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Parametric Optimization of Feruloyl Esterase Production from *Aspergillus terreus* Strain GA2 Isolated from Tropical Agro-Ecosystems Cultivating Sweet Sorghum

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A fungal strain, *Aspergillus terreus* strain GA2, isolated from an agricultural field cultivating sweet sorghum, produced feruloyl esterase using maize bran. In order to obtain maximum yields of feruloyl esterase, the solid state fermentation (SSF) conditions for enzyme production were standardized. Effective feruloyl esterase production was observed with maize bran as substrate followed by wheat bran, coconut husk, and rice husk among the tested agro-waste crop residues. Optimum particle size of 0.71–0.3 mm and moisture content of 80% favored enzyme production. Moreover, optimum feruloyl esterase production was observed at pH 6.0 and a temperature of 30°C. Supplementation of potato starch (0.6%) as the carbon source and casein (1%) as the nitrogen source favored enzyme production. Furthermore, the culture produced the enzyme after 7 days of incubation when the C:N ratio was 5. Optimization of the SSF conditions revealed that maximum enzyme activity (1,162 U/gds) was observed after 7 days in a production medium of 80% moisture content and pH 6.0 containing 16 g maize bran [25% (w/v)] of particle size of 0.71–0.3 mm, 0.6% potato starch, 3.0% casein, and 64 ml of formulated basal salt solution. Overall, the enzyme production was enhanced by 3.2-fold as compared with un-optimized conditions.

Keywords: *Aspergillus terreus*, feruloyl esterase, ferulic acid, maize bran, SSF

Ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) is one of the hydrocinnamic acids abundantly present in the hemicelluloses of plant cell wall. FA is covalently cross-linked at the 5' hydroxyl groups of the arabinoxylans by an ester linkage [18, 27]. It plays a functional role in structural integrity of the plant cell wall [14]. FA finds use in a wide range of applications in different industries; it is used in the food industry as a food preservative owing to its antioxidant and radical scavenging properties [28], as well as to enhance the viscosity or gelation properties of foods [17], and it can be enzymatically transformed to many value-added products like vanillic acid, vanillin, and vinyl guaiacol that are used as flavoring agents in foods, beverages, and perfumes [24, 38]. It is also used in paper and textile industries [31].

Feruloyl esterases (FAEs, E.C. 3.1.1.73), also known as ferulic acid esterases, are a subclass of carboxylic acid esterases (E.C. 3.1.1.1). They are classified into four types (A, B, C, and D) based on their amino acid sequence identity and substrate specificity towards mono- and di-ferulates [6, 15]. FAEs release ferulic acid and other cinnamic acids from plant cell walls [39], and function as accessory enzymes in combination with xylanases for breaking the diferulic bridges between xylan chains and releasing lignin from the biomass [34, 40]. Xylan, pectin, wheat bran, or sugar beet pulp are good substrates for these enzymes [12]. Additionally, FAEs have the ability to release caffeic acid, p-coumaric acid, and ferulic acid from agro-industrial by-products like coffee pulp, apple marc, and wheat straw [4]. FAEs were first identified in *Streptomyces viridosporus* [7]; later they were purified and characterized from fungi like *Aspergillus niger* [3], *Aspergillus flavipes* [25], *Aspergillus awamori* [13], *Penicillium* [5, 9] *Fusarium oxysporum* [36], and *Fusarium proliferatum* [33]. There is a growing interest in feruloyl esterases as promising biocatalysts in the processing of hemicelluloses [32], pulp bleaching [31], as well as in the production of phenolic acids such as ferulic, p-coumaric, caffeic, and sinapic acids as fine chemicals [11, 19] from natural sources. Moreover, recovery of ferulic acid from agricultural waste residues is important as it is a precursor for vanillin production, having huge global demand and high market value [38].

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The aim of this study was to screen feruloyl-esterase-producing fungi isolated from the agricultural fields of Medak district of tropical South India cultivating sweet sorghum that could eventually be used for the recovery of ferulic acid from agro-wastes. In addition, the solid state fermentation (SSF) conditions were optimized for *Aspergillus terreus* strain GA2 using maize bran as substrate that favors maximum feruloyl esterase production.

Materials and Methods

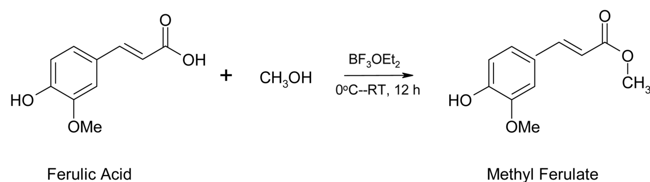
Chemicals

Boron trifluoride diethyl etherate (BF_3OEt_2) and ferulic acid was procured from Sigma, St. Louis, MO, USA. Dry methanol, dry ethanol, acetonitrile (HPLC grade), and all other chemicals and solvents were of analytical grade and were obtained from Rankem Fine Chemicals, New Delhi, India. The microbiological media and other medium ingredients like yeast extract, beef extract, proteose peptone, soyameal, casein, and urea were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Synthesis, Purification, and Analysis of Ferulic Acid Esters

Two ferulic acid esters, namely methyl ferulate (Scheme 1) and ethyl ferulate (Scheme 2), were synthesized in the laboratory *via* a single step using ferulic acid as substrates, since these substrates were not available commercially.

For performing these two reactions, ferulic acid (1 g) was dissolved in 10 ml of dry methanol and ethanol, respectively. BF_3OEt_2 (1.5 equivalent) was used as a catalyst and was added under cold conditions (4°C). The whole reaction was carried under inert conditions at room temperature (32°C) for 12 h. The conversion of ferulic acid to methyl and ethyl ferulate was confirmed by thin layer chromatography on silica gel 60 plates (F_{254}; Merck, Darmstadt, Germany) with ethyl acetate:hexane [1:1 (v/v)] as solvent. The purified ester compounds were separated on a silica gel (mesh size 100−200) column (25 × 2 cm) by using a solvent system of 3:20 (v/v) ethyl acetate:hexane. The esters were eluted gradually and concentrated on a Laborota 4000 rotary evaporator (Heidolph, IL, USA). The purity of ferulic acid esters was analyzed and confirmed by NMR on a Unity 400 MHz high field NMR spectrometer (Varian, USA) using tetramethylsilane as an internal standard. ^{1}H NMR spectra were measured in deuterated chloroform ($CDCl_3$) at room temperature. The chemical shifts were represented in δ values (ppm) referenced to the solvent proton at 7.25 ppm as an internal standard. ^{13}C NMR spectra were recorded in $CDCl_3$ using broad-band proton decoupling and the chemical shifts were represented in δ values (ppm) referenced to the carbon of the solvent at 77.0 ppm in $CDCl_3$. The Fourier transform infrared spectrum (FT–IR) was measured on a Thermo-Nicolet Nexus 670 FTIR spectrophotometer (Thermo Fisher Scientific Inc., Madison, WI, USA) using KBr pellets and the spectrum was collected at a resolution of 4 cm^{-1} in the wave number region of 400−4,000 cm^{-1}. The high-resolution mass spectra were recorded on a QSTAR XL Hybrid ESI-QTOF mass spectrometer (Applied Biosystems Inc., Foster City, CA, USA).



Scheme 1

Scheme 2

Microorganism and Culture Conditions

Aspergillus terreus strain GA2 was isolated from a soil sample collected from agricultural fields cultivating sweet sorghum, using a serial dilution method on potato dextrose agar and processed as reported previously [20]. Screening for feruloyl esterase was done on a minimal agar medium (pH 6.0) containing (per liter): $(NH_4)_2SO_4$ (1.5 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.25 g), $CaCl_2$ (0.05 g), $FeCl_3$ (0.01 g), Na_2HPO_4 (1.5 g), yeast extract (1.0 g), and bacteriological agar (20.0 g). The sterile medium was supplemented with ethyl ferulate (1%) in 5% (w/v) solution of dimethylformamide (DMF) [10]. The assay plates were inoculated with the fungal isolate from the fresh potato dextrose agar slants and were statically incubated at 30°C for 5 days. The clearing zones around the growing colonies indicated feruloyl esterase activity. This was further purified and cultured under submerged fermentation (SmF) conditions in a liquid minimal medium containing methyl ferulate as the main carbon source. The screening medium (pH 6.0) was of the following composition (g/l): $(NH_4)_2SO_4$ (1.5), KH_2PO_4 (0.5), $MgSO_4$ (0.25), $CaCl_2$ (0.05), $FeCl_3$ (0.01), and yeast extract (1.0), and supplemented with methyl ferulate (1%) in 5% DMF solution. The isolates were cultured in 250 ml Erlenmeyer flasks containing the screening medium (100 ml) and incubated at 35°C for 120 h in an incubator shaker at 150 rpm. Samples were withdrawn periodically at 24 h intervals and quantification of ferulic acid was done using HPLC.

Optimization of Production Conditions for Feruloyl Esterase

The initial solid state fermentation (SSF) experiments were conducted to evaluate the influence of pH and temperature under the pH conditions of pH 2.0 to 12.0 and temperature conditions of 25°C to 50°C on feruloyl esterase production using wheat bran as substrate [25% (w/v)] supplemented with basal salt solution (to achieve 80% moisture content) containing (per litre) $(NH_4)_2SO_4$ (1.5 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.25 g), $CaCl_2$ (0.05 g), $FeCl_3$ (0.01 g), Na_2HPO_4 (1.5 g). The flasks were inoculated with 2 ml of spore suspension (concentration of 1×10^8 spores/ml) of 5-days-grown-old culture, and were incubated at 30°C and pH 6.0 for 7 days. To select a suitable agro-waste substrate that supports maximum enzyme production, the different agro-waste crop residues examined were wheat bran, maize bran, rice bran, rice husk, green gram husk, red gram husk, coconut husk, corn cobs, cotton stalks, *Lantana camara* stalks, sweet sorghum bagasse, and sesame seed cake at a concentration of 25% (w/v). All

the agro-waste substrates were destarched by incubation in 0.5 M potassium acetate for 5 min at 100°C using the modified method of Topakas *et al*. [35]. The destarched agro-waste substrates were then washed with water and air-dried prior to use. Further SSF experiments for feruloyl esterase production were carried out using destarched maize bran supplemented with basal salt solution as mentioned above. Parameter optimization studies for feruloyl esterase production were carried out with regard to substrate particle size, moisture content, carbon and nitrogen sources, and C:N ratio. The effect of substrate particle size was evaluated for different particle sizes ranging from <0.21, 0.21−0.3, 0.3−0.71, and 0.71−1.4 mm. The influence of moisture content on feruloyl esterase production was studied by maintaining the initial moisture content ranging from 50% to 88% (w/w, wet weight basis). The flasks were incubated at 30°C in an incubator without further maintenance of moisture content during the fermentation process. Different carbon sources examined included D-glucose, D-fructose, sucrose, lactose, maltose, sorbitol, mannitol, maize starch, potato starch, and glycerol at a concentration of 0.5% (w/v), whereas nitrogen sources included organic sources like yeast extract, beef extract, proteose peptone, soyameal, casein, and urea, and inorganic sources like sodium nitrate, potassium nitrate, ammonium nitrate, and ammonium chloride, at a concentration of 1.0% (w/v). Furthermore, the effects of additives like potato starch and casein were studied individually at various concentrations ranging from 0.2% to 1.4% (w/v) by mixing with maize bran and then supplementing with basal salt solution before autoclaving. The C:N ratio on feruloyl esterase production was examined by simultaneous addition of potato starch and casein as carbon and nitrogen sources, respectively. In this regard, potato starch [0.6% (w/v)] was used and the amount of casein was varied to attain the desired C:N ratio. All experiments were carried out in duplicates and the presented results are average values of two independent experiments. The results were analyzed statistically and represented with a standard error.

Each flask was withdrawn periodically every 24 h and the contents of each fermented flask (250 ml) was processed individually by addition of 40 ml of 0.1 M acetate buffer (pH 5.0) and kept for shaking on an orbital shaker at 150 rpm at 30°C for 20 min. The solids were later separated by sieving through a muslin cloth and the recovered extract was further subjected to centrifugation (Sorvall RC 5C Plus; Kendro Lab Products, Ashville, NC, USA) at 8,000 rpm for 20 min at 4°C, and the particulate-free supernatant was used as a crude enzyme source for quantification of feruloyl esterase activity by HPLC.

Measurement of Feruloyl Esterase Activity by HPLC

Methyl ferulate was used as a standard substrate for determination of feruloyl esterase activity. A stock solution of 1% (w/v) methyl ferulate substrate was prepared in dimethylformamide (DMF, w/v). The reaction mixture was prepared by mixing 0.1 ml of substrate stock solution, 0.1 ml of 1 M acetate buffer (pH 5.0), and 0.1 ml of crude enzyme solution. The final reaction mixture volume was made up to 1 ml with distilled water and the mixure was then incubated at 40°C for 30 min. The reaction was terminated by boiling the reaction mixture for 10 min and the mixure was then diluted in the ratio of 1:10 with distilled water and then filtered through a 0.22 μm filter and the respective ferulic acid concentration was quantified by HPLC. The HPLC analysis was carried out on a reverse-phase HPLC (Waters, Milford, MA, USA) equipped with a C_{18} column (Spherisorb C_{18} ODS2, 4.6 × 250 mm; Waters, Milford, MA, USA). The sample size was 20 μl and the product (ferulic acid) and substrate (methyl ferulate) were eluted in an isocratic solvent system of acetonitrile:water:formic acid in the ratio 4:6:0.01 as the mobile phase at a flow rate of 1 ml/min for 12 min and monitored at 310 nm. The feruloyl esterase activity was computed from the ferulic acid standard curve. One unit of feruloyl esterase activity was defined as the amount of enzyme required to release 1 μmole of ferulic acid per minute under the assay conditions of 40°C and pH 5.0.

RESULTS AND DISCUSSION

Synthesis of Ferulic Acid Esters

Facile and efficient chemical synthesis methods were developed to prepare methyl and ethyl ferulate esters, which are based on Lewis acid catalyzed esterification (Scheme 1). The structural confirmation of the prepared ferulic acid esters was done based on the analysis of ^{1}H and ^{13}C NMR (Supplementary data, Fig. S1−S2), FTIR (Supplementary data, Fig. S3), and high-resolution mass spectrometry (HR-MS) spectral data. The methyl and ethyl esters were compared with the published spectra of free acid [30]. In the case of methyl ester (Supplementary data, Fig. S1A), the characteristic chemical shifts were observed at 3.92 ppm (singlet, 3H, methoxy peak), 6.22 and 7.56 ppm (doublet, J = 16.1, 1H, olefinic protons), 5.84 ppm (singlet, 1H, phenolic hydroxyl group), and 7.09−6.83 ppm (multiplet, 3H, aromatic protons), and the methyl peak of the ester group was observed at 3.77 ppm (singlet, 3H). In the case of ethyl ester (Supplementary Fig. S1B), the characteristic chemical shifts were observed at 3.92 ppm (singlet, 3H, methoxy peak), 6.23 and 7.56 ppm (doublet, J = 15.8 Hz, 1H, olefinic protons), 5.83 ppm (singlet, 1H, phenolic hydroxyl group), and 7.08−6.85 ppm (multiplet, 3H, aromatic protons), and the methyl and methylene peaks of the ester group were observed at 1.33 ppm (triplet, J = 6.7 Hz, 3H) and 4.23 ppm (quartet, J = 6.7, 14.3 Hz, 2H). The specific assignments of the respective carbon atoms of the methyl and ethyl esters elucidated from the ^{13}C NMR spectral data (see Supplementary Fig. S2A and S2B) are depicted in Supplementary Table ST1. Based on the HR-MS analyses (data not shown), molecular ion peaks were observed at m/z 231.0633 ($C_{11}H_{12}O_4Na$) and m/z 245.0789 ($C_{12}H_{14}O_4Na$) for methyl and ethyl esters, respectively. In some previous reports, different synthetic substrates like 4-nitrophenyl, 4-methylumbelliferyl, 1-naphthyl and 2,2,2-trifluoroethyl esters of 4-hydroxy-3-methyoxycinnamic acid [23], nitrophenyl ferulate [22], and mono- and diferuloyl-butanetriol analogs [21] were employed for the screening of feruloyl esterases, involving multiple steps for synthesis, which are cumbersome and time-consuming. In comparison, the present developed methods are simple, efficient, single-step, and gave recovery yields of more than 98%.

Screening for Feruloyl Esterase Producers

A total of 150 fungi were isolated from 24 soil samples collected from the agricultural fields of Medak district, Andhra Pradesh, South India cultivating different monocot crops. The plate assay used for screening feruloyl-esterase-producing fungi employed ethyl ferulate and the formation of a clearing zone after static incubation at 30°C for 5 days around the point of inoculation indicated feruloyl esterase production. A total of 42 feruloyl-esterase-producing fungi were isolated using this screening procedure. Based on the cultural and morphological characterizations of the 42 isolates (data not shown), 6 different morphotypes were identified after dereplication. Two were only identified to the genus level such as *Aspergillus* sp. and *Sclerotium* sp. The remaining fungi identified up to the species level were *Aspergillus terreus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Lasiodiplodia theobromae*. Among these 42 isolates, the feruloyl esterase activity of *Aspergillus terreus* under SmF conditions was considerably high (6.7 U/ml), whereas the production ability of *Lasiodiplodia theobromae* and *Sclerotium* sp. was very low (1.27 and 0.96 U/ml, respectively). These results suggest that the occurrence of feruloyl-esterase-producing fungi in agricultural field soils is predominant, as these soils are rich in ferulic acid precursors [37].

Optimization of SSF Conditions for Production of Feruloyl Esterase

In our studies, we optimized the SSF conditions for production of feruloyl esterase from *Aspergillus terreus* strain GA2. It is generally believed that SSF is advantageous over SmF in view of its simplicity and closeness to the natural growth conditions, especially for fungi. Additionally, SSF has lower energy requirements, lower risk of contamination, absence of complex machinery, and sophisticated control systems. Besides achieving higher enzyme productivity levels, SSF enables increased stability of the excreted enzymes [26], low levels of catabolic repression [1], and secretion of low level of extracellular proteases [2, 8]. Furthermore, SSF cultures are not subjected to limited oxygen transfer rates as observed in the case of SmF cultures. The higher biomass observed in SSF, especially in *Aspergillus* species, may also be attributed to the close contact between the mycelium and substrate [3]. These were some of the deciding factors for the selection of SSF in our optimization studies for production of feruloyl esterase.

The pH and temperature conditions were initially optimized using wheat bran as substrate. Based on these initial optimization studies, the pH conditions needed for enzyme production by *Aspergillus terreus* strain GA2 was observed in the pH range of 2.0 to 12.0, with an optimum enzyme production of 518 U/gds (units/gram of dry substrate) noticed at pH 6.0 (Supplementary data, Fig. S4A). This value is similar to that observed for *Aspergillus niger* strain CFR 1105 [16]. Furthermore, it was observed that the enzyme production was very good in the pH range of 5.0–8.0; however, the enzyme titers reduced drastically at pH values of less than 5.0 and more than 8.0. Maximum feruloyl esterase production (533 U/gds) was demonstrated at a temperature range of 25–50°C and a temperature of 30°C was observed as the optimum (Supplementary data, Fig. S4B). At temperatures above 35°C and below 30°C, a decrease in enzyme production was observed. This optimum value corroborates with an earlier study on feruloyl esterase production from *Fusarium proliferatum* [33].

Among the different agro-waste residues screened, it was noticed that all the agro-wastes supported the growth and feruloyl esterase secretion by the fungus. Maize bran was identified as a most suitable substrate and exhibited a maximum enzyme production of 658 U/gds, followed by

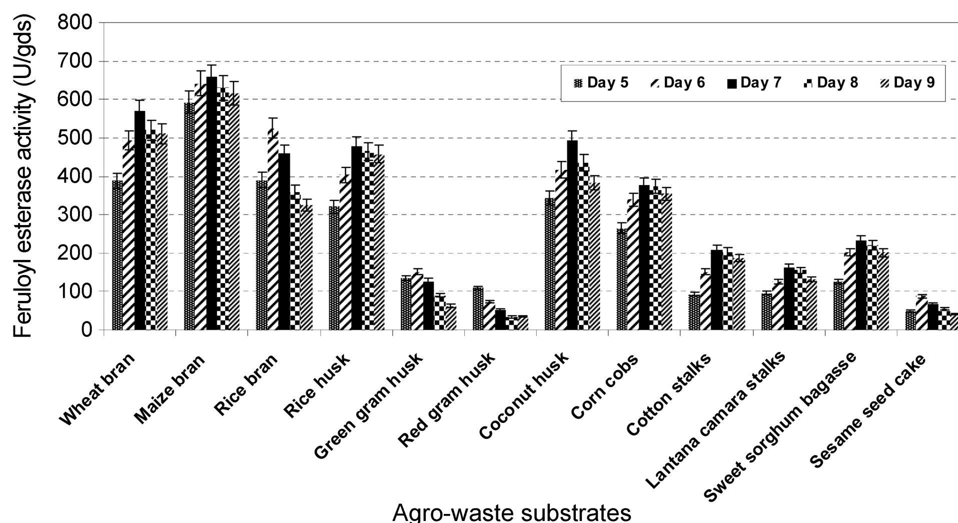


Fig. 1. Effect of different agro-waste residues as substrate [25% (w/v)] on feruloyl esterase production by *Aspergillus terreus* strain GA2.

wheat bran (enzyme titer of 569 U/gds) after 7 days of incubation, and further increase in the fermentation time resulted in slight decrease in enzyme production (Fig. 1). It was earlier reported that complex carbon sources that contain high amounts of esterified ferulic acid such as de-starched wheat bran and sugar beet pulp favored efficient microbial production of FAEs [37]. It is important to note that the selection of a suitable substrate for the SSF process depends on several factors mainly related with availability and low-cost, and thus may involve the screening of several agro-industrial residues. In the SSF process, the solid substrate not only supplies the nutrients to the fungal culture growing in it, but also serves as an anchorage for the growing mycelia. Further enzyme improvement studies for *Aspergillus terreus* were carried out using maize bran substrate.

Among the several factors that are important for microbial growth and activity in a particular substrate, particle size and moisture level are the most critical parameters [29]. An optimum particle size of 0.71–0.3 mm favored maximum enzyme production (661 U/gds), whereas smaller and bigger particle sizes did not effectively support the enzyme production (Supplementary data, Fig. S5A). This observation may be due to the fact that the optimum particle size provided effective support for attachment of the fungus and subsequent alteration in microbial metabolism. Generally, smaller substrate particles would provide a larger surface area for microbial attack and thus should be considered as a desirable factor. However, too small a substrate particle size may result in substrate agglomeration in most of the cases, which may interfere with microbial respiration/aeration, and thus may result in poor growth. At the same time, larger particles provided better respiration/aeration efficiency (due to increased interparticle space) but provide a limited surface for microbial attack. Thus, it would be mandatory to arrive at a compromised particle size for a particular SSF process. Additionally, in SSF processes, moisture content plays a vital role and influences fungal growth and subsequent metabolite production [35]. A moisture content of 80% was found to be optimal for maximum enzyme production (600 U/gds) for *A. terreus* strain GA2 (Supplementary data, Fig. S5B).

Carbon and nitrogen sources are essential constituents of the fermentation medium and play a major role in the overall cellular growth and metabolism. Among the carbon sources tested that favored enzyme secretion in *A. terreus* GA2, potato starch (860 U/gds) was observed as the most effective, followed by fructose (848 U/gds) and sucrose (839 U/gds) (Fig. 2). The effect of different nitrogen sources on feruloyl esterase production indicated that casein increased the enzyme titers (734.19 U/gds), followed by yeast extract (653 U/gds) and beef extract (580 U/gds) (Fig. 3). However, in an earlier study, it was reported that complex organic nitrogen sources like yeast extract and peptone did not support feruloyl esterase production [33]. When potato starch and casein were supplemented individually at various concentrations ranging from 0.2% to 1.4% (w/v) in the basal medium along with maize bran, the maximum feruloyl esterase production was observed on supplementation of 0.6% (w/v) potato starch (Fig. 4A) and 1.0% (w/v) casein (Fig. 4B). Moreover, *A. terreus* strain GA2 recorded a maximum enzyme production after 7 days when the C:N ratio was 5 (Fig. 5). Optimization of the SSF conditions has led to the formulation of the production medium (pH 6.0) containing maize bran 16 g [25% (w/v)] of particle size of 0.71–0.3 mm, potato starch 0.6% (w/v), casein 3.0% (w/v), and 64 ml of basal salt solution [(per liter): $(NH_4)_2SO_4$ (1.5 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.25 g), $CaCl_2$ (0.05 g), $FeCl_3$ (0.01 g), Na_2HPO_4 (1.5 g)] to achieve a moisture content of 80%. Using this formulated medium, the maximum feruloyl esterase production observed was 1,162 U/gds after 7 days of incubation. The overall enzyme production was increased by 3.2-fold as compared with un-optimized conditions. This formulated medium can be further explored for commercial production of feruloyl esterases.

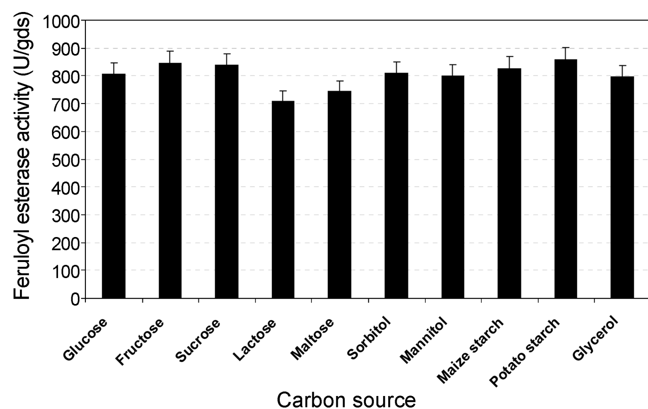


Fig. 2. Effects of carbon sources on feruloyl esterase production by *Aspergillus terreus* strain GA2.

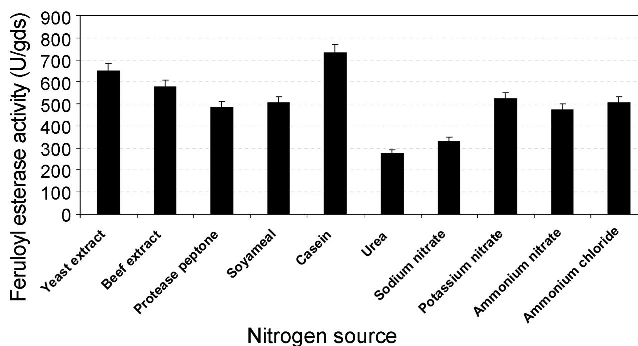


Fig. 3. Effects of nitrogen sources on feruloyl esterase production by *Aspergillus terreus* strain GA2.

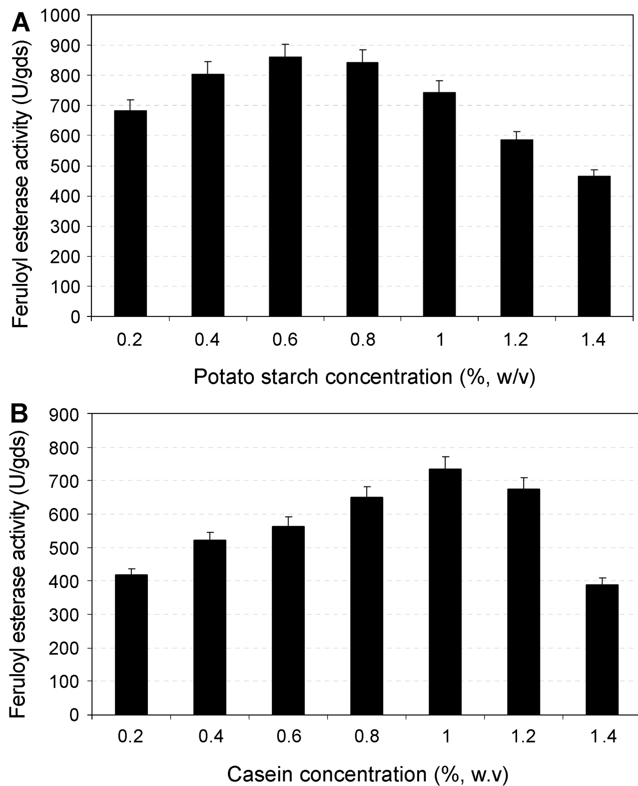


Fig. 4. Effects of (**A**) potato starch and (**B**) casein concentrations on feruloyl esterase production by *Aspergillus terreus* strain GA2.

In conclusion, these results suggest that the synthesized ferulic acid esters can be used for screening feruloyl-esterase-producing fungi by a simple plate assay method, and *A. terreus* strain GA2 proved to be a promising microorganism for feruloyl esterase production. Furthermore, among the fungal isolates identified in our study, *Aspergillus terreus*, *Lasiodiplodia theobromae*, and *Sclerotium* sp., to the best of our knowledge, are the three new species that are new additions to the list of feruloyl-esterase-producing fungi. No detailed studies on feruloyl esterase production by

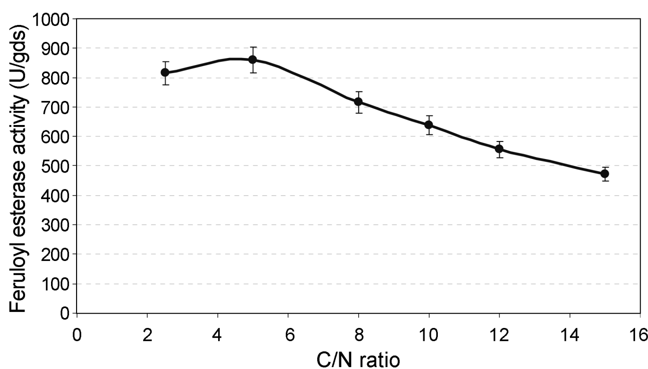


Fig. 5. Effect of C/N ratio on feruloyl esterase production by *Aspergillus terreus* strain GA2.

Aspergillus terreus using SSF has been published so far. The agro-waste crop residues used in this study have shown the importance of these substrates for feruloyl esterase production. Work is in progress to purify and characterize the enzymes produced by this fungus.

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