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Original Article

PARAMETRIC OPTIMIZATION OF FERULIC ACID ESTERASE PRODUCTION FROM *MUCOR HIEMALIS* NCIM837

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ABSTRACT

Objective: To optimize the parametric conditions for the production of ferulic acid esterase from *Mucor hiemalis* NCIM837 using HPLC for quantification.

Methods: The experiments were conducted to determine the influence of pH and temperature on ferulic acid esterase production. To optimize the suitable agricultural waste, carbon source and the nitrogen source, different agricultural wastes, nitrogen sources and carbon sources were examined. Released ferulic acid was confirmed using HPLC.

Results: Among the different agricultural waste residues screened, maize bran was identified as most suitable for ferulic acid esterase production with activity 143U/mg. Soyabean meal as the nitrogen source and sucrose as the additional carbon source was observed as the most effective in Ferulic acid ester production. Overall enzyme production increased by 2.2-fold as compared with un-optimized conditions. One unit of FAE was defined as the amount of enzyme required for release of 1µmole of FA per minute.

Conclusion: *Mucor hiemalis*, to the best of our knowledge, is the new addition in the list of FAE producing microorganisms. No detailed studies on FAE production using *Mucor hiemalis* have been published so far.

Keywords: Ferulic acid, Ethyl ferulate, Ferulic acid esterase, *Mucor hiemalis* NCIM837.

INTRODUCTION

Plant cell of Poaceae (Gramineae) family contains various hydroxycinnamic acid such as Ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid), covalently linked to 5' hydroxyl group of arabinoxylans by an ester bond (1). FA constitutes about 5% (w/w) of Corn kernel (2), 3.8% (w/w) of oat hulls (3), 3% (w/w) of maize bran (4), 1.4 % (w/w) of barley hulls (5). FA provides integrity to plant cell wall (6). There are enormous numbers of studies documented on the properties of FA such as antioxidant, antimicrobial and anticancer activity (7). FA is applicable in various ranges such as in pharmacy, bakery, cosmetic and food companies. Besides these applications FA is also applicable for the production of vanilla, the most flouring agent in food industries, perfumes and beverages (8, 9). Ferulic acid esterase (FAE, E. C.3.1.1.73) also known as cinnamic acid esterase, ferulic acid esterase or cinnamoyl esterase, an enzyme hydrolyze the ester linkage between sugar and hydroxycinnamic acids and releases FA from plant cell walls. Besides the release of FA, FAEs itself have a number of biotechnological applications in animal nutrition, pulp & paper industries (10, 11)and production of fuel ethanol. FAEs were first detected in the culture of *Streptomyces olivochromogenes* releasing FA from wheat bran (12), after that a number of microorganisms have been isolated and characterized for FAE activity such as *Pleurotuseryngii* (13), *Aspergillus flavus*(14)*Lactobacillus acidophilus* IFO13951 (15), *Talaromyces stipitatus* CBS 375.48 (16) and *Penicillium brevicompactum (17)*. FAE activity was also observed in various *Aspergillus* species such as *Aspergillus awamori* (18, 19), *Aspergillus nidulans* (20, 21). This study emphasizes the parametric optimization of FAE from *Mucor hiemalis* NCIM 837 using maize bran as agricultural waste that favors maximum FAE production. *Mucor hiemalis* NCIM837 showed Maximum FAE production in the presence of 0.1% (w/v) soyabean meal and 0.075% (w/v) sucrose.

MATERIALS AND METHODS

Chemicals

Ethyl ferulate (98%) and ferulic acid (99%) were procured from Sigma Aldrich, HPLC grade. Methanol and ethanol were purchased from Himedia; other chemicals were of analytical grade. The microbiological media and other medium ingredients like yeast extract, soyabean meal, beef extract, casein, urea, peptone and potato starch were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Microorganism and culture conditions

The laboratory strain *Mucor hiemalis* NCIM837 was used in the present investigation. The stock culture was maintained on potato-dextrose agar at 4°C. The minimal salt medium, used for the screening contained$(NH_4)_2SO_4$ 0.13% (w/v), KH_2PO_4 0.037% (w/v), $MgSO_4.7H_2O$ 0.025 % (w/v), $CaCl_2.2H_2O$ 0.007% (w/v), $FeCl_3$ 0.002% (w/v) and yeast extract 0.1% (w/v);pH-5. Sterilized media were supplemented with filter sterilized 1%(v/v) ethyl ferulate (1% w/v in Dimethyl formamide). The assay plate was inoculated with 6 days old culture of *Mucor hiemalis* from potato dextrose agar slants and incubated in a stationary condition at 30°C for 2-6 days. The clear halo zone around the point of inoculation indicated the enzyme activity. *Mucor hiemalis* was further cultured and purified in a liquid minimal medium containing ethyl ferulate as the main carbon source. For production of FAE, the minimal medium was used with composition (per liter) $(NH_4)_2SO_4$ 1.3g, KH_2PO_4 0.37g, $MgSO_4.7H_2O$ 0.25g, $CaCl_2.2H_2O$ 0.07g, $FeCl_3$ 0.02g, Yeast extract 1.0g, pH-6.8 supplemented with 1% (v/v) ethyl ferulate (1% w/v in Dimethylformamide). One ml spore suspension of *Mucor hiemalis* from a 6-day old culture, grown on PDA slopes at 30°C, was inoculated to 250 ml Erlenmeyer flasks each containing 100 ml of the above mineral media supplemented with ethyl ferulate. The flasks were incubated at 30°C for 4 days for mycelium production. An inoculum of mycelial suspension (4% v/v) was added to the enzyme production medium (EPM) in 100 ml of Erlenmeyer flasks containing 25 ml media and incubated at 30°C under the stationary condition for different period of time. Samples were withdrawn periodically at 48h intervals and contents of each were separated by sieving through muslin cloth. Removed extract was further centrifuged at 10,000 rpm for 10 min at 4°C, and the cell free supernatant was used as a crude enzyme for quantification of FAE activity. Samples were withdrawn periodically at 48h intervals and quantification of FA was done using HPLC.

Optimization of production conditions for ferulic acid esterase

The initial experiments were conducted to determine the influence of temperature and pH under the temperature conditions of 25°C to 55°C and pH conditions of 3.0 to 9.0 on FAE production. Different agricultural wastes were examined for maximum enzyme production, included wheat bran, rice bran, maize bran, rice husk and sugarcane baggase at a concentration of 4% (w/v). Different nitrogen sources examined included organic sources like soyabean meal, peptone, beef extract, yeast extract, casein, ammonium chloride, ammonium nitrate and urea at a concentration of 0.1% (w/v), whereas carbon sources included sucrose, dextrose, D-fructose, lactose, potato starch, maltose, glycerol and mannose, at a concentration of 0.1% (w/v). Furthermore, the effect of optimized additional carbon and nitrogen source was studied individually at various concentrations ranging from 0.025 to 0.2% (w/v). All the experiments were performed in duplicates and the presented results are average values of two independent experiments, the results were analyzed statistically and represented with a standard deviation (less than 14).

Measurement of ferulic acid esterase activity by HPLC

Ethyl ferulate was used as a standard substrate for determination of FAEactivity. Ethyl ferulate (substrate) was dissolved in the minimum volume of 100% ethanol followed by the dilution with sodium phosphate buffer (50 mM, pH 6) up to 6 mM concentration. The reaction mixture was prepared by mixing 0.1 ml of substrate solution and 0.5 ml of crude enzyme extract and incubated at 37°C water bath for 30 min. The reaction was terminated by boiling the reaction mixture at 100°C for 10 min followed by filteration through 0.2µm filter, respective released FA concentration was quantified by HPLC (Waters, Milford, MA, USA) equipped with RP_{18} column (XTerra, RP18 5µm, 4.6×150 mm Column). The sample size was 20µl and the FA (product) and ethyl ferulate (substrate) were eluted in an isocratic solvent system of mili Q water and 100% methanol in the ratio 40:60 as the mobile phase at a flow rate of 1 ml/min for 8 min and monitored at 310 nm. One unit of FAEwas defined as the amount of enzyme required for release of 1µmole of FA per minute.

RESULTS AND DISCUSSION

The assay plate used for screening of FAE employed ethyl ferulate and the formation of a clear halo zone around the point of inoculation after static incubation at 30°C for 5 days indicated FAE production. *Mucor hiemalis* was found with halo zone formation, therefore selected for further study. FAE activity was quantified by HPLC by using ethyl ferulate as carbon source, analysis was based on the measurement of released ferulic acid from the ethyl ferulate hydrolysis (Fig.5). Total retention time for ferulic acid was 3.0 min and for ethyl ferulate it was 5.4 min (Figure 1). One unit of enzyme activity was defined as the amount of enzyme releasing 1µmol of ferulic acid per minute under specific conditions. Determination of the specific activity requires total protein concentration that was quantified by the Bradford method [22].

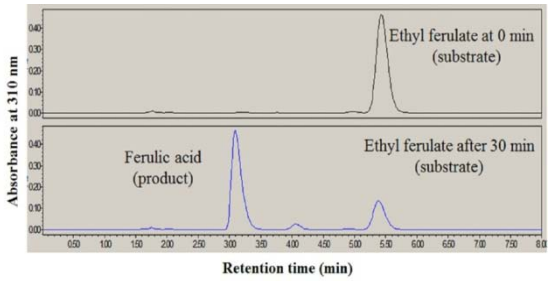

Fig. 1: Chromatogram for reaction mixture of culture medium containing ferulic acid esterase produced by *Mucor hiemalis*for 0 min and 30 min. of incubation time, respectively at 310 nm

Initially temperature and pH conditions were optimized. Based on the initial studies, the temperature conditions needed for enzyme production by *Mucor hiemalis* was observed in the range of 25°C to 55°C, with an optimum enzyme production of 78 U/mg noticed at temperature 30°C (figure 2) similar to *Fusarium proliferatum* (23). Enzyme showed 80% enzyme production in the range of 30°C to 35°C; however, above 35°C and below 30°C, a decrease in enzyme activity was observed.

Maximum enzyme production (76 U/mg) was demonstrated at optimum pH 5 (figure 3), corroborates with an earlier study on FAE production from *Aspergillus niger* strain CFR 1105 (24). Furthermore, It was observed that the enzyme production was very good in the range of 5.0 to 7.0; however, the enzyme reduced drastically at pH value less than 5.0 and more than 7.0

Among the different agricultural waste residues screened, it was noticed that all the agricultural waste supported the FAE secretion by the fungus. Maize bran was identified as a most suitable substrate for FAE production of 143 U/mg (8 days incubation) followed by rice bran 129 U/mg (6 days incubation), a further increase in the fermentation time resulted in a decrease in the enzyme production (figure 4). Earlier it was reported that the complex carbon sources such as wheat bran and sugarcane baggase favored efficient microbial production of FAE (25)

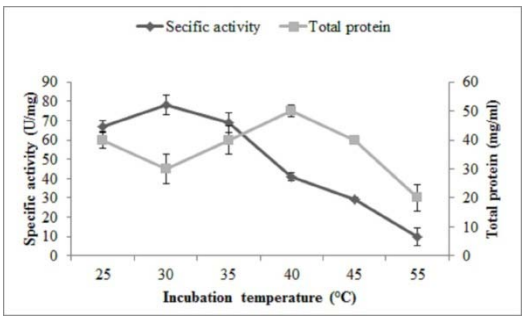

Fig. 2: Effects of Temperatureon ferulic acid esterase production by *Mucor hiemalis* NCIM 837

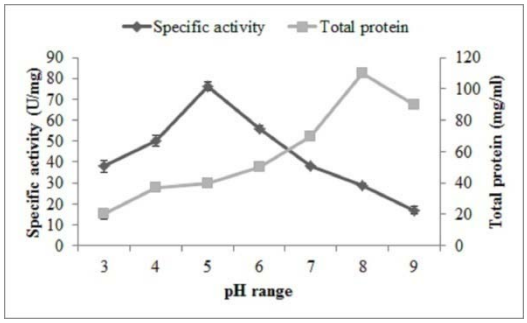

Fig. 3: Effects of pH on ferulic acid esterase production by *Mucor hiemalis* NCIM 837

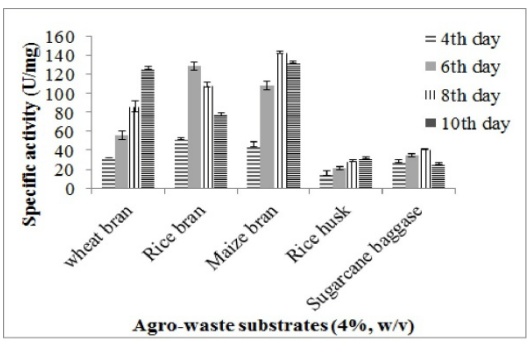

Fig. 4: Effect of different agro-waste residues as substrate inferulic acid esterase production by *Mucor hiemalis* NCIM 837

Nitrogen and carbon sources are essential elements of the enzyme production medium and play an important role in the fungal growth and metabolism. In earlier studies, it was reported that complex organic nitrogen sources like peptone did not support FAE production (26), fortifies this study as *Mucor hiemalis* did not support FAE production in presence of peptone as nitrogen source. Among the nitrogen sources tested that favored the enzyme production from *Mucor hiemalis* NCIM837, soyabean meal (127 U/mg) was observed as the most effective, followed by ammonium chloride (94 U/mg) and beef extract (89 U/mg) (figure 5). *Aspergillus terreus* Strain GA2 showed maximum enzyme activity with potato starch followed by fructose and sucrose (27),whereas this study emphasize that *M hiemalis* showed maximum enzyme production with sucrose (112 U/mg) followed by lactose (98 U/mg) (Fig. 6).

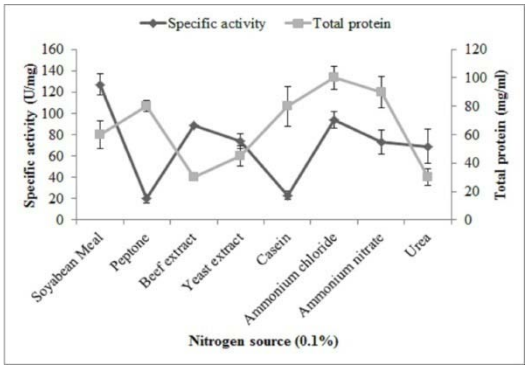

Fig. 5: Effects of nitrogen sources on ferulic acid esterase production by *Mucor hiemalis* NCIM 837

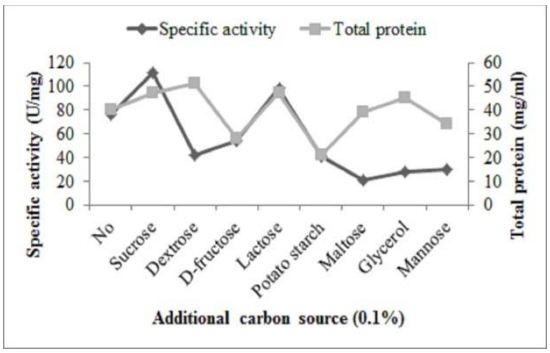

Fig. 6: Effects of carbon sources on ferulic acid esterase production by *Mucor hiemalis* NCIM 837

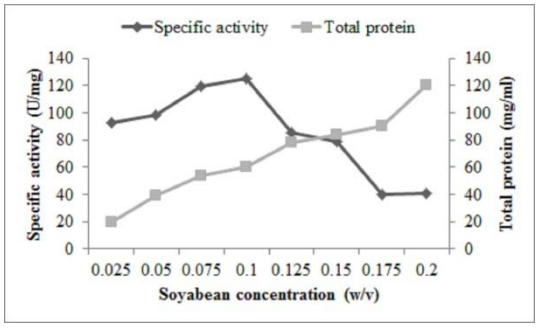

Fig. 7: effect of soyabean meal concentrations on ferulic acid esterase production by *Mucor hiemalis* NCIM 837

Furthermore when soyabean meal and sucrose were supplemented individually at various concentrations ranging from 0.025% to 0.2% (w/v) in the EPM along with 1% (v/v) ethyl ferulate (1%, w/v in DMF), the maximum enzyme production was observed on supplementation of 0.1 % (w/v) soyabean meal (figure 7) and 0.075% (w/v) sucrose (figure 8). The use of soyabean meal and sucrose aided in the biomass of *Mucor hiemalis* resulting FAE production was more rapidly than when grown in without sucrose and soyabean meal. Using this formulated medium, the maximum enzyme production observed was 172 U/mg after 8 days of incubation at 30°C. The overall enzyme production increased by 2.2 fold as compared the un-optimized conditions. This optimized medium can be further explored for commercial production of FAE.

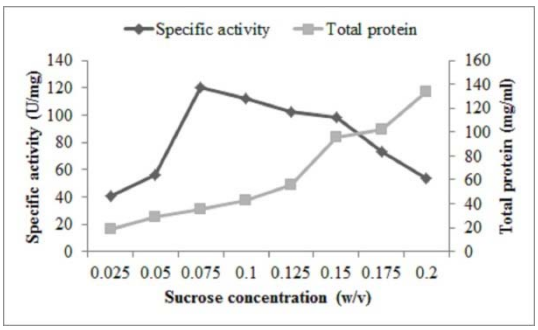

Fig. 5: Effects of sucrose concentrations on ferulic acid esterase production by *Mucor hiemalis* NCIM 837

CONCLUSION

In conclusion, result suggested that FAE producing microorganisms from a pool of microorganism can be screened by a simple plate assay method. *Mucor hiemalis* proved to be the promising microorganism for FAE production in screened microorganisms. *Mucor hiemalis*, to the best of our knowledge, is the new addition in the list of FAE producing microorganism. No detailed studies on FAE production using *Mucor hiemalis* has been published so far. Work is in progress to purify and characterize the enzyme produced by this fungus.

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CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Jeffries TW. Biodegradation of lignin carbohydrate complexes. Biodegradation 1990;1:163-76.
2. Barberousse H, Roiseux O, Robert C, Paquot M, Deroanne C, Blecker C. Review. analytical methodologies for quantification of ferulic acid and its oligomers. J Sci Food Agric 2008;88:1494-511.
3. Garleb KA, Fahey GC, Lewis SM, Kerley MS, Montgomery L. Chemical composition and digestibility of fiber fractions of certain by-product feedstuffs fed to ruminants. J Anim Sci 1988;66:2650-62.
4. Saulnier L, Marot C, Chanliiaud E, Thibault JF. Cell wall polysachharide interaction in maize bran. Carbohydr Polym 1995;26:279-87.
5. Tenkanen M, Schuseil J, Puls J. Production, purification and characterisation of an esterase liberating phenolic acids from lignocellulosics. J Biotechnol 1991;18:69-84.
6. Fry SC. Phenolic components of the primary cell wall. Biochem J 1982;203:493-504.
7. Kikuzaki H, Hisamoto M, Hirose K, Akiyama K, Taniguchi H. Antioxidant properties of ferulic acid and its related compounds. J Agric Food Chem 2002;50:2161-8.

8. Mathew S, Abraham TE. Ferulic acid: an antioxidant found naturally in plant cell walls and ferulic acid esterases involved in its release and their applications. Crit Rev Biotechnol 2004;24:59-83.
9. Walton NJ, Mayer MJ, Narbad A. Molecules of interest: Vanillin. Phytochem 2003;63:505-15.
10. Sigoillot C, Camarero S, Vidal T. Comparison of different fungal enzymes for bleaching high quality paper pulps. J Biotechnol 2005;115:333-43.
11. Tapin S, Sigoillot JC, Asther M. Ferulic acid esterase utilization for simultaneous processing of nonwood plants into phenolic compounds and pulp fibers. J Agric Food Chem 2006;54:3697-703.
12. MacKenzie CR, Bilous D. Ferulic acid esterase activity from *Schizophyllum commune*. Appl Environ Microbiol 1988;54:1170-3.
13. Nieter A, Hasse-Aschoff P, Linke D, Nimtz M, Berger RG. A halotolerant type Aferulic acid esterase from *Pleurotuseryngii*. Fungal Biol 2014;118348-57.
14. Zhang SB, Zhai HC, Wang L, Yu GH. Expression, purification and characterization of a ferulic acid esterase A from *Aspergillus flavus.* Protein Expr Purif 2013;92:36-40.
15. Wang X, Geng X, Egashira Y, Sanada H. Purification and characterization of a ferulic acid esterase from the intestinal bacterium *Lactobacillus acidophilus*. Appl Environ Microbiol 2004;70:2367-72.
16. Garcia-Conesa MT, Crepin VF, Goldson AJ, Williamson G, Cummings NJ, Connerton IF, *et al*. The ferulic acid esterase system of *Talaromyces stipitatus*: Production of three discrete ferulic acid esterases, including a novel enzyme, TsFaeC, with broad substrate specificity. J Biotechnol 2004;108:227-41.
17. Donaghy JA, McKay AM. Production of feruloyl/p-coumaroyl esterase activity by *Penicillium expansum, Penicillium brevicompactum* and *Aspergillus niger*. J Appl Bacteriol 1995;79:657-62.
18. Koseki T, Furuse S, Iwano K, Matsuzawa H. Purification and characterization of a ferulic acid esterase from *Aspergillus awamori*. Biosci Biotechnol Biochem 1998;62:2032-4.
19. Koseki T, Takahashi K, Fushinobu S, Iefugi H, Iwano K, Hashizume K, *et al*. Mutational analysis of a ferulic acid esterase from *Aspergillus awamori* involved in substrate discrimination and pH dependence. Biochim Biophys Acta 2005;1722:200-8.
20. Shin HD, Chen RR. A type b ferulic acid esterase from *Aspergillus nidulans* with broad pH applicability. Appl Microbiol Biotechnol 2007;73:1323-30.
21. Philippe D, Paul K, Jean-Marc J, Vincent P. Product patterns of a ferulic acid esterase from *Aspergillus nidulans* on large feruloyl-arabino-xylo-oligosaccharides from wheat bran. Bioresour Technol 2012;119:425-8.
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248-54.
23. Shin HD, Chen RR. Production and characterization of a type B ferulic acid esterase from *Fusariumproliferatum* NRRL 26517. Enzyme Microb Technol 2006;38:478-85.
24. Hegde S, Muralikrishna G. Isolation and partial characterization of alkaline ferulic acid esterases from *Aspergillus niger* CFR 1105 grown on wheat bran. World J Microbiol Biotechnol 2009;25:1963-9.
25. Topakas E, Vafiadi C, Christakopoulos P. Microbial production, characterization and applications of ferulic acid esterases. Process Biochem 2007;42:497-509.
26. Shin HD, ChenRR. Production and characterization of a type B ferulic acid esterase from *Fusariumproliferatum* NRRL 26517. Enzyme Microb Technol 2006;38:478-85.
27. Kumar CG, Kamle A, Mongolla P, Joseph J. Parametric optimization of ferulic acid esterase production from *aspergillus terreus* Strain GA2 Isolated from tropical agro-ecosystems cultivating sweet sorghum. J Microbiol Biotechnol 2011;21:947-53.