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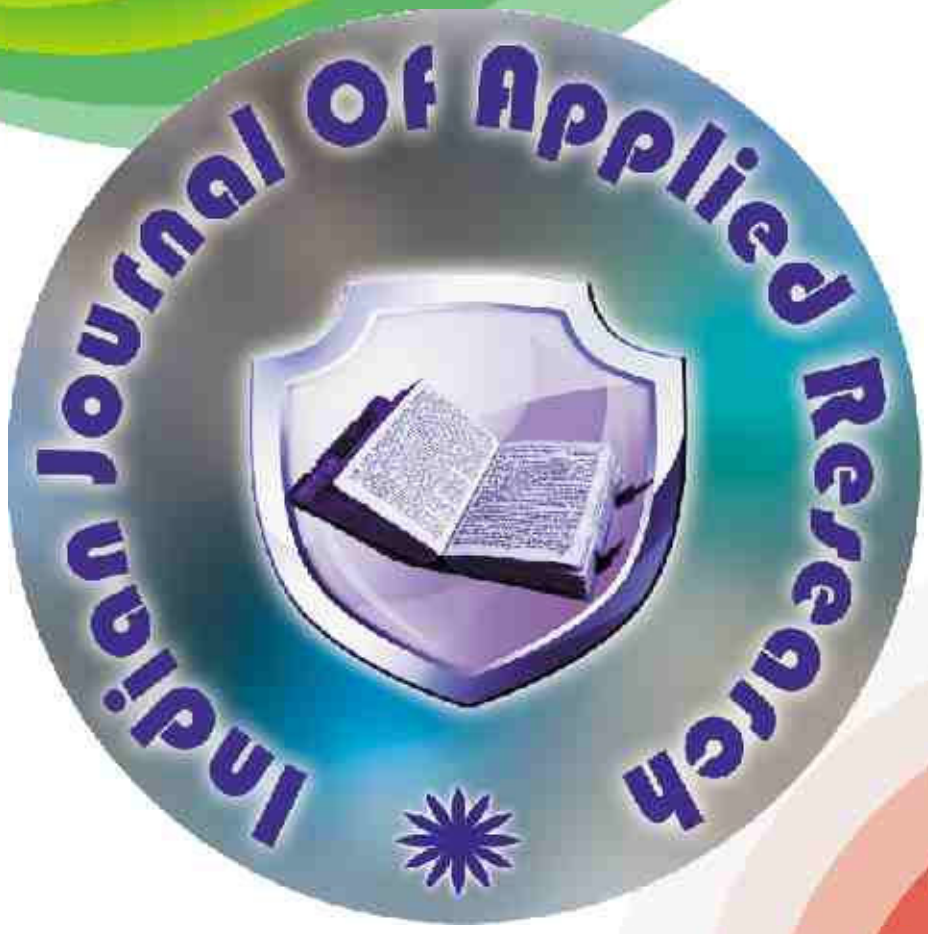

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Statistical Optimization Of Ferulic Acid Esterase Production In Aspergillus Niger Isolate Using Response Surface Methodology

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ABSTRACT

Ferulic acid esterases (FAEs) degrade major plant cell wall polysaccharides by hydrolyzing ferulate ester groups. Few strains of Aspergillus niger were isolated and evaluated on various substrates for production of extracellular FAEs. Response surface methodology (RSM) was used to optimize the media components to maximize FAE production in A. niger isolate. Model predicted a specific FAE activity of 2.98 nkatals/mg using optimized medium and via. experimental rechecking of the model a specific activity of 3.45 nkatals/mg was achieved. Ferulic acid was efficiently released from rice bran when it was cohydrolyzed with FAE and macerozyme of Paenibacillus macerans. FAE activity was induced using pure ferulic acid on rice bran cultures. Finally, hydrolyzed FA was analyzed by C-18 reverse phase HPLC. RSM facilitated optimization of chemical constituents and physical conditions which consisted of wheat bran 2.5 g/100ml, rice bran 2.5, cooked rice 5.0, KH_2PO_4 0.4, $NaNO_3$ 0.6, sucrose 0.375, tween-80 0.1%, inducer 10 µg/ml, pH 4.5, incubation time of 4 days and 33.5°C temperature to enhance FAE production in A. niger spp.

Keywords : Ferulic acid esterase, Aspergillus niger, Response surface methodology, Media optimization.

Introduction

Ferulic acid esterases (FAEs) (EC3.1.1.73) belong to a subclass of the carboxylic acid esterases which degrade plant cell wall by hydrolyzing ferulate ester groups in the lingo-cellulose (Lynd et al. 2002; Rosenbrock et al. 2004; Lee, 2005). Ferulic acid (FA) is a typical cinnamic acid derivative that has been identified in a variety of monocotyledonous and dicotyledonous plants. Ferulic acid is also present in roots of Helianthus annuus (Mehboob et al. 2000). With the intervention of biotechnological processes, Biotechnology Industry has started using FAEs for the improvement of food and feed quality and for the production of improved medicinal compounds (Williamson et al. 1998; Mathew and Abraham, 2004; Topakas and Christakopoulos, 2004). Its catalytic property has been exploited in pulp and paper process (Record et al. 2003; Sigoillot et al. 2005). FA has a great potential for commercial vanillin production (Lee, 2005). It has been listed as an oxidation inhibitor in the food additive list and expected to be used as anti-discoloration agent (Ernst, 1992). It absorbs the harmful long wave ultraviolet (UV) band. Vitamin E ester of FA decreases melanin generation and makes it effective for cosmetic use as a whitening agent and sunscreen (Sohn and Oh, 2003; Lin et al. 2005; Fazary and Ju, 2007). Substrate hydrolysis products of FAE have been used for the production of food biopreservatives (Khamidullina et al. 2006) and bio-ethanol (Fazary and Ju, 2007). Use of pure FA as anti-inflammatory agent and in various pharmaceuticals to treat breast, colon, lung, stomach and tongue cancer, Alzheimer's disease, hyper-cholesterolemia and as a bone tonic has been well documented in literature (Ou and Kwok, 2004; Fazary and Ju, 2007). FA provides significant protection in the alleviation of GalN induced hepatocellular injury (Shanmugarajan et al. 2008).

Statistical experimental designs provide an efficient approach to media optimization in various microbiological processes. Response surface methodology (RSM) is a powerful technique for testing multiple process variables in a single experiment. Significant interactions between process variables can be identified and quantified by this technique. It employs multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously. RSM has been increasingly used for an optimization process in fermentation (Buchanan and Philips, 1990; Prapulla et al. 1992; Haltrich et al. 1994). The Plackett-Burman design is especially suitable in accounting interactions and identifying more significant components in a medium formula generating a certain optimal response (Liu et al. 2005). To ensure that the experiments are maximally informative, a central composite design (CCD) can be applied for regression and graphical analysis (Zhao et al. 2007).

Keeping in view the great commercial potential of FAEs and pure FA, this study was undertaken with the aim to optimize culture medium for microbial production of ferulic acid esterases from A. niger S-4 isolate using response surface methodology. Role of FA in inducing enzyme activity and macerozyme in facilitating substrate hydrolysis was also studied.

Materials and methods

Isolation of fungal strains: Fungal strains capable of producing FAE were isolated from soil and rotten food articles. Pure cultures were isolated on PDA plates (an extract of finely chopped potatoes (20% w/v), 2.5% sucrose, 0.10% Tween-80 and 1.5% agar) after incubating for 3d at 30oC. Paenibacillus macerans MTCC2294 was procured from (MTCC, Chandigarh, India) and maintained in enrichment growth media (consisting of beef extract-1g, yeast extract-2g, peptone-5g, NaCl-5g, agar-15g, distilled water-1l) at 30°C for 24h.

Preparation of media: Potato Dextrose Broth (PDB) was prepared to study production of FAE under submerged static conditions. PDA, wheat bran, rice bran and cooked rice were employed to study solid state fermentation. Substrates (10%w/v) were suspended in distilled water and supplemented with 2.5% sucrose and 0.10% Tween-80. pH of media was adjusted to 5.6. Media was sterilized by autoclaving at 15psi for 20min.

Characterization of fungal strains: The method involves characterization of fungal colony and microscopic examination of sporulating bodies and spores. Sporulating fungi were stained with congo red and lactophenol cotton blue dyes and observed under the microscope. Fungal morphology including nature of hyphae (septate or nonseptate, hyaline or colored), length of conidiophores, shape and size of vesicles, number of cells present on the vesicle, shape, size and color of the conidia etc was studied. The above features were compared with descriptions given in the literature (Gilman, 1957; Subramanian, 1971).

Characterization of substrates: Complex carbon sources that contain high amounts of esterified FA and rich in inducing compounds and micronutrients viz. vitamins and minerals required for growth of microorganisms have been efficiently used for the microbial production of FAEs. Alkali extractable FA content of all substrates was determined by the standard method (Wang et al. 2005).

Preparation of fungal inoculums: PDB broth was inoculated with a loopful of fungal mat and incubated at 37˚C for 3d. Culture broth was vigorously shaken to suspend spores and fungal mat. Finely dispersed culture broth was used for further inoculations.

Medium optimization using traditional method of selecting most suited factor: Media formulations were inoculated with 1%v/v fungal inoculums and incubated at 30˚C for 3d under static conditions. After incubation, substrates were centrifuged at 7000rpm for 20minutes at 4˚C. Cell free supernatant was used as crude extracellular enzyme preparation to assay FAE activity. To measure intracellular FAE activity, entire fungal mat of a culture vessel was transferred to an oakridge tube and resuspended in 5 ml of 1% Tween 80 in normal saline and sonicated for 8min (with 30sec pulse on and 30sec pulse off, amplitude-45%). Lyzed material was centrifuged for 20min at 4˚C at 7000rpm. Supernatant was taken as crude intracellular enzyme preparation and FAE activity assay was carried out as prescribed by Hatfield et al. 1991 with little modifications. Amount of FA released was quantified from standard curve of pure FA (absorbance at 326.5nm).

Medium optimization using RSM: RSM was applied to optimize media components to enhance production of FAE from A. niger isolate S-4. Statistical experimental designs were made by using Design Expert software version 7.1.6. A Plackett-Burman designs was used to investigate the statistical significance of the factors viz. rice bran, wheat bran, cooked rice, KH2PO4, NaNO3, Tween-80, sucrose, pH, temperature, inducer, incubation time on FAE activity. This factorial design is important when large numbers of factors are to be considered for optimization. A total of 12 sets experiments were employed as given in Table 4. Each variable was represented at two levels, upper (+1) and lower (-1) levels of the range. This model does not describe the interaction among factors but it is used to screen and evaluate important factors that influence the response. From the regression analysis of the variables (ANNOVA), the factors having significant effect on the specific activity of FA were further optimized by CCD.

Based on the results of the Placket-Burman design, the experiment was further expanded to CCD which employed multiple regressions to estimate model coefficient of the four selected variables found to be significantly influence the process. The significant factors wheat bran, rice bran, sucrose and temperature were selected for further studies. Other factors, which did not influence FAE production such as cooked rice (5g/100ml), KH2PO4 (0.4), NaNO3 (0.6), tween-80 (0.1), pH (4), inducer (10µg/ml), incubation time (3d) were kept at constant level. The maximum and minimum levels of the independent variables that influence FAE response were selected on the basis of single variable experiments in the previous studies of our laboratory and by conducting some additional experiments. The CCD experimental plan consisted of 30 experiments in three blocks (Table 5). The average specific activity of FAE (nkatals/mg) was taken as dependent variable or response (Y). The three dimensional graphical representation of model equation represents the individual and interactive effect of the variable on the response.

Validation of the experimental designs: To confirm aforementioned results, FAE production was conducted in flasks in optimized culture medium designed by response surface analysis.

Statistical analysis of results: Results of the study were recorded as mean of the three analyses. All the results were expressed as mean ± S.E.M. The data of the test were statistically analyzed by using one-way ANOVA, followed by Turkey's multiple range test which was applied for FAE activity analysis. The data was considered to be statistically significant of the probability had a value of 0.05 or less (Lee et al. 2008). A second-order polynomial equation was then fitted to the data by multiple regression procedure (Pio et al. 2008). This resulted in the independent factors. All tests were performed in triplicates, and the data represented is a mean of the three. The model equation was:

$$Y = \beta 0 + \beta 1A + \beta 2B + \beta 3C + \beta 4D$$

Where, Y is the predicted response for specific activity of FAE; β0, intercept β1, β2, β3, β4, linear coefficients. The proportion of variance explained by the polynomial model obtained was given by multiple coefficient of determination, R2. The fitted polynomial equation was expressed as three dimensional response surface plots to find concentration of each factor for maximum specific activity of FAE.

Enhancing release of FA from solid substrates: Supernatant withdrawn from an overnight grown culture of Paenibacillus macerans MTCC 2294, was used as crude macerozyme preparation in the study. Reaction mixture consisting of 25µl of the FAE enzyme sample and crude macerozyme preparation from 10µl to 100µl in different eppendorfs was set up to quantify amount of FA released from the given substrate following Hatfield procedure (Hatfield et al. 1991).

Induction of FAE activity: Separate flasks containing rice bran substrate were substituted with pure FA at concentrations viz. 1ng/ml, 1µg/ml and 1mg/ml respectively for inducing FAE activity. One flask was kept as control without induction. Flasks were inoculated with 1 ml fungal inoculum and incubated at 30˚C for 3d. Media was centrifuged at 7000rpm at 4˚C for 20min and the FAE content was estimated as nkatals (Hatfield et al. 1991; Bonnin et al. 2002).

Partial purification of ferulic acid (FA) from hydrolyzed substrates: Rice bran substrate was inoculated with isolate no. S-4 and incubated at 30˚C for 3d. Media was centrifuged at 7000rpm at 4˚C for 20min. Supernatant was extracted in double volume of ethyl acetate by vigorous shaking, upper ethyl acetate layer was separated and solvent was evaporated to dryness using rotary vacuum evaporator to partially purify FA (Wang et al. 2005).

Analysis of FA released by C-18 reverse phase HPLC: To dry residue, 40µl of methanol/water (50:50,v/v) was added and its FA content was determined by HPLC (Wang et al. 2005). The extracted FA was analyzed on C18 reverse phase column by maintaining flow rate of 1ml/min at OD326.5 nm and compared with C-18 reverse phase chromatogram of pure FA.

Results and discussion

Isolation and characterization of fungal strains: Six fungal strains named from S-1 to S-6 were isolated from soil and spoiled food samples. The macroscopic features of the isolates and stained mounts of isolates were examined at 400 times magnification and dimensions of the mycelia, conidiophores and conidia were measured using ocular micrometer.

Cultures were identified on the basis of reports by Gilman (1957) and Subramanian (1971). Mycelium of all A. niger isolates is 6-8.2 µm in diameter, pale white in color, septate and thick walled. Conidiophores are 700-2500µm long, hyaline, aseptate, thick walled, bearing a globose vesicle at the tip as shown in Figure 1(a) to 1(f). Diameter of vesicle is 40-60µm that bears 6-8µm black, round and spiked conidia (Table 1 and Figure 1).

Characterization of the substrates: FA is extremely abundant in cell walls of many cereals and grasses. Maize bran (30g/kg), sugar beet (5-10g/kg), rice endosperm (9g/kg), wheat bran (6.6g/kg) and barley grains (1.4g/kg) are rich sources of esterified FA that can be released by strong alkali treatment (Mathew and Abraham, 2004). Ferulic acid was de-esterified and released from the lingo-cellulosics using alkaline treatment. Maximum FA release was observed in case of rice bran (61642nM of FA) and minimum in PDA (3221.6nM) as depicted in Table 2. Total alkali extractable FA content of rice bran was maximum that's why it was regarded as best substrate for growth of the A. niger isolates and production of FAE.

Medium optimization using traditional method of selecting most suited factor: FAE production in A. niger S-1 isolate was better under submerged static conditions in PDB broth (0.13 nkatals/mg) than solid state fermentation on PDA (0.011 nkatals/mg). It suggested that fungal culture conditions have a great influence on enzyme production. When other substrates were compared, maximum FAE activity was obtained in rice bran substrate which was 2.47 nkatals/mg followed by wheat bran 1.66 nkatals/mg. Intracellular FAE content was almost negligible in the isolate (0.0009-0.037 nkatals/mg). Other three Aspergillus isolates namely S-4, S-3 and S-5 produced very high concentration of extracellular FAE with specific activities viz. 2.47, 2.30 and 2.26 nkatals/mg respectively. Again, FAE activity was restricted to supernatant and very little intracellular FAE content was present in them. As in case of S-1 isolate, A. niger S-4 also gave more FAE activity under submerged static conditions in PDB as compared to PDA. Upon, comparative evaluation highest production of extracellular FAE was obtained in case of A. niger S-4 isolate in its rice bran cultures, supplemented with 2.5 % sucrose and 0.1% Tween-80 (Table 3). In cooked rice, maximum extracellular FAE (0.612 nkatals/mg) was reported in S-5 isolate when compared to other isolates. Alkali extractable FA content of cooked rice was almost one fourth compared to the rice bran. Thus, almost four fold lower FAE productions in S-5 isolates could be justified. FA content of rice bran was 1.6 fold higher than wheat bran and it supported 2.1 fold higher specific FAE activity as compared to wheat bran substrate. A. niger isolate S-4 showed very high specific activities on rice bran and wheat bran substrates in accordance with literature (Tatineni et al. 2007).

FAEs are promising tools in agro-industrial processes as they accelerate hydrolysis of cell wall polymers by acting synergistically with other lignocelluloses-degrading enzymes. The applications of FA and feruloyl esterase enzymes are many with wide spread industrial uses. Cinnamoyl esterases of A. niger and Pseudomonas strains have been commonly exploited to release both monomeric and dimeric FA from cereal derived materials (Faulds and Williamson, 1995; Bartolome et al. 1997). In accordance with these reports, authors are also able to release 88% FA (54855.6 nM) in rice bran and 77% FA (33777 nM) in wheat bran using FAE's produced is A. niger S-4 (Figure 2).

Medium optimization using RSM: RSM was applied to optimize media components for producing FAE of A. niger isolate S-4. First PlackettBurman design (Table 4) was used to identify critical factors, which were further incorporated in CCD experiments (Table 5). The positive coefficient terms indicate an increase in response with increase in the level variable while negative coefficients show decrease in response. The central (zero level) values chosen for the experiment design were wheat bran 2.5g/l, rice bran 2.5, sucrose 0.375 and temperature 33.5oC. The contour plots were used to select the range of different parameters as given in figure 3. Final composition of the optimized culture media consisted of wheat bran 2.5g/l, rice bran 2.5, cooked rice 5, sucrose 0.375, KH2PO4 0.4, NaNO3 0.6, Tween-80 0.1% (w/v) and inducer 10µg/ml. Physical culture parameters included pH 4.5, temperature 33.5oC and an incubation time of 4 days for optimal production. The coded values and FAE responses obtained in Plackett-Burman and CCD designs are given in table 4 and 5 respectively. This model predicted a specific FAE activity of 2.98 nkatals/mg and by experimental rechecking 3.45 nkatals/mg value was obtained.

The statistical significance of the first-order model equation was evaluated by the F test analysis of variance (ANOVA), which revealed that this regression was statistically significant ($P<0.05$) at 95% of confidence level (Table 6). The fitting of the first-order model was calculated to be 0.999 by the coefficient of determination R2, which indicated that 99.8% of the variability in the response could be explained by the model. The “adjusted R2” is 0.9983 and predicted R2 is 0.9947, indicating that this is a good statistical model. R2 should be in the range of 0-1.0, and the nearer to 1.0 the value is, the more fit the model is deemed to be. The “adequate precision value” of the present model was 127.5, suggesting that the model can be used to navigate the design space. The “precision value” is an index of the signal-to-noise ratio, and values of higher than 4 are prerequisites for a model to be a good fit.

Consequently, the data was fitted with a second-order polynomial function to get the following regression equation which is an empirical relationship between the logarithmic values of enzyme yields and test variables in coded unit:

$$Y = + 2.08 + 0.37 * A + 0.042 * B + 2.500 * E \ \ 0.03 * C + 0.016 * D$$

Where Y: specific activity of FAE (nkatals/mg), A: rice bran (g/100ml), B: wheat bran (g/100ml), C: sucrose (g/l), D: temperature (oC).

Rice bran, wheat bran, sucrose and temperature are the critical factors to influence FAE activity significantly. A. niger gave higher FAE activity in the presence of rice bran and wheat bran as shown in Fig 3(a) whereas temperature when studied against substrates did not show much effect on enzyme production. Same is the case of sucrose, when it was studied in combination with substrates, it did not show any increase in enzyme production as evidenced from Fig 3(b) to 3(e). But when substrates were studied together, temperature showed a slight increase in enzyme activity where as sucrose showed a constant effect as depicted in Fig 3(f). From these experiments it was concluded that out of four parameters, rice bran yields maximum FAE activity and sucrose gives the poorest FAE response. It might be due to the fact that some kinds of inducers were released upon hydrolysis of respective substrates.

Comparison of traditional and statistical methods of media optimization: Statistical experimental designs in medium optimization can improve product yield by assessing combined effect of all the physical and chemical factors, reduce process variability, maximize output response, reduce development time and overall costs of the biotechnological process as compared to conventional practices (Cochran and Cox, 1992; Hounsa et al. 1996). Scientists have started exploiting this wonderful method in order to optimize culture media for production of enzymes and industrially important metabolites (Dagbagli and Goksungur, 2008; Gupta et al. 2008; Juarez-Jimenez et al. 2008; Pio and Macedo, 2008; Sawale and Lele, 2009). Similar observations were made in this study where traditional method of media optimization was compared with statistical approach. Earlier, 2.47 nkatals/mg specific FAE activity was obtained on rice bran substrate following traditional approach (Table 3). While, RSM model predicted a specific FAE activity of 2.98 nkatals/mg and by experimental rechecking 3.45 nkatals/mg (Table 5) value was obtained. Use of RSM in optimizing media composition and physical growth factors is also supported in a number of previous studies on production of keretinase (Tatineni et al. 2007), cephalosporin (Lotfy, 2007), pyruvate oxidase (Zhao et al. 2007), fructanohydrolase (Ge et al. 2008), cutinase (Pio and Macedo, 2008), chitinolytic enzymes (Juarez-Jimenez et al. 2008), alkaline exo-polygalacturonase (Gupta et al. 2008), β-galactosidase (Dagbagli and Goksungur, 2008), cyclodextrin glycosyltrasferase (Ai-Noi et al. 2008) dextransucrase (Sawale and Lele, 2009) and chitinase (Akhir et al. 2009). Optimization of variables for batch degradation of phenols (Agarry et al. 2010), modeling of chromium (IV) accumulation (Berekaa et al. 2006), improvement of excretory overexpression of cyclodextrin glycosyltransferase (Lo et al. 2009) and ultrasonic extraction of polysaccharides from Chinese malted sorghum was also achieved using RSM (Claver et al. 2010).

Enhancing release of FA: FAEs work synergistically with other cell wall degrading enzymes. Upon mixed enzymatic hydrolysis, amount of released FA improved 70 folds after addition of 100µl macerozyme. As a mixture of FAE, macerozymes, cellulases, hemicellulases, xylanases, arabinoxylanases etc., in combination to improve the release of ferulic acid from agro industry wastes. Alkali extractable FA content of rice bran was 61642 nM (Table 2). When FAE activity of the rice bran was compared under single enzyme hydrolysis (extracellular FAE of A. niger isolate) and mixed enzyme hydrolysis (crude mixture of extracellular FAEs and macerozyme preparation) and it was observed that the FAE activity increased by 3.4 times to 156 nkatals after adding 100µl macerozyme (Table 7).

Induction of ferulic acid esterase (FAE) activity: It has been reported that FAE's are quorum sensing molecules which can be induced (Zhao et al. 2007; Ge et al. 2008). Induction was studied using pure FA on rice bran cultures of A. niger S-4 and quorum sensing nature of its extracellular FAE was deduced. 2.9 folds higher FAE activity i.e. 133.6 nkatals, was obtained as against control value of 45.9 nkatals, when pure FA was added to rice bran at a concentration of 1 mg/ml (Table 8). Even, addition of 1 ng of substrate (0.005 nM FA) could raise FAE activity (2.15 times). With increase in inducer concentration amount of FA hydrolysis showed a concordant elevation. The study indicated FA as a potent inducer of extracellular FAEs in Aspergillus species and a concentration of 0.005 nM inducer is sufficient for induction. Present study and literature citations highlights regulation of FAE expression by xylose, arabinose and FA. This also confirms the finding that extracellular cinnamoyl esterases from A. niger are inducible upon growth on cereal derived materials and almost 100% FA content of substrates is released when used in conjunction with xylanases as indicated in one of the earlier study (Faulds and Williamson, 1995). Results of the present study were recorded as mean of the three analyses. All the results were expressed as mean ± S.E.M. Statistical analysis of the results was performed by one way ANOVA which indicated a high significance of all the results obtained.

Analysis of FA released by C-18 reverse phase HPLC: Retention time of pure FA was 2.1 min on C-18 reverse phase column as shown in Figure 4b and retention time of extracted FA was 2.073 min as shown in Figure 4a and the extracted compound was confirmed as FA. As one additional peak at a retention time of 3.316 min was also observed, the extracted FA might have some impurities or its diferulate. Solvent extraction could only be used for partial purification of FA from solid state fermentated substrates and is not suitable for high scale purification purpose.

Conclusions

Few stains of A. niger capable of producing extracellular FAEs were isolated on potato dextrose agar and evaluated comparatively for enzyme production. Culture media consisting of wheat bran 2.5g/100ml, rice bran 2.5, cooked rice 5, sucrose 0.375, KH2PO4 0.4, NaNO3 0.6, Tween-80 0.1 % and inducer 10 µg/ml with a pH 4.5, incubation of 4 days and 33.5oC temperature was optimized using RSM. Specific activity of 3.45 nkatals/mg for the inducible extracellular FAEs was obtained using optimized physical and chemical parameters, which was much higher than the predicted value (2.98 nkatals/mg) under static conditions. Reverse phase HPLC also confirmed de-estarification and release of mono ferulic acid from rice bran substrate. Therefore, highly significant results are obtained in the study as tested by one way ANOVA. Further, scale up of the optimized production parameters would help production of FAE at the industrial level. Thus, A. niger S-4 isolate is a promising candidate for cost effective industrial level production of extracellular FAEs and/or FA from agricultural waste materials.

Table 1: Morphological features of A. niger isolates

Fungal Characteristics	*A. niger* isolates					
	S-1	S-2	S-3	S-4	S-5	S-6
Diameter of mycelium (µm)	6.2-7.8	6.0-7.0	6.4-7.2	6.8-7.9	6.2-7.8	6.7-8.2
Diameter of conidiophores (µm)	700-1200	900-1500	700-1800	1100-2200	750-1850	1600-2500
Diameter of vesicle (µm)	60	50	48	40	50	55
Diameter of conidia (µm)	8	7-8	6.5	6	6	8

Table 2: Alkali extractable FA content of substrates

Substrate	Alkali extractable FA content (nM)	
	Before autoclaving	After autoclaving
PDA	2344.6 ± 4.1	3220 ± 3.6
Wheat bran	33777.0 ± 7.2	40368.3 ± 4.0
Rice bran	54855.6 ± 6.1	**61642.0** ± 2.0
Cooked rice	4802.0 ± 4.5	15061.6 ± 6.1
n=3, results are shown as mean ± standard deviation, P-value < 0.05		

Table 3: Production of FAE on various substrates

Substrates	Specific FAE activity (nkatals/mg)	A. niger isolates					
		S-1	S-2	S-3	S-4	S-5	S-6
PDB	Extracellular	[illegible]	0.07 ± 0.02	0.11 ± 0.04	0.09 ± 0.04	0.09 ± 0.06	0.04 ± 0.03
	Intracellular	0.008 ± 0.004	0.009 ± 0.003	0.006 ± 0.005	0.008 ± 0.005	0.006 ± 0.008	0.007 ± 0.005
PDA	Extracellular	0.011 ± 0.004	0.009 ± 0.005	0.01 ± 0.004	0.008 ± 0.005	0.01 ± 0.007	0.019 ± 0.003
	Intracellular	0.0009 ± 0.006	0.0009 ± 0.006	0.0012 ± 0.006	0.0012 ± 0.008	0.0009 ± 0.006	0.0013 ± 0.006
Wheat bran	Extracellular	0.74 ± 0.08	1.30 ± 0.09	1.47 ± 0.08	1.17 ± 0.06	1.20 ± 0.05	1.66 ± 0.10
	Intracellular	0.0054 ± 0.001	0.014 ± 0.005	0.025 ± 0.006	0.013 ± 0.006	0.015 ± 0.008	0.021 ± 0.008
Rice bran	Extracellular	1.28 ± 0.09	1.54 ± 0.14	2.30 ± 0.08	2.47 ± 0.11	2.26 ± 0.13	1.88 ± 0.07
	Intracellular	0.032 ± 0.011	0.031 ± 0.006	0.023 ± 0.008	0.033 ± 0.012	0.027 ± 0.005	0.028 ± 0.005
Cooked rice	Extracellular	0.024 ± 0.008	0.036 ± 0.010	0.053 ± 0.010	0.054 ± 0.011	0.612 ± 0.024	0.035 ± 0.007
	Intracellular	0.020 ± 0.005	0.037 ± 0.008	0.012 ± 0.009	0.011 ± 0.007	0.015 ± 0.012	0.021 ± 0.012
n=3, results are shown as mean + standard deviation, P-value < 0.05							

Table 4: PlackettBurman experiment design with coded values and results

Run No.	Concentration of chemical constituents (g/100ml)							Inducer (µg/ml)	Physical Factors			Specific activity of FAE (nkatals/mg)	
	Rice bran	Wheat bran	Cooked rice	Sucrose	KH_2PO_4	$NaNO_3$	Tween 80		pH	Temp. (°C)	Time (days)	Predicted value	Experimental value
1	0	0	5	0.25	0.1	0.6	0.10	10.0	9	37	3	1.84	1.86
2	5	5	0	0.25	0.4	0.6	0.10	10.0	4	30	3	1.96	1.94
3	0	5	0	0.50	0.4	0.6	0.01	0.01	9	37	3	1.76	1.78
4	0	5	5	0.25	0.4	0.3	0.01	10.0	9	30	4	1.89	1.97
5	5	0	0	0.50	0.1	0.6	0.01	10.0	9	30	4	2.54	2.64
6	0	0	0	0.50	0.4	0.3	0.10	10.0	4	37	4	1.67	1.68
7	0	5	5	0.50	0.1	0.6	0.10	0.01	4	30	4	1.84	1.83
8	0	0	0	0.25	0.1	0.3	0.01	0.01	4	30	3	2.65	2.70
9	5	5	5	0.50	0.1	0.3	0.01	10.0	4	37	3	2.18	2.20
10	5	5	0	0.25	0.1	0.3	0.10	0.01	9	37	4	1.67	1.65
11	5	0	5	0.50	0.4	0.3	0.10	0.01	9	30	3	2.65	2.69
12	5	0	5	0.25	0.4	0.6	0.01	0.01	4	37	4	2.80	**2.81**

Table 5: Central Composite Design (CCD) with coded values and results

Run No.	Rice bran (g/100ml)	Wheat bran (g/100ml)	Sucrose (g/100ml)	Temp. (°C)	Specific activity of FAE (nkatals/mg)	
					Predicted value	Experimental Value
1	0.0	5.0	0.50	37.0	1.85	1.86
2	-2.5	2.5	0.37	33.5	1.75	1.76
3	2.5	2.5	0.37	33.5	1.98	1.94
4	7.5	2.5	0.37	33.5	2.64	2.65
5	5.0	0.0	0.25	37.0	2.44	2.45
6	2.5	2.5	0.37	33.5	1.97	1.97
7	2.5	-2.5	0.37	33.5	2.35	2.34
8	0.0	5.0	0.50	30.0	1.87	1.87
9	2.5	2.5	0.37	33.5	1.97	1.98
10	5.0	0.0	0.50	37.0	2.76	2.78
11	2.5	2.5	0.37	33.5	1.54	1.55
12	2.5	2.5	0.37	33.5	1.79	1.78
13	5.0	5.0	0.50	30.0	2.35	2.34
14	0.0	5.0	0.25	37.0	1.93	1.94
15	0.0	5.0	0.25	30.0	1.90	1.95
16	2.5	2.5	0.37	26.5	1.78	1.79
17	2.5	2.5	0.37	40.5	1.78	1.78
18	2.5	7.5	0.37	33.5	2.19	2.20
19	5.0	0.0	0.50	30.0	2.30	2.32
20	2.5	2.5	0.12	33.5	2.27	2.29
21	5.0	5.0	0.25	30.0	2.38	2.39
22	0.0	0.0	0.25	30.0	1.21	1.22
23	0.0	0.0	0.50	37.0	1.31	1.32
24	5.0	5.0	0.25	37.0	2.36	2.34
25	0.0	0.0	0.50	30.0	1.42	1.43
26	5.0	0.0	0.25	30.0	2.53	2.65
27	2.5	2.5	0.37	33.5	2.98	**3.45**
28	2.5	2.5	0.62	33.5	2.43	2.44
29	0.0	0.0	0.25	37.0	1.54	1.55
30	5.0	5.0	0.50	37.0	2.65	2.67

Table 7: Enhanced release of FA using FAE and Paenibacillus macerozyme

Volume of crude macerozyme added (µl)	Total FAE activity (nkatals)	Total FAE activity (mU)	Specific activity (nkatals/mg)
0	45.4 ± 3.5	2726 ± 2.2	2.2 ± 0.2
10	63.8 ± 4.1	3832 ± 3.7	3.1 ± 0.3
20	71.3 ± 4.2	4278 ± 1.8	3.4 ± 0.4
30	82.6 ± 5.1	4955 ± 1.3	4.0 ± 0.2
40	95.7 ± 5.6	5746 ± 3.5	4.6 ± 0.3
50	111.5 ± 6.7	6690 ± 2.4	5.4 ± 0.2
60	122.8 ± 3.2	7367 ± 2.9	6.0 ± 0.5
70	131.4 ± 2.7	7885 ± 1.7	6.4 ± 0.2
80	137.2 ± 2.1	8235 ± 2.2	6.7 ± 0.2
90	145.9 ± 2.6	8753 ± 2.8	7.1 ± 0.5
100	**156.0 ± 3.9**	**9360 ± 2.5**	**7.6 ± 0.3**
n=3, results are shown as mean ± standard deviation, P-value < 0.05			

Fig. 1: Morphological features of A. niger isolates under 400 times magnification a) S-1; b) S-2; c) S-3; d) S-4; e) S-5; f) S-6.

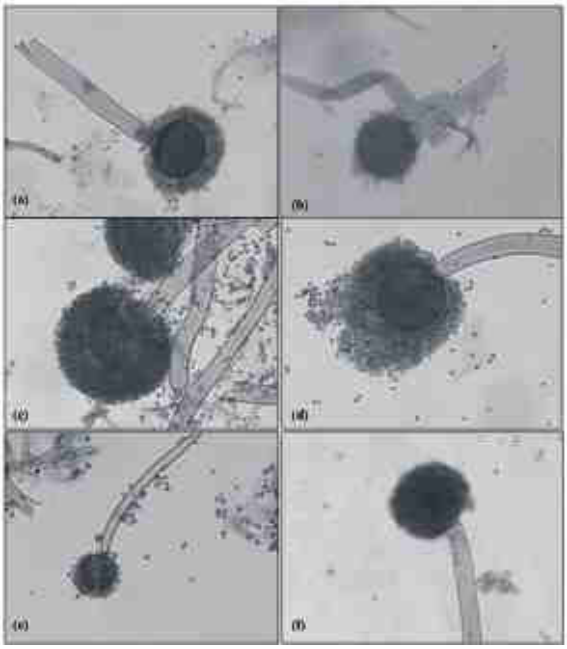


Table 6: ANNOVA results of FAE responses

Source	Sum of Square/ Mean Square	Degree of Freedom	f-value	p-value
Plackett Burman Model				
Model	13442.7	5	1164.2	0.038
A (Rice bran)	11675.5	1	1173.5	0.003
B (Wheat bran)	2987.4	1	154.6	0.076
C (Cooked rice)	23658.8	1	256.8	0.065
D (Sucrose)	32.8	1	382.9	0.054
E (KH_2PO_4)	3674.9	1	548.7	0.057
F ($NaNO_3$)	1083.8	1	456.4	0.085
G (Tween 80)	456.9	1	765.4	0.034
H (Inducer)	3944.7	1	568.3	0.072
I (pH)	453.6	1	502.7	0.056
J (Temp.)	3874.5	1	1267.3	0.027
K (Time)	1820.6	1	443.3	0.056
Residual	83.4	1		
CCD Quadratic Model				
Model	1673.4	10	1283.8	0.054
A (Rice bran)	3434.5	1	1446.8	0.073
B (Wheat bran)	1673.6	1	1621.7	0.047
C (Sucrose)	1193.7	1	1327.4	0.062
D (Temp.)	1773.7	1	1427.3	0.073
Residual	114.8	12	1793.6	0.082
Lack of Fit	2.2	10	1562.7	0.027
Pure Error	0.54	7		
Corrected Total	5.7	16		

R2 0.9981; Adj R2 0.9983; Pred R2 0.9947; Adeq Precision 127.5; Model f-value (Plackett Burman) - 1164.2; Model f-value (CCD) 1283.8; Lack-of-fit value 2.2

Table 8: Induction of FAE on rice bran using pure FA

Amount of FA added	Total FAE activity (nkatals)	Total FAE activity (mU)
Control	45.9 ± 2.5	2754 ± 2.5
1ng/ml	99.0 ± 1.8	5943 ± 3.7
1µg/ml	112.4 ± 3.2	6747 ± 2.3
1mg/ml	133.6 ± 4.1	**8019** ± 1.9

N=3, results are shown as mean + standard deviation, P-value < 0.05

Fig. 2: Release of FA using FAE
Results are shown as mean of three experiments + standard deviation

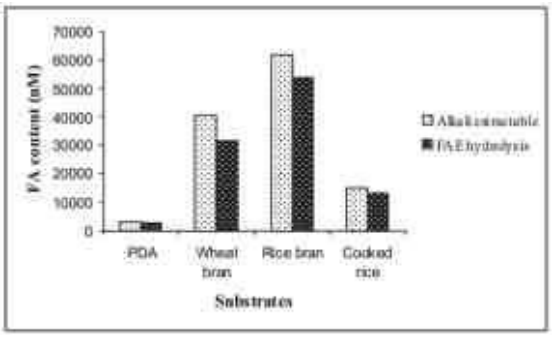


Fig. 3: Response surface plots showing relative effects of critical parameters on production of FAE by Aspergillus niger isolate S-4 while keeping others at constant levels.

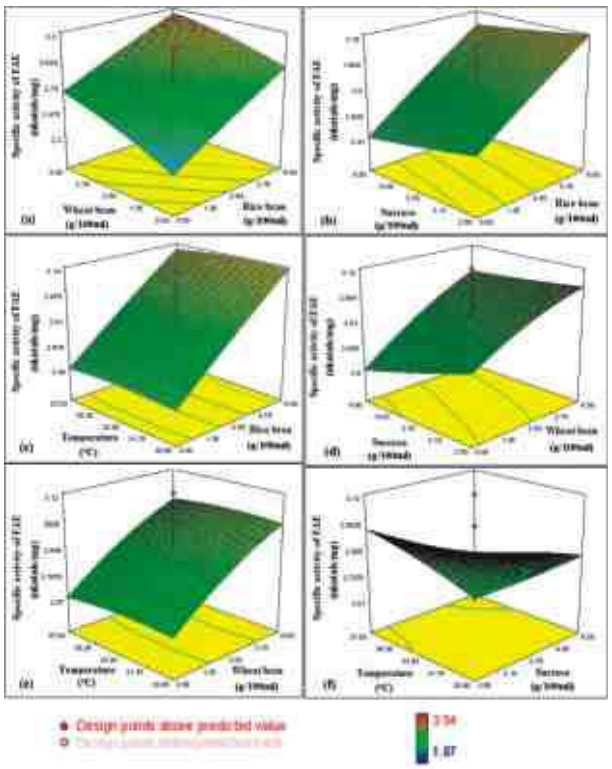
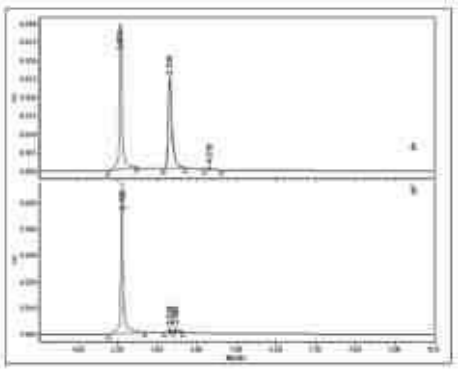


Fig. 4: C-18 Reverse phase chromatogram of a) extracted ferulic acid; b) pure ferulic acid



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