to crude. The maximum protein content from 80% ammonium sulphate concentration was found to be 67.75 mg/ml and the least was found to be with 40% saturation 48.25 mg/ml respectively.

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The above picture depicts the saturation of crude extract from 30%-80% anddeep freezing at 4°C (Ammonium sulphate precipitation)

DIALYSIS

The maximum activity and maximum protein was determined by Invertase Enzyme assay and Lowry's method of Protein estimation for all the precipitated samples from 30% ammonium sulphate saturation to 80% ammonium sulphate saturation and the maximum enzyme activity was exhibited by 80% saturated sample (3.95 x 10^{-3}IU/ml) and the total protein content was 72.5 mg/ml. The dialysed sample was later diluted with acetate buffer and used for Biochemical characterization study.

The above picture depicts the Dialysis of 80% Ammonium sulphate saturatedsample

LOWRY'S METHOD

The Lowry's method was used for the determination of total Protein content from crude and ammonium sulphate saturated samples of mushroom and total protein content at every consecutive step was compared with the total enzyme

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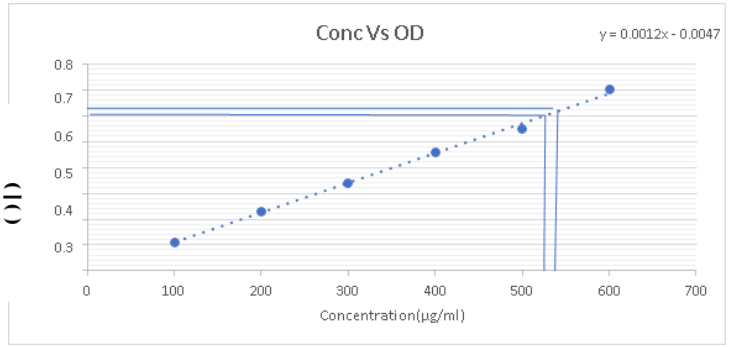
activity.



The OD values of the crude test samples were determined from colorimeter and was found to be 0.60 nm and 0.65 nm and the graph was plotted OD Vs concentration withthe standard values. The graph was found to be linear and from the graph. The total protein content was found to be 81.5 mg/ml in crude.

Table 2: Determination of total protein in Ammonium sulphate saturated samples

% OF SATURATION	TOTAL PROTEIN (mg/ml)
30% ammonium sulphate precipitation	48.25
40% ammonium sulphate precipitation	55
50% ammonium sulphate precipitation	61.5
60% ammonium sulphate precipitation	62.5
70% ammonium sulphate precipitation	63.25
80% ammonium sulphate precipitation	67.75
Dialysed 80% ammonium sulphate saturated sample	72.5

DETERMINATION OF ENZYME ACTIVITY OF INVERTASE ENZYME

The OD values of the Enzyme activity of Dialysed 80% ammonium sulphate saturated test samples were determined from colorimeter and was found to be 0.60 nm and 0.68 nm from the sample preparation graph and the graph was plotted OD Vs concentrationfrom the standard values. The graph was found to be linear and from the graph the specific enzyme activity of the crude sample was found to be 0.045 IU/ml.

Table 3: Determination of enzyme activity from Ammonium sulphate saturatedsamples

% OF SATURATION	TOTAL ENZYME ACTIVITY (U)	SPECIFIC ENZYME ACTIVITY(IU/ml)
30% Ammonium sulphate precipitation	$1.35x10^{-3}$	0.021
40% Ammonium sulphate precipitation	$1.225x10^{-3}$	0.025
50% Ammonium sulphate precipitation	$1.30x10^{-3}$	0.0236
60% Ammonium sulphate precipitation	$1.8x10^{-3}$	0.0289
70% Ammonium sulphate precipitation	$2.675x10^{-3}$	0.043
80% Ammonium sulphate precipitation	$3.025x10^{-3}$	0.045
Dialysed 80%saturated sample	$3.95x10^{-3}$	0.054

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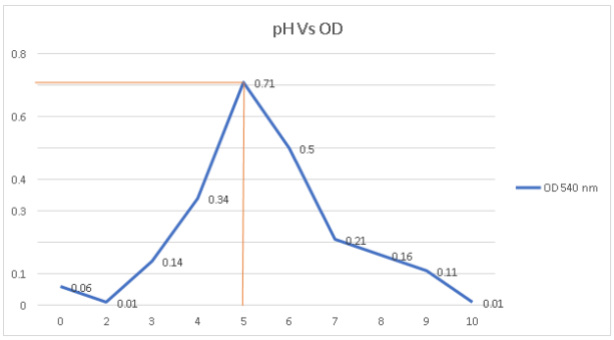
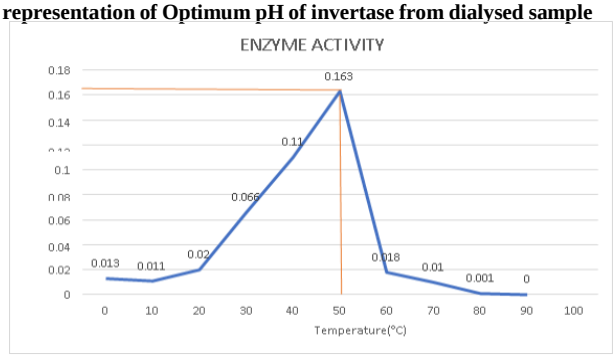
BIOCHEMICAL CHARACTERIZATION OF ENZYME INVERTASE & ENZYME KINETICS

Determination of Optimum pH and Temperature of enzyme invertase and its enzyme activity

From the Biochemical characterization assays, the enzyme activity was calculated forevery pH and Temperature to understand the stability of invertase and was found to be very less at pH 2 with 19.28 IU/ml and at 70°C with 10.12 IU/ml. So, it was understood that invertase enzyme is inactive at very acidic condition showing no or very least activity at very extreme temperatures



Graphical representation of Optimum pH of invertase from dialysed sample

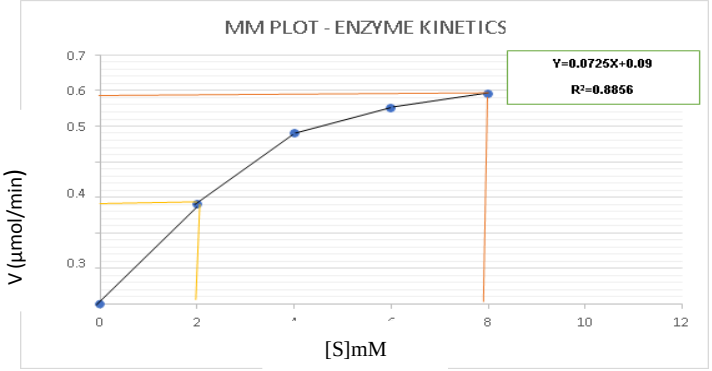


Graphical representation of Optimum Temperature of invertase from dialysed sample

Determination of Optimum Substrate concentration of enzyme invertase and itsenzyme activity

Table 4: Determination of Enzyme kinetics of invertase by MM plot

[S] mM	VELOCITY (µmol/min)
2	0.28
4	0.48
6	0.55
8	0.59
10	0.31



Graphical representation of enzyme kinetics of invertase by MM plot

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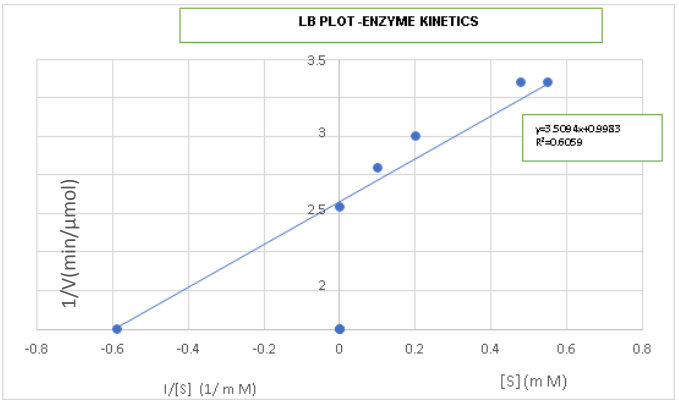
The sucrose was used as the main substrate to determine the invertase maximum activity at different concentrations from 2 mM - 10 mM. The maximum activity of 139.85 IU/ml was reported at 8 mM Concentration with OD 0.132nm and the apparent valueswere used to study enzyme kinetics.
From the MM plot, the value of Km and V of the enzyme invertase was found to be2mM and 0.467 µmol/min respectively. The maximum velocity Vmax was found to be 0.59 µmol/min from which the ½ V max was found to be 0.295 µmol/min.

Table 5: Determination of Enzyme kinetics of invertase by double reciprocal method

[S] mM	Velocity (µmol/min)	1/[S]	1/V
2	0.28	0.5	3.5714
4	0.48	0.25	2.0833
6	0.55	0.16667	1.8181
8	0.59	0.125	1.6949
10	0.56	0.1	1.7857



Graphical representation of Enzyme kinetics of invertase by Doublereciprocal plot

From the MM plot, the inverse values of [S] and V of the enzyme invertase were calculated and double reciprocal LB graph was plotted. The maximum velocity Vmax was found to be 0.55 µmol/min which was found from the reciprocal ½ Vmax value 1.797 µmol/min and 1/Km was found to be 0.602 1/Mm from graph respectively.

SUMMARY & CONCLUSION

The enzyme β – *fructofuranosidase (invertase)* was extracted from white edible mushroom *Agaricus bisporus* by homogenization method. The crude was subjected to Lowry's protein estimation and Invertase enzyme activity and was found to have 81.5 mg/ml and 0.028 IU/ml respectively. Then partially purified byAmmonium sulphate precipitation method and Dialysis where the protein content gradually decreased (67.75 mg/ml) after saturation when compared to crude. The enzyme activity gradually increased was found to be 0.045 IU/ml. The specific activity was found with respect total protein at every stage of saturation and dialysis. The Biochemical characterization of the enzyme invertase showedoptimum pH at 5 and optimum temperature at 50°C with enzyme activities about 144.92 IU/ml and 254.07 IU/ml respectively. The Km and Vmax values were found from Optimum substrate concentration, sucrose as substrate to be 2.1 Mm and 0.59 µmol/min respectively by Michealis Menten kinetics. The apparent values were used to plot double reciprocal Lineweaver Burk graph were the 1/Km and 1/Vmax values were found to be 0.602 (1/mM) and 1.797 (min/µmol) respectively.

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