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CHARACTERIZATION OF ESTEROLYTIC ACTIVITY FROM EDIBLE MUSHROOM *HYPSIZYGUS ULMARIUS*

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ABSTRACT

Characterization of esterase activity from the edible mushroom *Hypsizygusulmarius*were investigated. The extract prepared from the mushroom sample showed the esterolytic activity. The extract had the highest activity in 2mM of 1-napthyl acetate as a substrate. pH and temperature optima were found to be at 6.0 and 30^0C. The pH and temperature stability profiles show that this enzyme is more stable under acidic, neutral and basic conditions and stable up to 40^0C. The K_m and V_{max} of esterase enzyme acting on 1-napthyl acetate were 0.33 and 0.47mM. The effect of alternate product (p-nitrophenol) on esterase activity were found to be at 10mM. The effect of metals on activity of esterase increased with the addition of $CuSO_4$ and decreased with $FeSO_4$.

Keywords: *Mushroom, Characterization, Napthyl acetate, Hypsizygusulmarius, Esterase.*

No of Figures: 09 **No of References: 37**

INTRODUCTION

Esterases (EC 3.1.1.1) are enzymes thatcatalyzesthe hydrolysis of various types of both endogenous and exogenous esters. These are widely distributed in nature, found in animals, plants and microorganisms(Slim cherif*et al*, 2010). They occur in multiple molecular forms and exhibit a number of unique enzyme characteristics such as substrate specificity, regiospecificity and chiral specificity (Jung *et al.*, 2003). The functions of these enzymes have also been implicated in carbon source utilization, pathogenicity and detoxification (Ewis*et al.*, 2004). These enzymes preferably catalyze the hydrolysis of esters composed of short chain fatty acids, but they also can catalyze ester synthesis and transesterification (Bornscheuer, 2002). Particularly, the potential application of these enzymes for the synthesis of short chain esters has attracted the interest of a broad range of industrial fields like foods, pharmaceuticals and cosmetics. Among these flavor acetates from primary alcohols constitute compounds with a great application due to their characteristic fragrance and flavor (Romero *et al.*,2005). The different types of esterase are also involved in fruit ripening, abscission, cell expansion, reproduction as well as hydrolysis of ester containing xenobiotic molecules.Other significant functions of the esterase include metabolism and subsequent detoxification of many agrochemicals, pharmaceuticals (Redinbo and Potter, 2005; Potter and Wadkins, 2006), metabolism of a number of therapeutics (Williams, 1985), including the cholesterol-lowering drug, lovastatin (Tang and Kalow, 1995), the antiinfluenza drug, Oseltamivir (Tamiflu) (Shi *et al.*, 2006), the narcotic analgesic meperidine (Demerol) (Zhang*et al.*, 1999), cocaine and heroin (Pindel*et al.*, 1997), and resolution of racemic mixtures by transesterification, or the enantioselective hydrolysis of esters for obtaining optically purecompounds (Bornscheuer, 2002). Esterase activity is also used extensively in soft- and pro-drug design (Bodor and Buchwald, 2000, 2003, 2004).

Microbial esterase are of considerable interest because of their potential application in biotechnology. Mushrooms that belong to the class of fungi are not only valuable foods but also good enzyme sources. So it is interesting to study enzyme activities from different mushroom species for identifying different enzyme characters. *Hypsizygusulmarius*commonly called as elm oyster mushroom is a highyielding edible mushroom for which commercialcultivation technology has been released andis gaining popularity. Previous reports suggeststhat the mushroom is rich in antioxidants andproved for its antidiabetic activity(Meera*et al.*, 2011). The presence of laccase enzyme, their purification and characterization was reported by Ravikumar*et al.*, 2012. The effect of laccase from *Hypsizygusulmarius* in decolorization of different dyes is reported (Ravikumar*et al.*,2013.)

MATERIALS AND METHODS

All reagents and chemicals used were of analytical grade, purchased from Sigma Chemical Co. (St. Louis, MO).

Hypsizygusulmarius mushroom were grown in association with vinayaka mushroom cultivators, Hebbel, Bangalore Karnataka.

Enzyme Extraction

The fresh fruiting bodies of Mushroom were isolated and freeze dried. Crude enzyme extracts were prepared as reported previously by Colak*et al.*, 2007; Kolcuoglu*et al.*,2007;Ertunga*et al.*,2009. Frozen fresh fruiting body of the mushroom was ground to a fine powder in liquid nitrogen for 15 min using a pre-chilled ceramic mortar and pestle. The cold mushroom sample (80g) were separately homogenized in 85ml of 0.15M NaClusing blender and left overnight on stirrer. The homogenate were centrifuged at 10,000 rpm for 20 min at 4^0C. The supernatant was measured and saved as crude for further analysis.

Protein Determination

Protein concentration was determined according to the method of Lowry *et al.*,1951, using bovine serum albumin (BSA) as standard.

Enzyme Assay

Esterase activity was assayed according to the method of Gomori*et al.*, 1953 as modified by Van Asperen*et al.*,1962. The assay mixture consisting of 5ml of 0.3 Mm 1-naphthyl acetate (a stock solution of 30 mM 1-naphthyl acetate prepared in acetone and diluted in 0.05 M sodium phosphate buffer pH 7.0) and 10–100µg of enzyme was incubated at 27^0C for 15 min. The reaction was stopped by addition of 1ml of DBLS reagent (2 parts of 1% diazo blue B and 5 parts of 5% sodium lauryl sulphate). In the control, enzyme was inactivated by DBLS prior to incubation with substrates. The absorbance of the developed color was measured at 600nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1mol of product per min at pH 7.0 and 27^0C.

Characterization of Enzyme

Effect of pH on esterase activity and pH stability

The effect of pH on esterolytic activity was determined by using the following buffers. 0.1M citrate phosphate buffer (pH 3.0 & 4.0), 20mM acetate buffer (pH 5.0), 50mM phosphate buffer (pH 6.0, 7.0 & 8.0) and Tris–HCl buffer (pH 9.0). The assay was performed by assaying the enzyme with different buffers of pH-3.0-9.0 at room temperature using alpha naphthyl acetate.All the experiments were performed in triplicate and the mean was taken for statistical analysis. The optimum pH obtained was used for determining thermal properties and other parameters. The same buffers were used to determine pH stability. The extract was preincubated with different buffersfor 24h at RT and the standard enzyme assay at optimum pH was performed. The percentage relative activities were calculated by comparison with unincubated enzyme (Faiz*et al.*, 2007).

Effect of temperature on esterase activity and thermal stability

To determine the optimum temperature for esterolytic activity, enzymatic reactions at various temperatures over the range

from10–80^{0}C were performed by using the procedure described above.In order to determine the thermal stability, aliquots of enzyme in Eppendorf tubes were preincubatedat different temperaturesbetween 10 and 80^{0}C for different intervals of time followed by rapid cooling in ice bath. The enzyme was then assayed at RT. All the experiments were performed in triplicate, and the mean was taken for statistical analysis. The percentage relative activities were calculated by comparison with unincubated enzyme (Faiz*et al.,* 2007).

Effect of metal ions on esterase activity

The effects of metals such as Na^+, Zn^{2+}, Li^+, Ni^{2+},Mg^{2+}, Cu^{2+}, $Fe^{2+}$and Ca^{2+} on the esterolytic activity were investigated. Final concentration of each metal ion in the reaction mixture was 10 mM. The percentage residual activities were determined by comparison with the standard assay mixture with no metal ion added (Lee *et al.,* 1999).

Effect of alternate product on esterase activity

Alternate product inhibition experiments were performed by varying the concentration of p-nitrophenol from 1-35mM for 30mins at RT and then assayed for esterase activity using1-naphthyl acetate as substrate.

Substrate specificity and enzyme kinetics

Stock solution ofalpha napthyl acetate were separately prepared at a concentration of 0.1 mM to 3mM. The activity was determined as described above. The kinetic data were plotted as reciprocals of activities versus substrate concentrations. The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) values were determined as the reciprocal absolute values of the intercepts on the Xand Yaxis respectively, for the linear regression curve (Lineweaver& Burk, 1934).

RESULT AND DISCUSSION

Recent report on culinary medicinal mushroom *sparassiscrispa* that secreted alkaliphilic esterase (Gayathri C*et al.*,2011)and on the mycelium culture of edible mushrooms that secreted a ferulic acid esterase was published which indicates applications for the functional foods and fermentation industries (Xie*et al.*,2010).

Protein content and enzyme activity

The protein and enzyme activity were found to be 3.57mg/mland 2.25 µmole/mlof crude mushroom sample used (Graph-A & B).

Effect of pH on esterase activity and stability

The effect of pH on esterase activity and pH stability was tested using α-napthyl acetate. As shown in Graph-C, the optimal pH were found to be 6.0. The pH-stability of the esterase enzyme (Graph-D) was found to be optimally stable over a wide pH range of3-9. It appears that the esterase is quite stable between these pH values. The stability of the enzymes in acidic, neutral and basic pH is important in terms of industrial applications Similar results were reported earlier for esterase from G. thermoleovorans YN (Soliman*et al.*, 2007), Mucor sp. (Abbas *et al.*, 2002) and Cucurbitapepo (EII)

(Fahmy*et al.*, 2007).

Effect of temperature on esterase activity and thermal stability

Temperature-activity is shown in Graph-E for *Hypsizygusulmarius*. Esterasewere most active at temperature 30^0C with relative activity of 100% similar to the esterase from *Cucurbitapepo*Elc and EII with an optimum temperature between 30-40^0 C (Fahmy*et al.*,2007), *Lactobacillus casei subsp. casei* (Lee & Lee, 1990) and *Lactobacillus fermentum* (Gobbetti, *et al.*,1997) at 30–35^0 C. Its optimum temperature is lesser than 70^0C as reported for *Anoxybacillusgonensis*(Faiz*et al.*, 2007) and *Avenafatua*(Mohamed *et al.*,2000) at 65–75^0 C.Graph-Fshows the loss of activity of the enzymes incubated for different lengths of time at various temperatures. The enzyme remained comparatively stable at temperature 20-40^0C when incubated for 1h. However, they are unstable at temperatures above 40^0C. The activities were fully lost between 50 and 80^0C.

Effect of metal ions on esterase activity

Enzyme enhancing or decreasing activity may be attributed to the metal ion's properties towards protein ligands (Colak*et al.*,2009).Various metal ions at 10 mM concentration were tested on *Hypsizygusulmarius* esterase. The overall metal ions study revealed that the esterase activity was inhibited completely withaddition of $FeSO_4$showing residual activity of 4.02% and increased with addition of $CuSO_4$showing relative activity of 110.90% (Graph-H) as compared with other mushroom esterase, *S. crispa*(Gayithri C*et al.*, 2011). The metal ions such as $MgCl_2$, $NiSO_4$, and NaCl maintained enzyme activity with relative activity of 96.18%, 94.54% and 92.36%.

Effect of alternate product on esterase activity

Increasing concentration of p-nitrophenol(1-35mM) drastically affected the activity of the enzyme. The plots of percent activity versus concentration of p-nitrophenol showed theinhibitionat 10mM with relative activity of -10.45% activity and complete inhibition at 35mM(Graph-G).

Substrate specificity and kinetics

The purified esterase was tested on different concentration of substrate, alpha napthyl acetate. The measured velocity increased as concentration of substrate increased. The activity of the esterase decreased as the number of carbon atoms increased. The highest activity was observed with 2mM concentration with K_m and $V_{max}$of 0.33mM and 0.47mM (Graph-I).The reported esterase from mushrooms species, such as *A. vaginatavar. vaginata, T. terreum*(Ertunga*et al.*, 2009) and *L. perlatum*(Colak*et al.*, 2009), showed specificitytowards short chain fatty acids such as p-nitrophenyl butyrate andpnitrophenyl acetate. Apparently, this specificity study suggeststhat the enzyme is an esterase not a lipase.

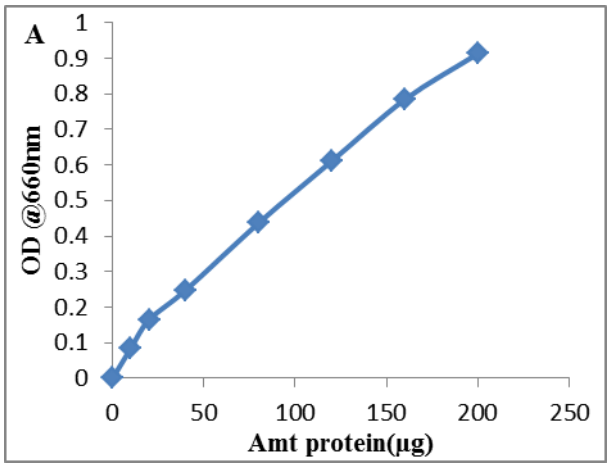

Fig1: *Graph A-Standard graph to determine total protein content (µg)*

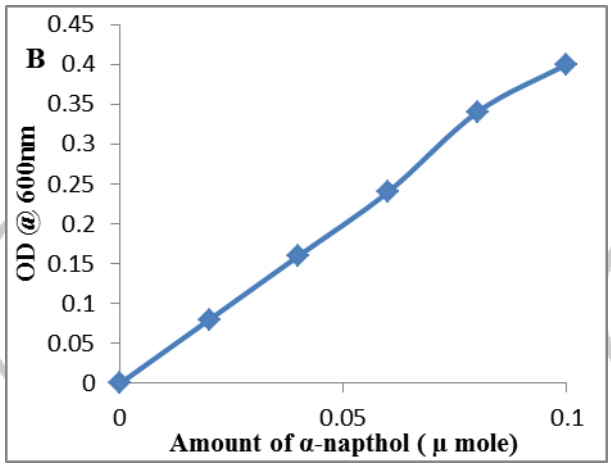

Fig 2: *Graph B- Esterase activity graph (µmole)*

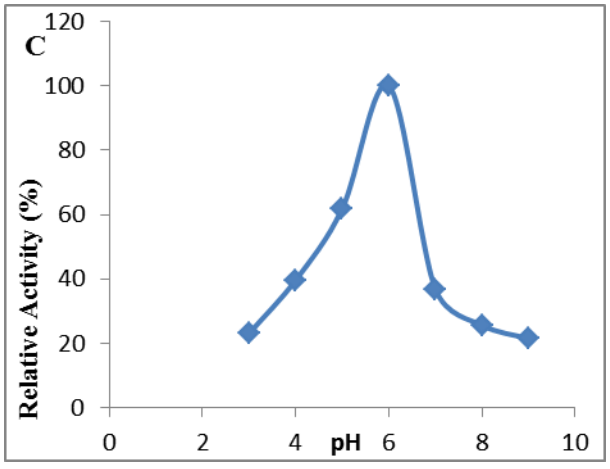

Fig 3: *Graph C- Effect of pH on esterase activity of Hypsizygusulmarius.*

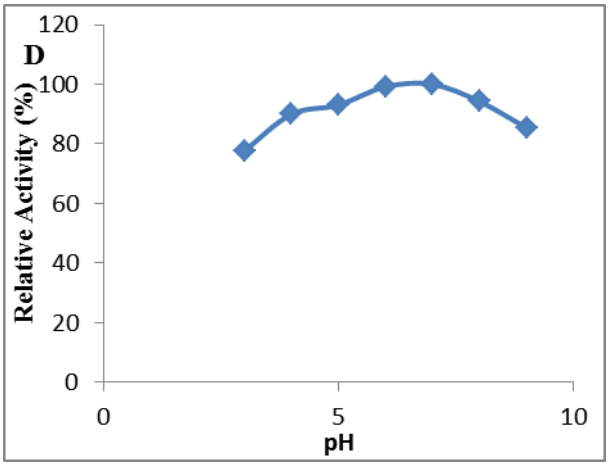

Fig 4: *Graph D- pH stability profile on esterase activity of Hypsizygusulmarius.*

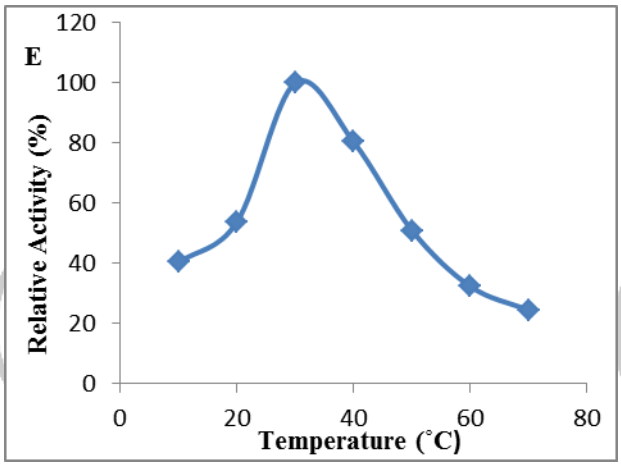

Fig 5: *Graph E- Temperature effect on esterase activity of Hypsizygusulmarius.*

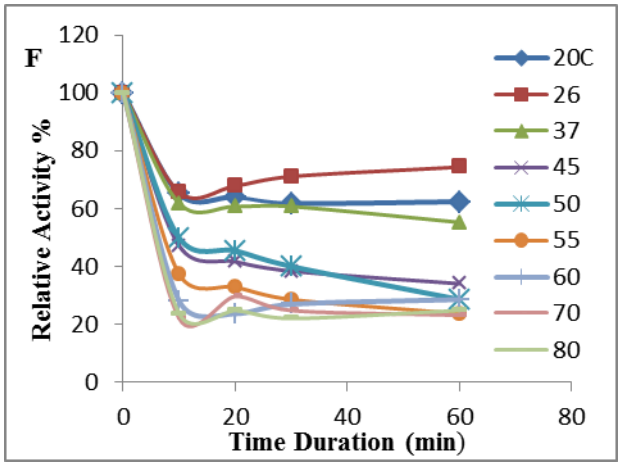

Fig 6: *Graph F- Thermal stability profile of Hypsizygusulmarius esterase.*

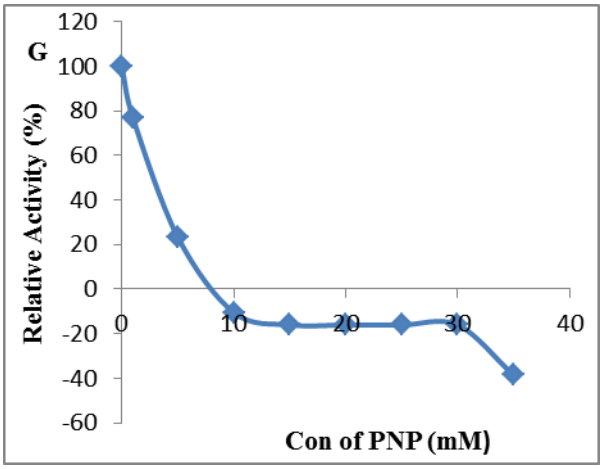

Fig 7: *Graph G- Effect of pnitrophenol on the activity of esterase from Hypsizygusulmarius.*

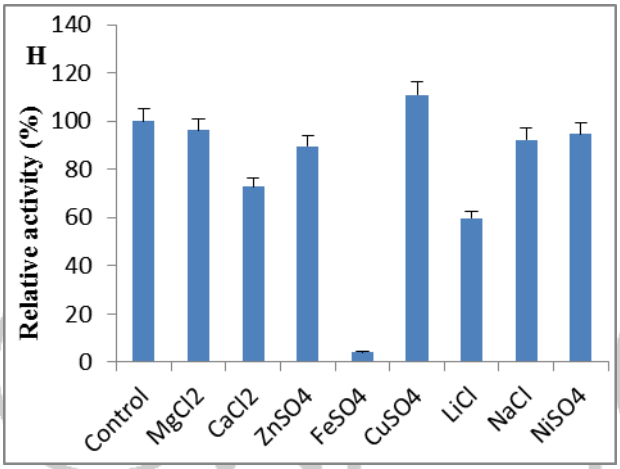

Fig 8: *Graph H- Effect of metals on the activity of Hypsizygusulmarius esterase.*

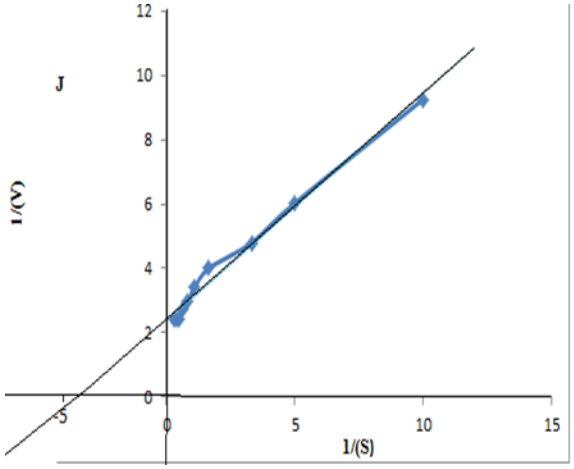

Fig 9: *Graph I- Determination of Km and VmaxbyLineweaver-Burk plot for Hypsizygusulmarius esterase*

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