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Original Research Article

Studies on Feruloyl Esterase Enzyme from *Pleurotus Ostreatus* Hp-1: Production, Statistical Optimization, Purification and Biochemical Characterization

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Abstract

An extracellular feruloyl esterase (FAE) was produced from *Pleurotus ostreatus HP*-1 through solid state fermentation using banana pseudostem. Statistical optimization increased the enzyme production by 1.22 fold on the 9th of fermentation with an activity of 3651U/gm. The FAE enzyme was purified using size exclusion chromatography and confirmed using native and SDS-PAGE in which enzyme showed the monomeric protein with an estimated molecular mass of 28 KDa. The maximum catalytic activity was obtained at pH-6 at 30°C. FAE exhibited pH stability in the range of 5-7 with temperature tolerance up to 40°C. Enzyme also showed the tolerance in presence of various metal ions in which Co+2, Mn+2, and Ni+2 influenced its activity by 1.01, 1.34 and 1.47 times at 10 mM concentration. The enzyme also exhibited higher activity in presence of non-polar organic solvents i.e., benzene, hexane and chloroform with increment of 105.49%, 72.37% and 67.28%, respectively while polar solvents reduced (methanol, ethanol) the enzyme activity by 90%.

Keywords: *Pleurotus ostreatus* HP-1, Feruloyl Esterase, Statistical Optimization, Solid State Fermentation, Purification

Introduction

The continuous development of bio-sustainable and renewable resource based technology is an extremely important step to reduce the environmental impact. The key technology is to convert lignocellulose into carbon source in a low molecular weight. The molecular architectural of the plant cell wall contains many structural polysaccharides like pectin, xylan, cellulose and lignin. The complete hydrolysis of this plant cell wall material requires an array of enzymes which are the subjects of great fundamental and practical practices. Due to the presence of cross-linked network like structure of plant cell wall its degradation requires synergistic action of different cellulase and hemicellulase. FAE (E.C. 3.1.1.73) is also known as a ferulic acid esterase (FAE), cinnamic acid esterase and cinnamoyl esterase. FAE represent the subclass of carboxylic acid esterase (E.C.3.1.1) which widely distributed in the plant cell wall and microorganisms [1]. FAE enzyme catalyzes the ester bond between the ferulic acid and sugar present in the plant

cell wall and release high value added products from the lignocellulosic waste. [2-4]

During the process of hydrolysis, FAE releases a high value added products in a form of ferulic acid from different lignocellulosic substrate including banana pseudostem [5]. The ferulic acid released from lignocellulosic is widely distributed in grains, fruits, vegetables and agroindustry derived by-products. Ferulic acid (Hydroxycinnamic acid, 3-methoxy 4-hydroxycinnamic acid) is the most abundant naturally occurring phenolic acid molecules, which is covalently attached to the other components of the plant cell wall using ester bonds which can be degraded by FAE enzyme [6]. It plays a functional role to provide a structural rigidity to the plant cell wall which partially limits the biodegradation of the plant cell wall [7]. As FAE helps in the release of ferulic acid from lignocellulosic feed stalks without the use of alkali treatment, the process in general is termed as a "green process". Due to the biotechnological importance, there is a growing interest in FAE which have been isolated and purified from different microorganisms [8]. Ferulic acid esterase has been classified into four types on the basis of the primary amino acid sequences and structural specificity. Based on the specificity against synthetic substrates such as methyl ferulate (MFA), methyl sinapate (MSA), methyl caffeate (MCA), methyl ρ-coumarate (MρCA), FAE have been classified in type A, B, C and D [9]. FAE have been considered for bio-catalytic conversion of ferulic acid into styrene, polymers, epoxide, vanillic acid derivatives and vanillin.

In the present work, a systematic study was conducted to optimize the production, purification, and characterization of the FAE enzyme produced by white rot fungus *Pleurotus ostreatus* HP-1(Gen Bank Accession No. EU420068). The properties investigated in this study included molecular mass, optimum temperature, time and pH, stability of the purified enzyme and effect of different metal ions and solvents on purified enzyme. Furthermore, the purified enzyme was utilized for the extraction of ferulic acid.

Materials and Methods

Chemicals

3-(N-morpholino)propanesulfonic acid (MOPS), dimethyl sulfoxide (DMSO), DMF (Dimethylformamide), malt extract agar, potato dextrose agar and all other used in experiments were of analytical grade with highest purity procured from Hi-Media Laboratories, Mumbai, India. Banana pseudostem was collected locally and used as lignocellulosic substrate. Ethyl ferulate, methyl ferulate, methyl sinapate, methyl caffeate, methyl ρ-coumarate were procured from Sigma Aldrich.

Enzyme assay and protein determination

FAE activity was evaluated according to procedure described by Ralet et al., (1994) [10]. The decrease in absorbance related to the reduction of MFA was measured spectrophotometrically at 335 nm for 10 min. The reaction system contained 800µl of 100 mM MOPS buffer (pH 6.0), 100 µl of 1 mM MFA and 100µl of enzyme extract. One unit of enzyme activity (U) is defined as the amount of enzyme that catalyzed the reduction of 1 µM of MFA/min under standard reaction conditions. The Folin-Lowry's method (Lowry et al., 1951) [11] was used for the detection of protein content in the crude and purified samples.

Optimization methodology for the feruloyl esterase production

The selection of moistening medium is an important parameter for the maximum production of FAE. To select a suitable moistening medium for the maximum production of enzyme, the six different moistening mediums were screened out (Minimal medium, Enzyme Production medium, Olga medium, Asther's medium, Christakopoul's medium and Mineral salt medium). Five grams of substrate (banana

pseudostem) was moistened with 20ml of different moistening medium. The solid state fermentation was carried out for FAE production by inoculating five agar plugs (8mm diameter) of actively growing culture of *Pleurotus ostreatus* HP-1 in each flask and incubated under thermostatically controlled incubator at 30°C for 2-15 days. The solid substrates were filtered by muslin cloth and enzyme content was collected. The extracted crude enzyme was centrifuged at 10000 rpm for 12 min at 4°C and supernatant collected and analyzed for feruloyl esterase enzyme activity.

In single factorial methodology, effect of moisture content was assessed by using various combinations of solid material to moistening agent ratio. The ratio of solid substrate to moistening agent used in the present study was 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 and 1:10 (w/v). Banana pseudostem was used as a solid substrate with Olga medium as a moistening agent. The production flasks were incubated at 30°C for 9 days under thermostatically controlled condition.

The effect of the inoculum size on the production of feruloyl esterase enzyme was also examined. The agar plugs (3 to 14 of 8mm in diameter) of freshly growing *Pleurotus ostreatus* HP-1 were added into the production flasks. For the selection of pH of Olga medium for the production of feruloyl esterase, the pH of the moistening medium was adjusted in the range of pH 3.0- pH11.0. The production flask was moistened with the 25 ml of olga medium. The solid state fermentation was carried out under optimized condition for 9 days.

The selection of the temperature for the production of FAE by *Pleurotus ostreatus* HP-1 was varied from 15°C to 45°C under optimum condition. The different carbon sources were screened to enhance the production of feruloyl esterase. The different carbon sources such as glucose, starch, fructose, maltose, lactose, mannitol, xylose, mannose and sucrose (1% w/v) were incorporated into the production flasks. The solid state fermentation was carried out with incubating the experimental and control flasks (devoid of any co-substrate) at 30°C for 9 days. In the screening of organic and inorganic nitrogen sources, soybean, peptone, ammonium sulfate, yeast extract, ammonium chloride, ammonium nitrate, urea, L-asparagine, Thiamine-HCl were used. The effect of nitrogen sources was evaluated by mixing the nitrogen source with the Olga medium. The fermentation was carried out at 30°C for 9 days.

Statistical optimization for FAE production

Screening of medium constituents by Plackett-Burman design

The statistical analysis of the components of Olga medium for the production of feruloyl esterase enzyme was evaluated by the plackett-Burman design. For the analysis eight (8) different moistening medium components (KH2PO4, K2HPO4, MgSO4, MnSO4, FeSO4, ZnSO4, Yeast extract and Glucose) (k=8) were screened in the combination of [12] according to the Plackett-Burman design. The following equation was used to calculate the effect of each variable.

$$E(xi)=2(\Sigma Mi\text{^}+-Mi\text{^}-)/N \quad (1)$$

Where, E(xi) = the concentrated effect of the tested variable, Mi+ and Mi- = feruloyl esterase production from the trials where the variable (xi) measured was present at high and low concentrations, respectively and N= number of trials.

The experimental error was evaluated by calculating the variance among the dummy variables as follow:

$$V\_eff=\Sigma(E\_d)\text{^}2/n \quad (2)$$

Where, Veff = concentration effects of variance, Ed= concentration effect of the dummy variable and n = number of the dummy variables. The standard error of the concentration effect was a square root

of variance of an effect and the significance level (p-value) of each concentration effect was evaluated using the student's t-test:

$$t(xi)=E(xi)/SE \quad (3)$$

Where, E(xi) is the effect of variable xi.

Optimization of screened components by RSM

The further validation of the components of Olga medium was performed by Response Surface Methodology using central composite design (CCD). The effect of four components on feruloyl esterase production was examined at five different levels: -α, -1, 0, +1, +α. The response of the independent variable was analyzed by using the quadratic equation (4):

$$Y=\beta 0+\Sigma\beta ixi+\Sigma\beta ijxixj+\Sigma\beta iixi2 \quad (4)$$

Where, Y= predicted response, β0= offset item, βi= linear offset coefficient, βii = squared offset coefficient, βij = interaction effect and xi = the dimensionless code of the Xi.

Time course study of feruloyl esterase enzyme production

The time course study for FAE production was done before and after optimization of the cultivation parameters. 5 g of banana pseudostem was moistened with 25 ml of optimized and unoptimized Olga medium in 250 ml of production flasks, each production flask was inoculated with five agar plugs of freshly growing mycelia of *Pleurotus ostreatus* HP-1 (8 mm diameter) from ME plate. The production flasks were incubated under optimized conditions for 15 days. The content of flasks was extracted periodically every 24 h then supernatant was collected after centrifugation (10000 rpm for 12 min).

Purification of FAE

The FAE production by *Pleurotus ostreatus* HP-1 was purified by two step process. The feruloyl esterase enzyme produced by solid state fermentation using banana pseudostem and extracted, centrifuged at 10000 rpm for 12 min (4°C). The Supernatant obtained after the centrifugation was used for the total protein precipitation by using ammonium sulphate. The ammonium sulphate precipitation was performed in the range of 0-80% saturation. After the ammonium precipitation, dialysis was performed to remove the salts from the fraction. The active fraction with highest feruloyl esterase activity was dialyzed against the 50 mM Potassium phosphate buffer (pH-7.0) for 24 h. The buffer was changed every 6 h. The purification of FAE enzyme was carried out on size exclusion chromatography. The column was equilibrated with 50 mM Potassium phosphate buffer (pH-7.0). Total of thirty fractions of 1 ml were collected in eppendrof tubes at the flow rate of 0.3 ml/min. All fractions were analyzed for FAE activity using MFA as a substrate. The protein content was determined by Folin-Lowry method [11].

The investigation of purity of an enzyme and molecular weight was done by sodium dodecyl sulfate (SDS) Polyacrylamide gel electrophoresis (PAGE) and non-denaturing Polyacrylamide gel electrophoresis (native-PAGE). SDS-PAGE was performed by preparing 12% resolving gel and 4% stacking gel. The molecular weight was investigated by protein marker containing Myosin rabbit muscle (205 KDa), phosphorylase b (97.4 KDa), bovine serum albumin (66.0 KDa), ovalbumin (43.0 KDa) and carbonic anhydrous (29.0 KDa). The zymogram study was performed by sandwich method according to the process described by kumar et al., (2013) [12]. The gel of Native-PAGE sandwiching between the two layers of zymogram medium. Zymogram medium contain a warm solution of 2% (w/v) agarose in 0.5 M acetate buffer (pH-6.0), added with 1% ethyl ferulate in 5% (w/v) DMF solution.

Characterization of purified FAE
Purified FAE was analyzed for optimum pH and temperature. The optimum pH was evaluated in the range of pH 3.0 to pH 9.0 using methyl ferulate as a substrate. Optimum temperature was analyzed by pre-incubation of feruloyl esterase enzyme in the range of 20-70°C for 30 min. The temperature stability was determined by evaluating the residual activity of FAE at temperature range 20-60°C. The residual activity for thermal stability was calculated after 24 h and 48 h. The pH stability was examined in the range of pH 5-7. The residual activity of FAE was calculated after 24 h. The effect of various metal ions on the activity of purified FAE was analyzed. At a concentration of 10 mM and 100 mM, metal ions Co+2, Cu+2, Hg+2, Mg+2, Mn+2, Fe+2, Ni+2, Ca+2, and Zn+2 were incubated with FAE for 30min at 30°C. The residual activity was calculated for FAE enzyme. The effect of various solvents on purified FAE activity was tested. The FAE was pre-incubated with different solvents such as methanol, ethanol, DMSO, acetone, ethyl acetate, benzene, n-hexane, chloroform, isopropyl alcohol and butanol with different concentrations (10% v/v, 25% v/v and 50% v/v) for 30 min at 30°C. The four different substrates such as MFA, MCA, MρCA, MSA were used to evaluate the effect of substrate concentration on FAE activity. The effect of different substrate concentration on FAE was examined at 37°C in 100 mM MOPS buffer (pH-6) by making proper dilution of enzyme and different substrate at various concentrations between 100 µM to 1000 µM. The value of Km and Vmax for all the four substrates were calculated from the Lineweaver-Burkplot.

Result and Discussion

Single factorial optimization for FAE production

The selection of moistening medium is one of the most important parameter for the maximum production of feruloyl esterase under SSF. The experiment was performed to determine the maximum FAE production using six different moistening mediums. The fungal mycelia of *Pleurotus ostreatus* HP-1 appeared on the surface of the banana pseudostem substrate after 24-48 h and gave highest production of FAE (2076 U/gm) on 9th day of incubation in Olga medium (Fig 1). However, any further incubation did not increase the enzyme production which may be attributed to the variation in a pH due to the accumulation of the certain organic acids [13].

Moisture ratio is another important parameter in SSF as it affects the growth of fungus and production of FAE. Solid materials swollen in the presence of moisture content which provides the better availability of the nutrient substrate for the organism. Lower moisture ratio (1:3) showed the less enzyme activity (1787.31 U/gm) because the lower moisture ratio affects the growth of the microorganism, swelling of solid material and enzyme stability. The FAE activity reached maximum (2239.31 U/gm) at the ratio of 1:5 (Fig 2a). At this ratio, highest FAE production was achieved because of the cell membrane allows the entry of the nutrient components which may be supports the high FAE production. However, further increase in moisture ratio, affect the FAE production. High moisture content will lead to the clumping of the solid substrates which reduce the inter-particle space thereby affecting availability of the nutrients [14-15].

In the present study, 3-14 agar discs of *Pleurotus ostreatus* HP-1 were used to examine the effect of inoculum size on the production of feruloyl esterase. (Fig.2b) shows the highest FAE activity (2378.60 U/gm) was obtained in the production flask incorporated with 5 agar discs. However, higher inoculum size (above 5 agar discs) showed adverse effect on the feruloyl esterase production. This may be due to the fast depletion of the nutrient

components which affect the metabolic activity due to the competitive inhibition [16].

The pH of the production medium affects the enzyme production and avaibility of nutrients. (Fig2c) represent the effect of pH of the production medium on feruloyl esterase enzyme activity. The maximum production of the enzyme was obtained at pH-5 (2590.13 U/gm). Furthermore, it also observed that enzyme production was increased between the pH ranges 4-8. However, the enzyme production reduced drastically between the pH-9 to pH-11. Microorganisms are very sensitive to change in pH which affects the growth and production of enzyme [17]. Similar findings were also reported by Hegde and Muralikrishna (2009) [18] during their study on alkaline FAE from *Aspergillus niger* grow on wheat bran.

The effect of temperature is a most significant parameter in SSF because during the fermentation process there is a general rise in the temperature of fermenting mass due to the respiration of microorganisms. The effect of temperature on FAE production was examined in the range of 15°C to 45°C. (Fig 2d) shows the influence of the temperature on the production of feruloyl esterase by *Pleurotus ostreatus* HP-1. The results showed that the FAE production increased with increasing temperature and maximum production of the FAE enzyme was obtained at 30°C (2638 U/gm). At temperature higher than 30°C did not support the growth of the microorganisms and production of the enzyme. This can be attributed to the fact that at the higher temperature, the substrate gets dried and does not support the growth and so also at higher temperature metabolic activity of the organism adversely affected there by leading to denaturation of key enzyme [19].

Both carbon and nitrogen sources are important constituents in any production media and play an important role in overall metabolism and cellular growth of the microorganisms. In the present study, Nine different carbon sources were evaluated which support the feruloyl esterase secretion from the *Pleurotus ostreatus* HP-1. The (Fig 2e) shows that among these nine carbon sources, glucose (2637.25 U/gm) enhanced the production of FAE followed by lactose (2456 U/gm), fructose (2337 U/gm), xylose (2335 U/gm) and mannitol (2278 U/gm). Kumar et al., (2011) [20] found that potato starch was preferable carbon source for the production of FAE from *Aspergillus terreus* strain GA2.

The effect of different nitrogen sources on the production of FAE is shown in (Fig 2f). Among the different nitrogen sources, yeast extract (2882 U/gm) was found to be the most efficient nitrogen source for FAE production, followed by peptone (2456 U/gm), soybean (2336 U/gm) and ammonium nitrate (2205 U/ gm). The results obtained by us are in contradiction to the result obtained by Shin and Chen (2006) [21] who reported that complex nitrogen sources like peptone and yeast extract does not support the production of FAE. Another study reported that, casein (734 U/gm) was most effective nitrogen source for FAE production from *Aspergillus terreus* GA2 [20]. However, in our enzyme study the results obtained were completely opposite. This may be due to the organism used, as we have used a white rot fungi *Pleurotus ostreatus* HP-1 as compared to *Aspergillus*.

Statistically optimization of nutrient components

The Plackett-Burman Design was performed for the evaluation of the important components of Olga medium for FAE production by *Pleurotus ostreatus* HP-1. The two level of the concentration of each medium component for 12 trials shows the corresponding FAE production in term of unit per g of solid substrate in (Table S1). The variable denoted as X1-X8 represent the medium components and D1-

D3 are dummy variables. FAE of *Pleurotus ostreatus* HP-1 produces 2976.11 U/gm of dry substrate from solid state fermentation. The production of FAE was enhanced by statistically optimized moistening medium. (Table 1) shows the effect, standard error, t (xi), p value and confidence level of each component based on the unit per gram of dry substrate. The component of moistening medium was screened at a confidence level of 95% on the basis of their effect. Component shows positive and negative effect on the production, positive effect enhances the enzyme production while negative effect reduces the enzyme production. Among eight medium components, MnSO4, yeast extract and glucose showed CI level of 98%, 97% and 98% respectively. The results indicates that the significance of Plackett-Burman design in evaluating the significant medium components which enhance the FAE production. The further optimization of three significant components were evaluated by central composite design and 3D surface plot represent the data on FAE production (Fig 3). The data was examined by the quadratic multiple regression using a Design expert version 10.0.8. The data from the ANOVA analysis (Table S2) represent that the F-value (7.74) and p-values were less than 0.05, which indicate the quadratic model was significant at 95% confidence level. The data support that the independent variable showed significant effect on the enzyme activity of FAE.

Time course study

The time course study of feruloyl esterase production by *Pleurotus ostreatus* HP-1 was performed before and after the optimization of production medium under SSF. The statistically optimized medium increased 1.22 fold production of feruloyl esterase enzyme. The maximum production (3651 U/gm) of FAE was obtained at 9th day of incubation (Fig 4).

Purification of FAE

The extracellular feruloyl esterase enzyme was produced using *Pleurotus ostreatus* HP-1 by solid state fermentation. FAE enzyme was partially purified by the ammonium sulphate precipitation followed by size exclusion chromatography. (Table S3) summarize the results of FAE purification using methyl ferulate as a substrate. The crude enzyme was subjected to 0-80% saturation by ammonium sulphate precipitation. The maximum FAE activity containing precipitates was found in 0-50% saturation by ammonium sulphate precipitation. The precipitates were resuspended in the minimum volume of 50mM potassium phosphate buffer and dialyzed against 50mM potassium phosphate buffer. After the ammonium sulphate precipitation and dialysis, 635.95 U/mg specific activity with 1.43 fold purification and the yield was 24.32% obtained. The further purification of FAE was performed by the size exclusion chromatography (Fig S1). The first six fractions did not show any FAE activity. The FAE activity started from the fraction 7 attaining highest activity in the fraction number 13. After the size exclusion chromatography, 3.24 fold purification was obtained with a specific activity of purified FAE was 1433.69 U/mg and a yield of 1.50%.

Determination of molecular weight and activity staining

Purity and molecular weight of feruloyl esterase enzyme was confirmed by SDS-PAGE and native PAGE with silver staining. Purified FAE showed single band on the SDS-PAGE after the silver staining which indicate the purity of enzyme and the enzyme is a monomeric. The approximate molecular weight of the purified FAE of *Pleurotus ostreatus* HP-1was estimated 28 KDa (Fig 5). The zymogram study also showed the zone of hydrolysis of a single activity band which indicates that this is an active protein. Topakas et al., (2003) [22] found 27 KDa esterase from Fusarium oxysporum. Romeo-Borbon et al. (2018) [3] also found monomeric protein (34 KDa) after

the purification from *Aspergillus ochraceus*.

Characterization of purified enzyme

Partially purified FAE was characterized for optimum pH and temperature as well as for the effect of different metal ions and solvents.

Effect of temperature and pH on purified enzyme

The purified feruloyl esterase enzyme was evaluated for its maximum activity at various temperature and pH and their relative activities exhibited in (Fig 6a). The effect of temperature on purified FAE was studied in the range of 20-60°C. The reduction rate of methyl ferulate increases as the temperature is raised from 20°C to 60°C. However, the maximum relative activity was obtained at 30°C. The loss of enzyme activity was observed at 40°C and drastic decrease in enzyme activity was noticed above 50°C. Huang et al. (1994) [23] reported the optimum temperature of FAE purified from Pseudomonas flurescense in the range of 27 to 30°C.Faulds and Williamson (1991) [24] found 30°C temperature optima for FAE of Streptomyces olivochromogenes. Temperature stability (Fig 6b) was performed using methyl ferulate as substrate. The results revealed that the 75-85% residual activity was retained after 2 h in the temperature range 20-40°C. After 48 h of pre-incubation, the purified FAE at 20°C showed 40% active enzyme remaining. As the temperature increased the stability decreases, <50% activity was retained at 50-60°C after 1h. There was a drastic decrease in the activity after the further incubation at 50-60°C. Increase in temperature a steady decrease of the enzyme activity, suggest the denaturation of the enzyme. A gradual increase in the temperature leads to an increase in reaction rate as heat allows the preparation of the groups that react and reached the transition state at a given time. However, when the temperature is increased to 30-60°C the heat results in denaturation of the enzyme. Most of the proteins are marginally stable, denaturation occurs resulting in sharp decline in enzyme activity [25]. FAE of *Aspergillus terreus* shows thermal stability up to 55°C [12].

The optimum pH of the purified enzyme was analyzed in the range of pH 3.0-9.0(Fig 6c). The optimum pH of the purified enzyme was obtained at pH 6.0. (Fig 6d) shows the pH tolerance of feruloyl esterase. The maximum residual activity 78.39% was obtained at the pH 6.0 and more than 50% activity was observed for purified FAE in the range of pH 5.0-7.0. However, the enzyme stability was decreased in the alkaline pH. It also suggests the potential to acidic environment. FAE (FAE-III) from *Aspergillus niger* shows pH optimum at 5 [26]. Similar results were also observed forFAE purified from Fusarium proliferatum. The FAE of Fusarium proliferatum was also stable in the range of pH 5.0-9.0 [21].

Effect of metal ions and organic solvents

The purified FAE enzyme was incubated with a range of metal ions with the different concentration to assess the effect of metal ions on enzyme (Table S4). Among nine different metal ions, Co+2, Mn+2, Ni+2 stimulate the enzyme activity 101%, 134% and 147% respectively at the concentration of 10mM. The increase in the FAE activity in the presence of some metal ions which may be due to the stability of the enzyme substrate complex by maintaining the active site of the enzyme and it also protect from the thermal denaturation [27-28]. On the contrary, Cu+2, Hg+2, Fe+2, Ca+2 showed inhibitory effect on the purified enzyme. At the concentration of 100 mM, Cu+2, Hg+2, Fe+2 and Ca+2 reduced the activity of enzyme to 86%, 87%, 88% and 86% respectively. These metal ions may affect to the enzyme catalysis activity, which reduce the enzyme activity. Mercury ions react with thiol group and it

converts them to the mercaptides, it also reacts with histidine and tryptophan residues of the protein. Another observation found that Hg+2 interact with the –SH and –SS bond of the protein and change the structural and functional property [29]. Similar behavior was observed by the Wang et al.,(2004)30 for FAE of Lactobacillus acidophilus. The metal ions Cu+2 and Fe+2 reduced the 97.25% and 94.80% FAE activity, respectively at a concentration of 5 mmol/liter.

The activity of FAE of *Pleurotus ostreatus* HP-1 was evaluated in the absence and presence of a range of organic solvent at different concentration (10%, 25% and 50% v/v) (Table S5). FAE exhibit different tolerance for different solvents. Among ten different organic solvents, benzene, n-hexane and chloroform enhanced the residual activity (179.32%, 100.86%, and 103.66% at 10%v/v), but the enzyme's stability was decreased as the concentration of the n-hexane and chloroform increased (25 and 50% v/v). The increase in the enzyme activity in the presence of these solvents which may be due to the solvent act as a homogenous co-solvent and enhanced the solubility [31]. However, ethanol and isopropyl alcohol inhibit the enzyme activity at 50%v/v concentration and showed 8.75% and 12.94% relative activity, as alcohol reduce the hydrolytic efficiency of the enzyme.

Substrate specificity

The data of substrate specificity and kinetic parameters for FAE of *Pleurotus ostreatus* HP-1 is shown in (Table 2). The substrate specificity was evaluated against four different substrates (MFA, MCA, MρCA, MSA). FAE catalyze all the four substrates and among these four different substrates enzyme shows highest substrates specificity against MFA followed by MCA, MρCA, and MSA. The purified FAE form *Pleurotus ostreatus* HP-1exhibits the lowest km value (0.555mM) with methyl ferulate which indicates the high affinity for methyl ferulate under optimized conditions. The purified FAE also shows the highest catalytic efficiency (1.2 × 106) with MFA compared to other substrates. Similar results have been observed by Wang et al.,(2014) [4] for FAE of Russula virescens. The feruloyl esterase of Russula virescens exhibited the highest specificity against methyl ferulate with 0.19 mM km value.

Conclusion

The present study proved that white rot fungus *Pleurotus ostreatus* HP-1 is a promising microorganism for the production of feruloyl esterase enzyme. The present work also proved that the utilization of lignocellulosic banana pseudostem waste as a low-cost and easily available solid material for the production of FAE under solid state fermentation. The production of feruloyl esterase by *Pleurotus ostreatus* HP-1 was also optimized by the method "one factor-at-a-time". After the optimization the production of feruloyl esterase enhanced by 1.4 fold compared to unoptimized conditions. Further, the feruloyl esterase production by *Pleurotus ostreatus* HP-1 was statistically optimized. This statistically optimized study significantly increased the production of feruloyl esterase by 22.68% on the 9th day of incubation. The FAE of fungus *Pleurotus ostreatus* HP-1was purified and characterized to explore the characteristic of the enzyme. The purified feruloyl esterase enzyme was a monomeric protein with an approx. molecular weight of 28 KDa. The purified enzyme showed a wide range of pH stability and moderately thermo stability. This purified FAE enzyme can be a greener route of production of ferulic acid.

Table 1: Statistical analysis of component of Olga medium for FAE production as per Plackett-Burman design

Factors	Medium component	Effect Exi	t(xi)	p-value	CI (%)
X1	KH_2PO_4	-54.67	-0.00317	0.878	12.15
X2	K_2HPO_4	176.81	0.010249	0.212	78.83
X3	$MgSO_4$	83.73	0.004853	0.724	27.61
X4	$MnSO_4$	396.43	0.022978	0.012	98.81
X5	$FeSO_4$	4.85	0.000281	0.999	0.096
X6	$ZnSO_4$	234.92	0.013617	0.085	91.461
X7	Yeast extract	-316.53	-0.01835	0.028	97.16
X8	Glucose	348.63	0.020208	0.02	98.04

Table 2: Substrate specificity for *Pleurotus ostreatus*HP-1 feruloyl esterase

Substrate	Specific activity (U/mg)	Km (mM)	Kcat (S^{-1})	Kcat/Km (S^{-1} M^{-1})
Methyl ferulate	511.24	0.555	6.7×10^5	1.2×10^6
Methyl ρ-coumarate	301.84	1.281	8.7×10^5	0.7×10^6
Methyl caffate	387.86	0.892	7.8×10^5	0.8×10^6
Methyl sinnapate	0.981	2.165	2.5×10^5	0.1×10^6

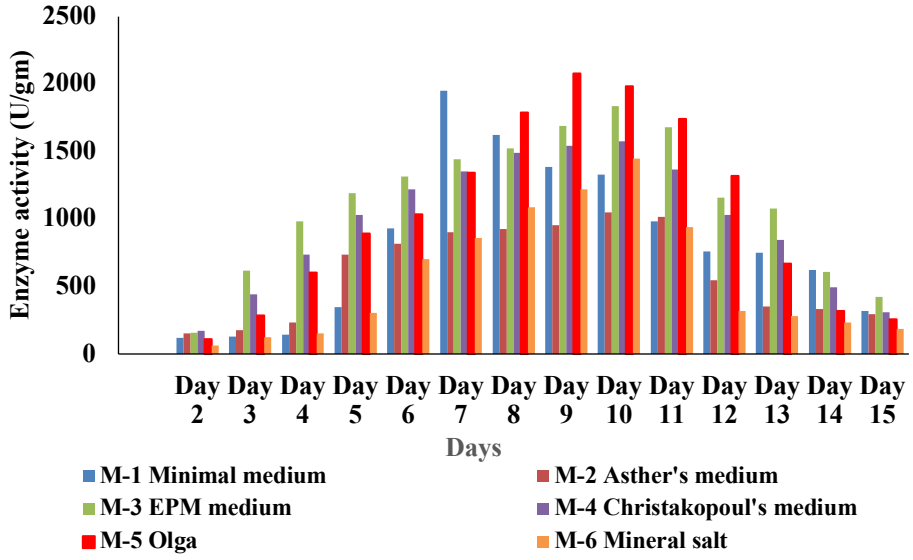

Fig. 1: Optimization of different moistening medium for feruloyl esterase production

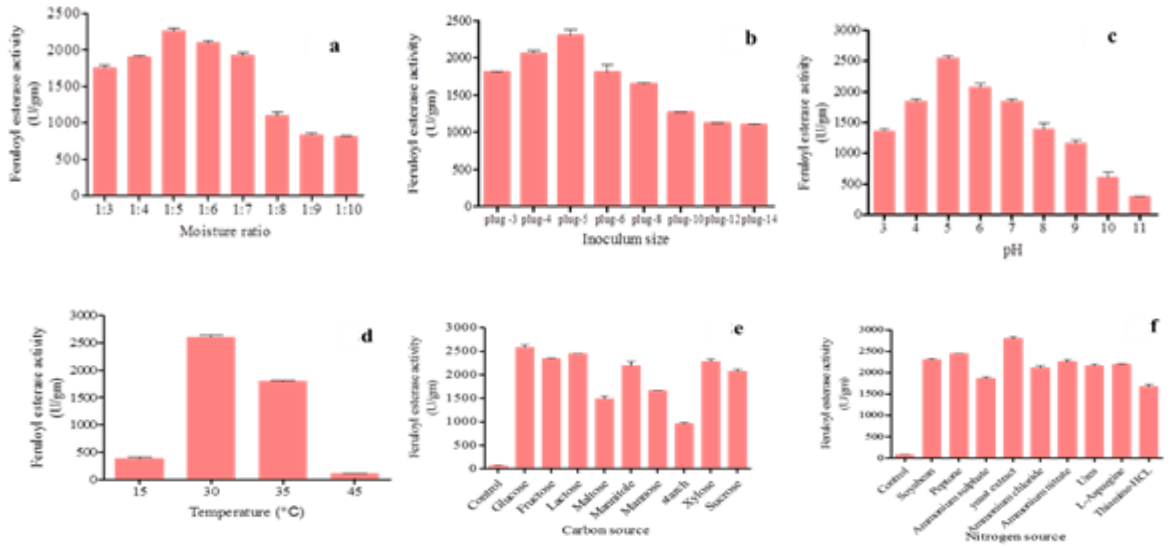

Fig. 2: Effect of substrate to moisture ratio (a), inoculum size (b), pH (c), temperature (d), carbon source (e) and nitrogen source (f) on FAE production

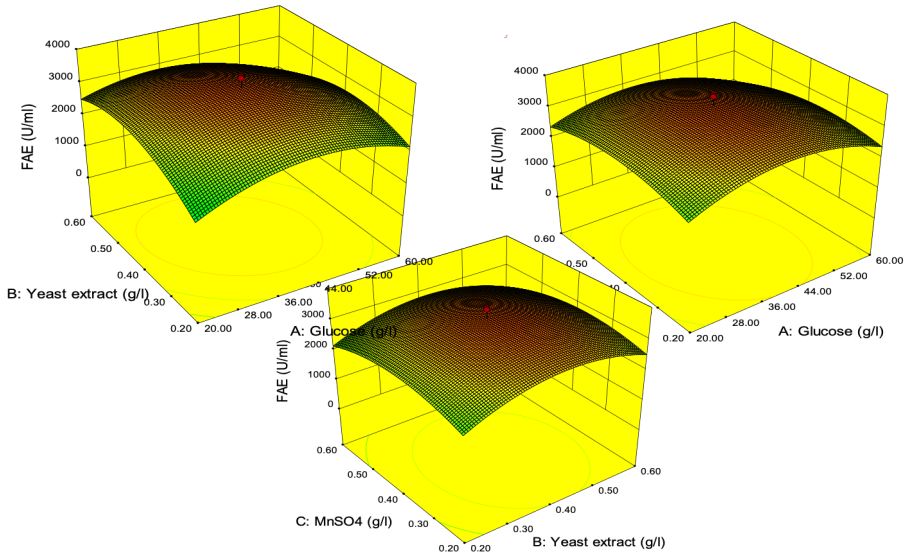

Fig. 3: Contour plot showing the effect of glucose and yeast extract (a), $MnSO_4$ and glucose, (b) $MnSO_4$ and yeast extract (c)

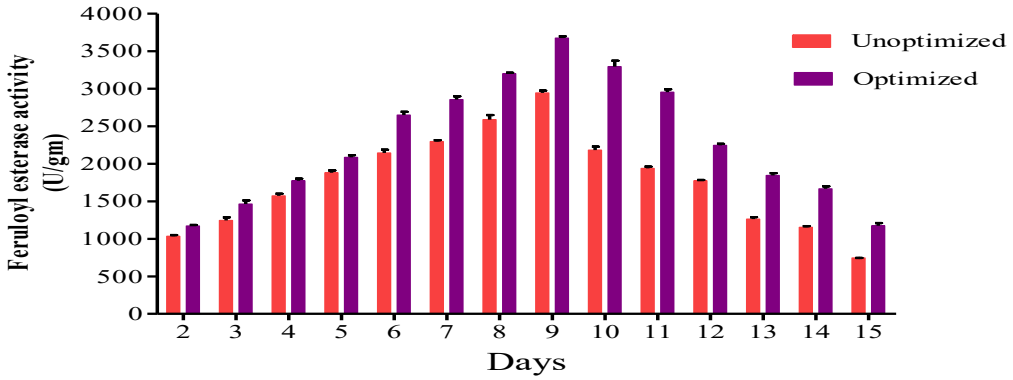

Fig. 4: Time course study of feruloyl esterase production by *Pleurotus ostreatus* HP-1 using unoptimized medium and statistically optimized Olga medium

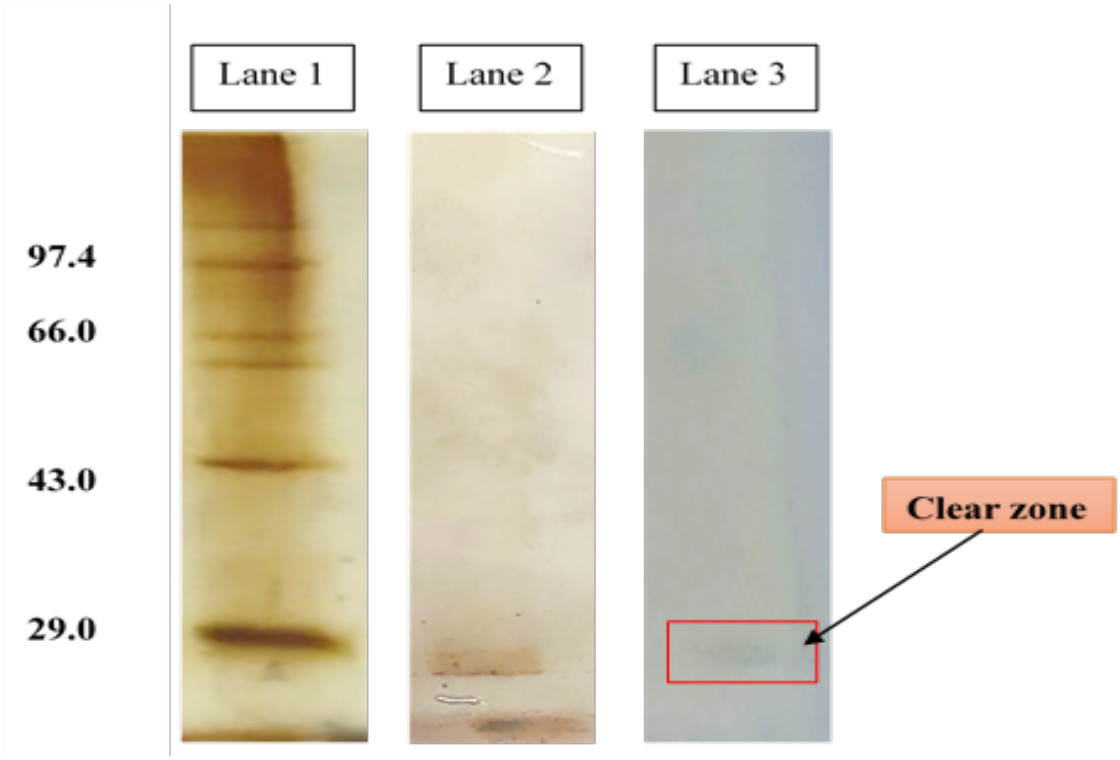

Fig. 5 Molecular mass determination of purified through SDS-PAGE, Lane 1 molecular weight marker (KDa), Lane 2 purified feruloyl esterase from Sephadex G-75, Lane 3 Active staining

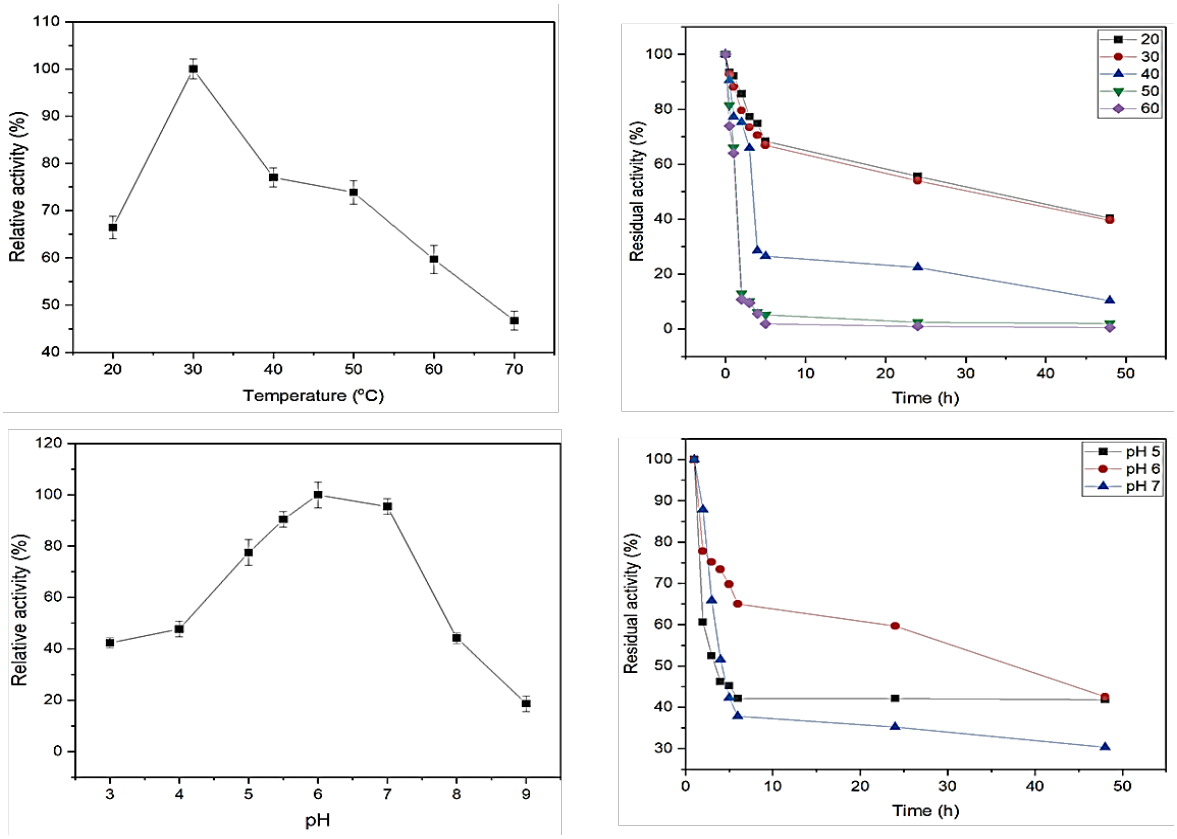

Fig. 6: Optimum temperature (a), temperature stability (b), pH optimum (c) and pH stability (d) of purified from *Pleurotus ostreatus* HP-1

Table S1: Plackett-Burman matrix of eight variables (X1-X8) and three dummy variables (D1-D3) along with response

Trial	KH_2PO_4 X1	K_2HPO_4 X2	$MgSO_4$ X3	$MnSO_4$ X4	$FeSO_4$ X5	$ZnSO_4$ X6	Yeast extract X7	Glucose X8	D1	D2	D3	Enzyme activity (U/m)
1	+	+	-	+	+	+	-	-	-	+	-	1293.78
2	-	+	+	-	+	+	+	-	-	-	+	476.33
3	+	-	+	+	-	+	+	+	-	-	-	1180.08
4	-	+	-	+	+	-	+	+	+	-	-	1204.67
5	-	-	+	-	+	+	-	+	+	+	-	1314.48
6	-	-	-	+	-	+	+	-	+	+	+	762.13
7	+	-	-	-	+	-	+	+	-	+	+	322.67
8	+	+	-	-	-	+	-	+	+	-	+	1105.10
9	+	+	+	-	-	-	+	-	+	+	-	531.65
10	-	+	+	+	-	-	-	+	-	+	+	1346.03
11	+	-	+	+	+	-	-	-	+	-	+	829.74
12	-	-	-	-	-	-	-	-	-	-	-	487.60

Table S2: Analysis of variance (ANNOVA) for the quadratic model

Source	Sum of squares	Df	Mean square	F-value	p-value prob>F	
Model	2.663E+007	14	1.902E+006	7.74	0.0002	**Significant**
A-Glucose	1.456E+005	1	1.456E+005	0.59	0.4532	
B-Yeast extract	2.347E+005	1	2.347E+005	0.96	0.3438	
C-MnSO4	1.550E+005	1	1.550E+005	0.63	0.4393	
D-ZnSO4	1.171E+005	1	1.171E+005	0.48	0.5005	
AB	1.201E+006	1	1.201E+006	4.89	0.0429	
AC	2.531E+005	1	2.531E+005	1.03	0.3262	
AD	6.925E+005	1	6.925E+005	2.82	0.1138	
BC	31745.44	1	31745.44	0.13	0.7242	
BD	34287.00	1	34287.00	0.14	0.7139	
CD	3491.92	1	3491.92	0.014	0.9067	
A^2	1.255E+007	1	1.255E+007	51.09	< 0.0001	
B^2	1.085E+007	1	1.085E+007	44.19	< 0.0001	
C^2	5.201E+006	1	5.201E+006	21.18	0.0003	
D^2	4.274E+006	1	4.274E+006	17.40	0.0008	

Residual	3.684E+006	15	2.456E+005			
Lack of Fit	2.014E+006	10	2.014E+005	0.60	**0.7681**	**Not significant**
Pure Error	1.670E+006	5	3.339E+005			
Cor Total	3.031E+007	29				

Table S3: Purification of feruloyl esterase from *Pleurotus ostreatus*HP-1

Steps	**Total enzyme activity (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Purification fold**	**Yield**
Crude enzyme	65887.2	148.8	442.25	1	100
Ammonium precipitation and Dialysis	16026.01	25.2	635.95	1.43	24.32
Size Exclusion Chromatography	989.25	0.69	1433.69	3.24	1.50

Table S4: Effect of various metal ions on purified feruloyl esterase enzyme

Metal ions	**Residual activity (%)**	
	10mM	**100mM**
Control	100	100
Co^{+2}	101.40	89.60
Cu^{+2}	60.99	14.49
Hg^{+2}	66.21	13.58
Mg^{+2}	90.59	48.97
Mn^{+2}	134.27	83.04
Fe^{+2}	36.39	12.67
Ni^{+2}	147.96	97.96
Ca^{+2}	38.23	14.23
Zn^{+2}	55.76	19.34

Table S5: Effect of various solvents on purified feruloyl esterase of *Pleurotus ostreatus* HP-1

Solvent	**Residual activity (%)**		
	10% (v/v)	**25% (v/v)**	**50% (v/v)**
Methanol	83.63	69.78	17.79
Ethanol	90.19	79.2	8.75
DMSO	82.39	80.18	27.14

Acetone	85.95	79.99	49.47
Ethyl acetate	94.52	83.64	52.84
Benzene	179.32	115.31	105.49
n-hexane	100.86	85.56	72.37
Chloroform	103.66	87.48	67.28
Isopropyl alcohol	94.81	78.04	12.94
Butanol	67.94	43.88	35.21

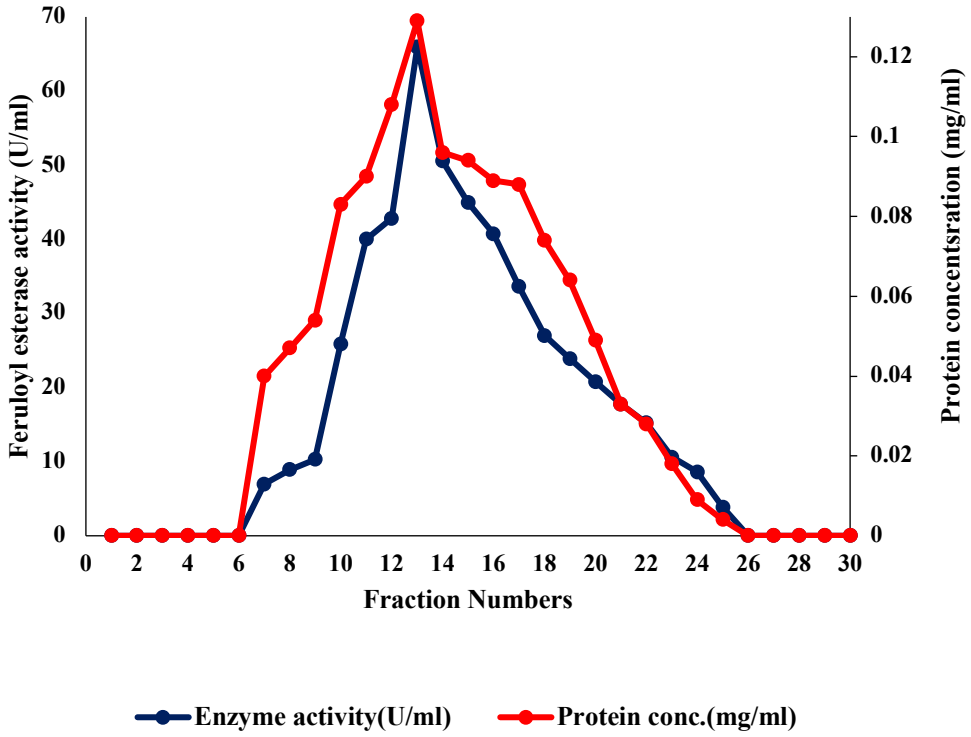

Fig.S1: Elution profile of feruloyl esterase on Sephadex G-75 gel permeation Chromatography

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