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Production of Ferulic Acid Esterase from Trichophytonajelloi MTCC 4878

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Abstract: Ferulic acid esterase is the enzyme, hydrolyze the ester bond between the ferulic acid and polysaccharides present in plant cell wall and release ferulic acid from agro residues. The production of ferulic acid esterase activity by bacterial and fungal strains was detected in agar plate assay. The assay involves ethyl ferulate as a sole carbon source in specific media. Of the examined strains Trichophyton ajelloi MTCC 4878 showed the highest level of ferulic acid esterase activity 397 U/mg at the optimum pH and temperature of 5.0 and 30°C. Supplementation of soyabean meal (1%) as nitrogen source and sucrose (0.1%) as additional carbon source enhances enzyme production.

1. INTRODUCTION

Ferulic acid (FA) is a phenolic acid which is most abundantly found in the plant cell wall polysaccharides such as arabinoxylans. FA and dihydroferulic acid are component of lignocelluloses and confer rigidity to plant cell wall by cross-linking the Lignin and polysaccharides [1]. This phenolic acid and their derivatives such as *p*-coumaric acid (4-hydroxycinnamic acid) are esterified to the 5'-OH group of arabinose residues which present as side chain of xylans. Synergistic action between main chain degrading enzymes such as endo-*β*-(1,4)-xylanases and ferulic acid esterase require for the complete degradation of plant cell wall structure which remove FA.

FA confers potential applications in various industries such as pharmaceutical, foods and cosmetics due to its antioxidant property. It also possesses wide physiological and therapeutic properties including anti-inflammatory, anti-diabetic, anti-microbial, anti-cancer and anti-ageing effects [2]. The free radical scavenging activity of ferulic acid is provided by three distinctive structural motifs present in it. Presence of 3 – methoxy and 4 -hydroxy electron donating groups provide it the ability to stop free radical reaction. The presence of carboxylic acid group with an adjacent C-C double bond prevents addition attack of free radical to the membrane. The carboxylic acid helps ferulic acid to bind with lipid bi-layer and thus protects against lipid per-oxidation [2]. Ferulic acid is also involved in the natural production of vanillin (a flavouring compound have application in foods, pharmaceuticals, beverages and perfumes) through its microbial biotransformation.

Wide application of ferulic acid in various industries leads to focus the research on its release from the walls of agro-residues in large scale. The release of ferulic acid from agricultural waste can be achieved by either chemical treatment such as acid or alkaline hydrolysis or by enzymatic method. Chemical treatment methods for the release of FA from agricultural waste provides cheaper alternative to enzymatic method but is chemically very harsh and involves the use of very toxic, corrosive and hazardous chemicals such as strong acids and base. Alkaline hydrolysis method cannot be applicable if the lignin content of agro-residue is higher than 26%. The enzymatic method for the release of FA from agro-residues involves the use of microbial carboxylic acid esterases enzymes such as ferulic acid esterase (FAE). Enzymatic method provides environment friendly and very mild method for the release of FA [3]. Ferulic acid esterases (FAE; E.C. 3.1.1.73) are a subclass of carboxylic acid esterases that play a key role in degrading the plant cell wall structure by cleaving the ester bond between hydroxycinnamic acids esterified to arabinoxylans and pectins present in plant cell walls [4]. This study focused on the release of ferulic acid from agro redues using ferulic acid esterase enzyme produced by *Trichophyton* and other microorganisms.

2. MATERIAL AND METHOD

2.1 Microbial strains and culture conditions

All microbial cultures were obtained from Departmental culture collection of Department of Bio-Engineering, Birla Institute of Technology, Mesra Ranchi. The sub-culturing of bacterial strains was done on the slants of nutrient agar (NA)

and that of fungal strains was done on the slants of potato dextrose agar (PDA). After 24 hour of incubation this bacterial culture was transferred into the nutrient broth and incubated at 37ºC, which was used as seed culture. The 6 days old fungal culture from slant was further transferred into the potato dextrose broth (PDB) and again allowed to grow for 6 days at room temperature which was then used for the further inoculation of media.

2.2 Screening of the ferulic acid esterase producing microorganisms

Total of 12 bacterial and 16 fungal cultures were screened for FAE activity. Bacterial cultures were screened on minimal agar plate consisting of (g/L) $(NH_4)_2SO_4$ (1.3), KH_2PO_4 (0.37), $MgSO_4.7H_2O$ (0.25), $CaCl_2$ (0.07), $FeCl_3$ (0.03), yeast extract (1.0) and bacteriological agar (20) and pH 6.5 [5]. Media was supplemented with syringe filtered 1% (*v/v*) ethyl ferulate (10% *w/v* in dimethylformamide)at the plate-pouring stage. Dried agar plates were inoculated with cultures from nutrient agar slants (24 h) and incubated at 37°C. Fungal media contain (g/L), $(NH_4)_2SO_4$ (1.5), KH_2PO_4 (0.5), Na_2HPO_4 (1.5), $MgSO_4.7H_2O$ (0.25 g), $CaCl_2$ (0.05 g), $FeCl_3$ (0.01 g), yeast extract (1.0 g), and bacteriological agar (20.0 g). The sterile medium was supplemented with syringe filtered ethyl ferulate (1%) in 10% (*w/v*) solution of dimethylformamide (DMF) [5]. Assay plates were inoculated with the fungal isolate from the fresh potato dextrose agar slants and were statically incubated at 25℃ for 5 days. FAE activity was confirmed with formation of halo-zone around the point of inoculation.

2.3 Production of enzyme

In the screening assay the fungal strain *Trichophytonajelloi*MTCC 4878 was observed with the maximum FAE activity. T ajelloi was selected for further study, inoculated in potato dextrose broth for 6 days at 30°C which then transferred into 250ml flask containing 50 ml of EPM (enzyme production media) having following composition (g/L), $(NH_4)_2SO_4$ (1.5), KH_2PO_4 (0.5), Na_2HPO_4 (1.5), $MgSO_4.7H_2O$ (0.25 g), $CaCl_2$ (0.05 g), $FeCl_3$ (0.01 g), yeast extract (1.0 g), pH 5.0 and supplemented with 1% ethyl ferulate as C-source dissolved in 1% (*w/v*) dimethylformamide. Media containing fungal culture were then incubated at 25 ℃ for 4-10 days. EPM inoculated with culture was filtered through filter paper after 4-10 days of incubation to remove fungal mycelium and spores. Quantification of enzymeactivity was done by HPLC analysis.

2.4 Determination of FAE activity

FAE activity was quantified by HPLC using ethyl ferulate as substrate. HPLC reaction was prepared by 0.1 ml of substrate (6mM ethyl ferulate) and 0.5 ml of crude enzyme solution, incubated at 37 ºC water bath for 1h. After incubation the reaction was terminated by adding equal volume of stop solution (methanol: acetic acid, 4:1), filtered through a 0.2 µm syringe filter and quantified by HPLC. HPLC analysis was done on a reverse-phase HPLC equipped with a C_{18} column (Waters XTerra RP18 5 µm, 4.6×150 mm). HPLC setup includes a waters 1525 binary HPLC pump, waters 717 plus auto-sampler and waters 2996 photodiode array detector. The analysis of result was done using the Empower Pro software. The sample volume injected was 20 µl and the elution of product (ferulic acid) and substrate (ethyl ferulate) was done in an isocratic solvent system of water: methanol (40:60) ratio as the mobile phase at the flow rate of 1 ml/min for 10 min and the peak of product and substrate was monitored at 254 and 310 nm. Ferulic acid esterase activity was calculated from the ferulic acid standard curve. One unit of ferulic acid esterase activity was defined as the amount of enzyme (FAE) required releasing 1 µmol of ferulic acid per minute and specific activity was given in units/mg of protein. Total protein concentration was executed by Bradford's method.

2.5 Optimization of production conditions for FAE

The fungal strain *Trichophytonajelloi*MTCC 4878 which was observed to give the maximum ferulic acid esterase activity was selected for the further optimization process. Optimization of media components (C-source and N-source) and environmental factors (days, pH and temperature) was performed to enhance the ferulic acid esterase production.

2.6 Optimization of environmental factors (days, pH and temperature)

Initially the effect of days was optimized by incubating the culture for 4-10 days and quantified the enzyme activity. Effect of pH on the ferulic acid esterase activity was examined by preparing minimal media with ph range of 4-8. In order to examine the effect of temperature with respect to days on FAE production the media was incubated at temperature range of 25- 45 ºC for 4-10 days. Then the HPLC analysis and Bradford's assay of crude extract of enzyme was done to calculate specific activity of enzyme.

2.7 Optimization of media components (Carbon source and nitrogen source)

In the submerged fermentation by *Trichophyton ajelloi*, the effect of different C-source on FAE activity was examined included dextrose, potato starch, D-fructose, sucrose and lactose at a concentration of 1% (w/v). Effect of nitrogen sources on FAE production was examined by using different nitrogen sources such as soya-bean meal, yeast extract, peptone, beef extract and casein having 0.1% (w/v) concentration.

3. RESULT AND DISCUSSION

Total 12 bacterial and 15 fungal strains were screened for FAE activity, *T. ajelloi* was observed with the maximum enzyme activity.

3.1 Optimization of environmental factors (days, pH and temperature)

In our studies, fermentation conditions were optimized for the production of ferulic acid esterase using*Trichophyton ajelloi*. Here we used submerged fermentation (SF) for FAE production and optimization of environmental factors (days, pH and temperature) and medium components (carbon source, nitrogen source with their concentration in media) was performed to study the effect of these factors on the activity of FAE and to enhance its production.

3.1.1 Optimization of incubation period

T.ajelloi indicates the presence of maximum enzyme activity at 30°C for 6 days incubation. However comparatively low FAE activity was detected at 4 days, 8 days and 10 days ofincubation (figure 1).

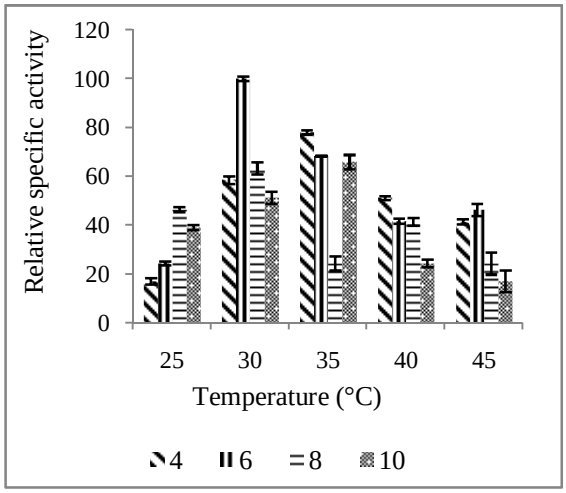

Figure 1 Effects of temperature/Incubation period on FAE production by *Trichophyton ajelloi*

3.1.2 Optimization of pH

The production of FAE from *Trichophyton ajelloi* was observed in the range of 3.0 to 10.0, with the optimum pH of 5.0 (figure 2). The optimal pH value obtained is very similar to that obtained for *Aspergillus niger* strain CFR 1105 [6]. There was significant enzyme production in the pH range of 5.0-6.0; however the specific activity of enzyme was very low above pH 7. Requirement of slightly acidic condition for the better growth of fungus *Trichophyton ajelloi*incorporated in good production of FAE in pH 5.

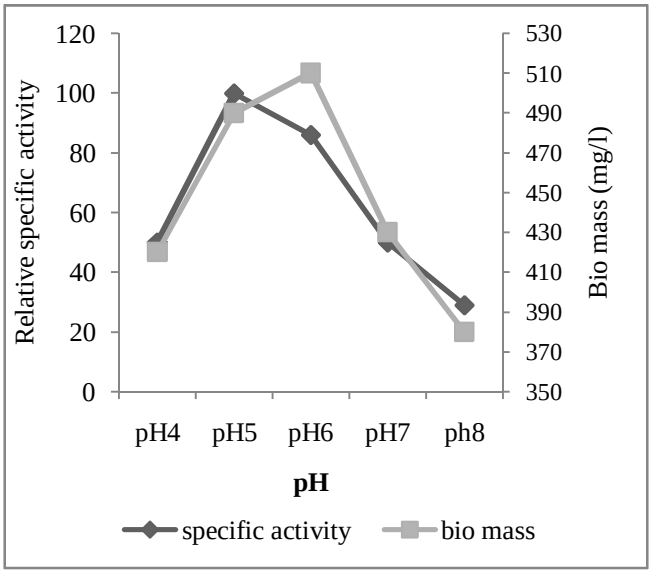

Figure 2 Effects of pH on FAE production by *Trichophyton ajelloi* (temperature 30°C, nitrogen source yeast extract)

3.1.3 Optimization of temperature

The optimization of temperature for production ferulic acid esterase from *T.ajelloi* was performed using 1% ethyl ferulate as substrate and pH of the medium was kept at optimum value 5. The temperature range studied was from 25 °C to 45°C. Maximum enzyme production was observed in temperature range of 25-35°C and 25°C was observed as optimum temperature for the production of FAE from *T.ajelloi* showing specific activity of enzyme 54.2 U/mg of protein (Figure 3). The specific activity of enzyme decreased significantly after temperature of 35°C that may be due to the loss of enzyme activity because of degradation of enzyme's secondary structure at high temperature.

The temperature range observed by Kumar et al., 2008 for the maximum production of FAE was 25-50°C with the optimum temperature of 30 °C which shows sufficient similarity with result obtained in our study. They also observed decrease in

enzyme production at temperature above 35 °C and below 30 °C.

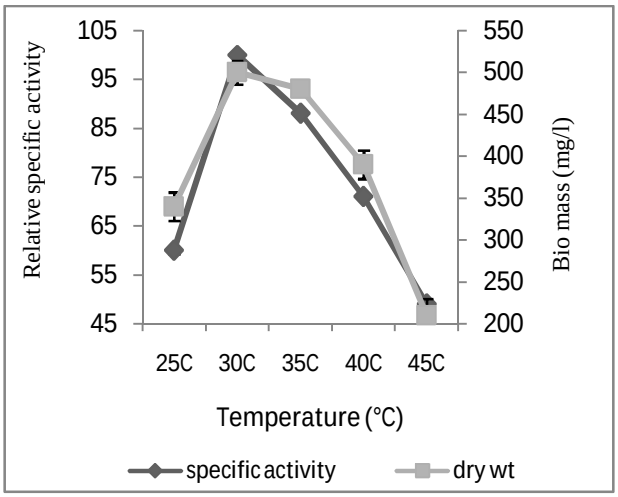

Figure 3 Effects of temperature on FAE production by *Trichophytonajelloi*

3.2.Optimization of media components

3.2.1. Optimization of carbon source

Proper overall cellular growth and metabolism require good carbon and nitrogen source which are essential constituents of the fermentation medium. In this study 1% (*w/v*) ethyl ferulate was used as sole carbon source which required for the production of FAE. Apart from ethyle ferulate additional C-source was added to enhance the growth of fungal culture and thus increase the production of FAE. To test the effect of different additional carbon source on FAE production, 1% (*w/v*) of this carbon source was supplemented in culture media along with ethyl ferulate. Sucrose was observed as the most effective carbon source showing maximum specific activity of enzyme FAE (100 U/mg of protein) produced by *T.ajelloi*. However lactose, fructose, potato starch and dextrose show specific activity of 57.64, 45.12, 40.34 and 41.87 U/mg of protein respectively. Figure4 is showing the effect of different C-sources on FAE production.

Potato starch (860 U/gds), fructose (848 U/gds) and sucrose (839U/gds) were observed as suitable carbon sources that favoured ferulic acid esterase production that shows relevance with this study [7].

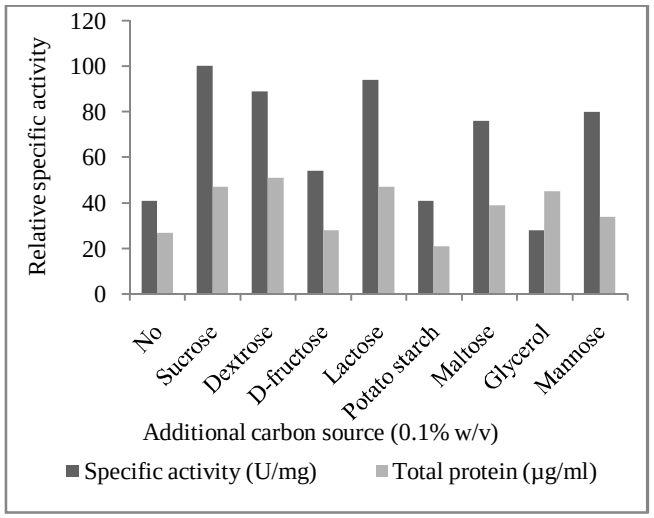

Figure 4 Effects of various carbon source on FAE production by *Trichophytonajelloi*

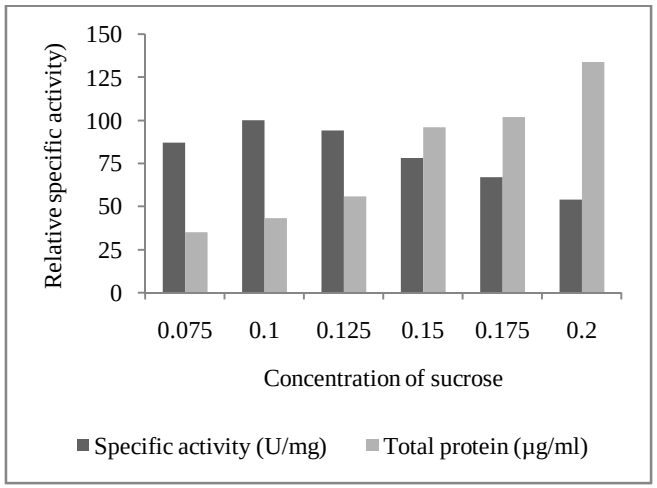

Figure 5 Effects of concentration of sucrose on FAE production by *Trichophytonajelloi*

3.2.2. Optimization of nitrogen source

Nitrogen source are also very essential constituents of fermentation medium required for the proper growth of microorganism. Among all the nitrogen sources selected for optimization process (soya-bean meal, yeast extract, beef extract, peptone and casein), highest specific activity of ferulic acid esterase was observed for 0.1 % (*w/v*) of soya-bean meal that was 121.42 U/mg of protein followed by beef extract and yeast extract which showed comparatively lesser specific activity that was 65.94 and 53.03 U/mg of protein. Casein and peptone showed minimum specific activities of 53.8 and 51.86 U/mg of protein respectively. Effect of different nitrogen sources on FAE activity has been shown in Figure 6 .

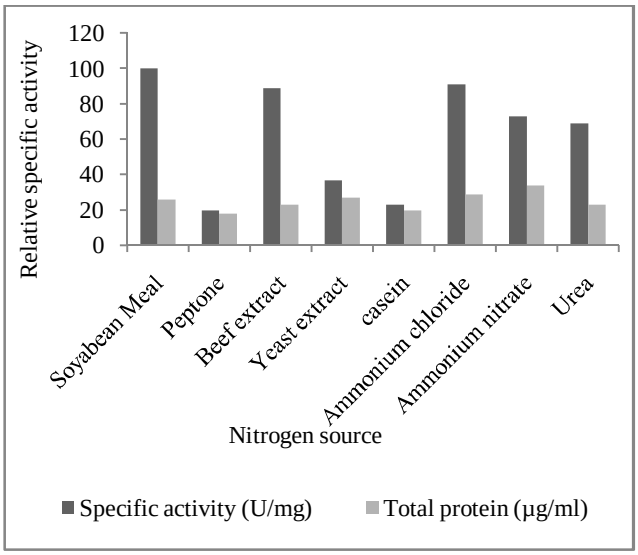

Figure 6 Effects of nitrogen source on FAE production by *Trichophytonajelloi*

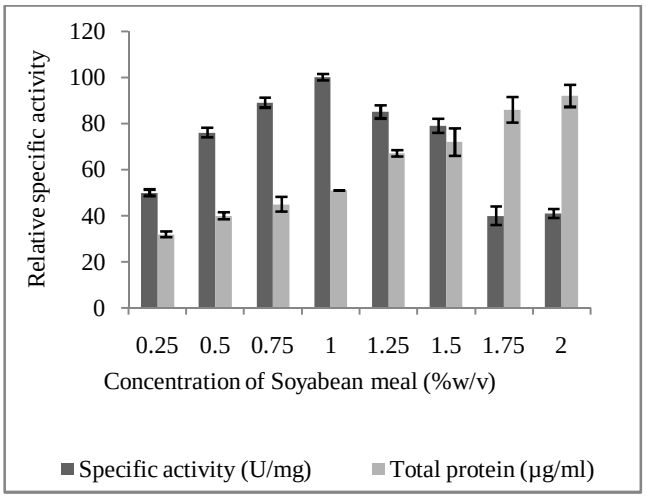

Figure 7 Effect of different concentrations of soyabean meal on ferulic acid esterase production.

In earlier study carried out by Shin and Chen, 2006, it was found out that complex nitrogen sources such as yeast extract and peptone do not support FAE production as observed in this study too. That shows that yeast extract and peptone are not suitable nitrogen sources for FAE production.

We also studied the effect of concentration of soya-bean meal that was observed as most appropriate C-source for ferulic acid esterase production, by adding different concentration (0.25-2.0%, *w/v*) of soya-bean meal into the minimal media. Maximum specific activity was observed when 1% (*w/v*) soya-bean meal was used as nitrogen source (Figure 7). sHowever, very low FAE activity was observed when 1.75 and above percentage was used. Thus it shows that high concentration of nitrogen source doesn't support ferulic acid esterase production [7].

4. CONCLUSION

As reported, FAE have potential application in baking and allied industries and their importance in food industries and pharmaceutical industries has fueled much of interest in the enzyme. In this study, number of strains was assayed for their ability to produce FAE and found that *Trichophytonajelloi* MTCC 4878 demonstrated highest activity. The work is in progress to enhance the production of FAE from *Trichophytonajelloi*along with purification and characterization of enzyme ferulic acid esterase.

5. ACKNOWLEDGEMENT:

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