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Production of Ferulic acid from Oryzanol Degradation during the Fermentation of Black Rice Bran by Ferulic acid esterase producing *Aspergillus oryzae* HP

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Rice bran (RB) is the known source for phenolic acids, anthocyanins, flavonoids, steroidal compounds, polymeric carbohydrates, proteins and essential nutrients like vitamin B, E, γ-oryzanol, ferulic acid and inositol. Ferulic acid (FA), an ingredient for therapeutics, can be produced from oryzanol by ferulic acid esterases (FAE). The present study deals with the screening and production of FA from RB by *Aspergillus oryzae*. About 21 strains were screened through plate assays and spectrophotometric analysis. *A. oryzae* HP 36 has been identified as potent FAE producer (3.20 ± 0.10 mm) among the tested strains and optimum incubation up to 72 h yielded maximum of ~ 2.16 ± 0.05 μmol/ h of FAE. RB fermentation by *A. oryzae* HP 36 resulted in the production of 311.18 ± 15.6 μg of FA/ 5 g of RB. High-performance liquid chromatography (HPLC) analysis of FA and oryzanol content in fermented samples also supports the formation of FA from oryzanol during fermentation. This primary study, represent the efficiency of ferulic acid esterase of *A. oryzae* in naive condition and use of RB as substrate for FA production. Further, characterization of FAE and standardization of fermentation conditions are under progress to achieve improved FA production.

Keywords: *A. oryzae*, Ferulic acid esterase, Ferulic acid, γ-oryzanol, Rice bran.

Rice is the principal food crop of Asia that provides essential nutrients like vitamin B, E, γ-oryzanol, ferulic acid (FA) and inositol[1, 2]. About 10 % of the rice weight is attributed to bran, consists of phenolic and cinnamic acids, anthocyanins, flavonoids, steroidal compounds, polymeric carbohydrates and proteins[3]. Ferulic acid derivatives are used as ingredients for therapeutic agents. Profitable innate FA is majorly produced from rice bran oil because of cross-links with polysaccharides. FA can be unconfined from sugar links by ferulic acid esterases (FAEs) and alkaline hydrolysis[4]. FAE can be a solo catalytic unit, in addition as a part of a multi-modular protein. Some of the FAEs have carbohydrate-binding modules[5]. The catalytic ability of FAEs is attributed to the firm binding of carbohydrate binding module to a catalytic domain[6]. FAEs are classified based on the substrate specificity, sequence similarity and other criteria as detailed by Fazary and Ju[7].

Some of the *Fusarium* species such as *F. oxysporum*, *F. proliferatum* were also reported for their ability to produce FAE[8-10]. *B. subtilis* can be found as FAE producer by Donaghy et al. [11]. Some studies have been reported the FAE producing nature of *Streptomyces*, such as *Streptomyces olivochromogenes*[12], *Streptomyces avermitilis*

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CECT 3339[13], Streptomyces S10[14], *Streptomyces ambofaciens*[15]. Some of the recent studies explain about FAEs producing lactic acid bacteria[16]. Donaghy et al.[11] have reported the FAE producing *Bacillus* spp. and *lactobacilli*. FAE producing *Lactobacillus acidophilus* was isolated, and the enzymes were purified and characterized[17, 18].

Screenings of FAE have been reported in several previous studies[19]. Ferulic acid from wheat bran was extracted by FAE of *Aspergillus niger* [20]. Koseki et al[21], and Rumbold et al[22] have reported the production of FAE like enzyme by *Aspergillus oryzae* and *Aureobasidium pullulans*, respectively. Some studies provided the evidences for FAE like enzymes in *A. oryzae* and its expression[23, 24]. Likewise, a study by Udatha et al[25] revealed that *A. oryzae* encodes the FAE like enzyme with structural and substrate specifications. Best of our knowledge, there is no detailed study on conversion of rice bran γ-oryzanol to FA by *A. oryzae*.

In the present study, we screened *A. oryzae* strains for the identification of high content of FAE producing strain and investigated the ability of *A. oryzae* HP to produce ferulic acid from rice bran (RB) and assessment of γ-oryzanol degradation during fermentation.

MATERIALS AND METHODS

Raw materials, Strains and Screening of FAE producing *A. oryzae*

Black rice bran was received from Maerim district and Chiang Mai province and used as a substrate for fermentation. About seven *A. oryzae* strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR) Culture Collection and 14 strains were received from health product research and development unit, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. *A. oryzae* strains were maintained on Potato dextrose agar (PDA) or Sabouraud Dextrose Agar (SDA) and spores were collected by growing on PDA for 5 days at 30 °C. SDA plates were prepared with 1% (v/v) of filter (0.22 µm) sterilized ethyl ferulate (substrate of FAE, carbon source) and fungal strains were inoculated and incubated at 30 °C for 3-5 days. Clear zone formation around the fungal colonies indicates the FAE producing ability and the zone was measured by vernier caliper. Screening was repeated five times to ensure the FAE activity of the strains.

Preparation of crude FAE from selected fungal strains

The minimum salt medium (0.1% (w/v) yeast extract, 0.10% (w/v) $(NH_4)_2SO_4$, 0.008% (w/v) $CaCl_2.2H_2O$, 0.04% (w/v) KH_2PO_4, 0.025% (w/v) $MgSO_4.7H_2O$ and 0.002% (w/v) $FeCl_3$; pH 7.0 ± 0.2) was prepared with the supplementation of 1% of ethyl ferulate. Ethyl ferulate (1%) stock solution was made in dimethylformamide. Spores were collected by growing the selected strains on PDA for 5 days at 30 °C and spore suspensions were prepared in sterile distilled water, and the concentration of spore solution was estimated by hemocytometer based counting method. About 10^6 spores/ml of medium was used as inoculum and culture flasks were incubated at 30 °C on a rotary shaker (200 rpm) for 7 days.

Samples were collected every 24 h, and fungal mycelium were removed through a muslin cloth followed by centrifuged at 10,000 rpm for 15 min at 4 °C. FAE activity of the crude enzyme was determined by spectrophotometric assay[26].

FAE assay

FAE activity was determined by measuring the rate of hydrolysis of 4-Nitrophenyl ferulate (4NPF, Merck, Germany)[26]. Crude FAE samples were mixed (9: 1 ratio) with assay solution (10.0 mM of 4NPF in DMSO, 2.5% Triton X-100 and 0.1 M potassium phosphate buffer (pH 6.8 ± 0.3)) and incubated at 37 °C for 10 min. After incubation, the amount of 4-nitrophenol from 4NPF released was evaluated by measuring at 405 nm using multimode spectrophotometer (DTX 880, Beckman Coulter Inc., UK). The extracellular FAE enzyme activity was defined as the amount of FAE enzyme that releases 1 µmol of 4NP from the substrate 4NPF per hour under the condition of the analysis. The assay was repeated five times with independent replicates.

Fermentation of RB

100 g of rice bran used as a substrate was sterilized and mixed with 100 ml of filter (0.45 µm, nitrocellulose membrane from Millipore) sterilized nutrient solution (2 g/l KH_2PO_4, 1 g/l $MgSO_4$, 8 g/l $(NH_4)_2SO_4$ in 0.4 N HCl) (27, 28). *A. oryzae* HP spore suspension was added at an initial concentration of 7.8 x 10^6 spore/g of bran. Then mixture was incubated at 30 °C for 5 days. Every 24 h, samples

were collected for analysis and stored at 20 ° C until processing.

Extraction of oryzanol

Oryzanol extraction from fermented rice bran was executed by hexane. Five gram of fermented rice bran was stirred with 50 ml of hexane for 30 min three times at room temperature (RT). Samples were extracted trice to recover maximum yield. The extracts were filtered through Whatman no.1 filter paper, and residual hexane was evaporated by rotary evaporator at 50 °C under reduced pressure and stored at 20 °C.

Extraction of FA

Phenolic compounds of fermented rice bran were extracted using 80% ethanol. Five gram of bran was bran was allowed to shake at RT for 1 h at RT for 1 h with 200 ml of 80% ethanol. Samples were extracted trice to recover maximum yield. The extracts were filtered through Whatman no.1 filter paper, and residual ethanolic part was evaporated by rotary evaporator at 60 °C under reduced pressure and stored at 20 °C.

Analysis of g-oryzanol

γ-oryzanol content of rice bran extracts were analyzed by HPLC. Preparing in 10 mg/ml of extracts in dichloromethane and filtered through a 0.45 µm syringe filter. A reverse-phase C18 column (Shodex C18-4E (5 µm), 4.6 X 250 mm) purchased from Showa Denko America, NY, USA was used. The mobile phases of the system consist of 50% (v/v) methanol, 44% (v/v) acetonitrile, 3% (v/v) dichloromethane and 3% (v/v) acetic acid. 20 µl of samples were injected, and flow rates were adjusted to 1.4 ml/min. Fraction detection was achieved with Ultraviolet-Visible detector at 330 nm. Quantitative data was obtained by external standards of

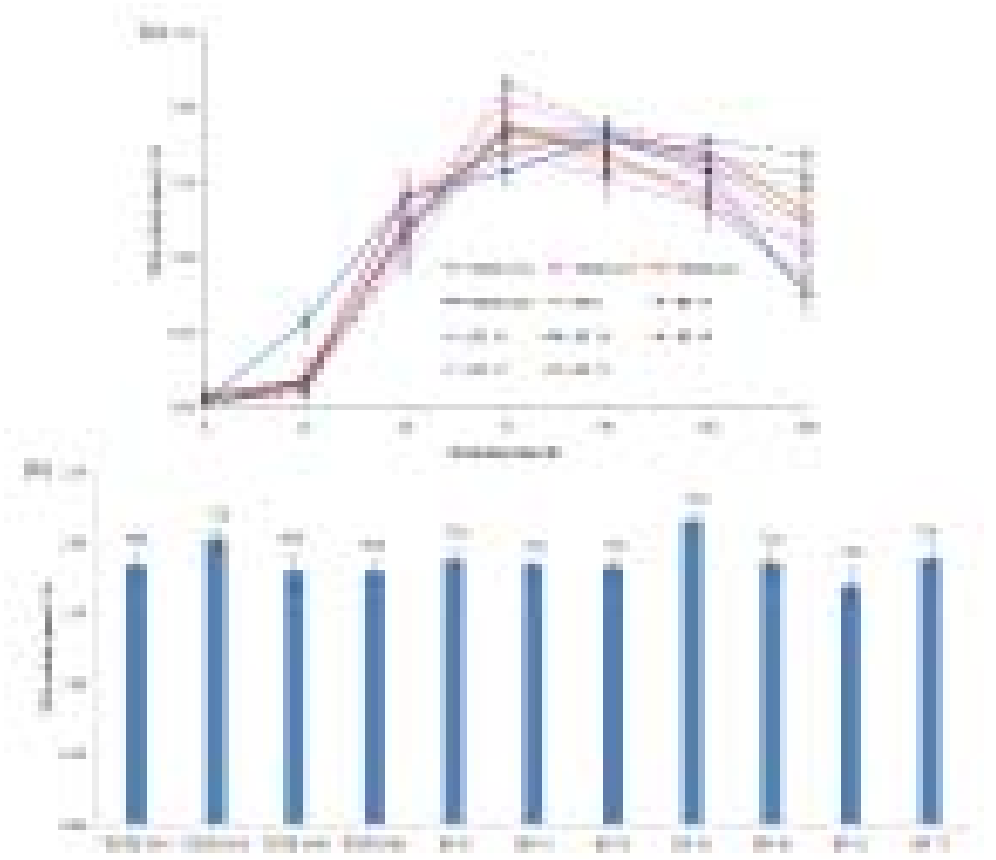


Fig. 1. (a) Kinetic analysis of FAE activities (µmol/hour) of 11 *Aspergillus* strains in medium at various incubation times. (b) Graphical representation of optimum incubation time of tested strains with maximum FAE activity. Values on each bar represents the incubation time. All the data are outcome of three independent assays and represented as mean ± SD and are significantly different ($p < 0.05$).

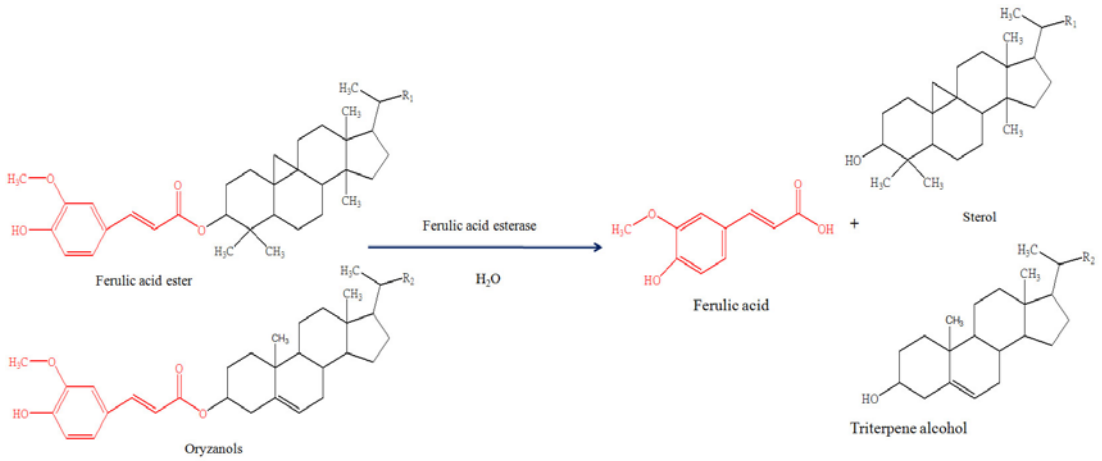


Fig. 2. Schematic representation of FAE mediated transfer of ferulic acid esters and oryzanol to ferulic acid, sterol, and triterpene alcohol. R group of the substrate and by products are varied.

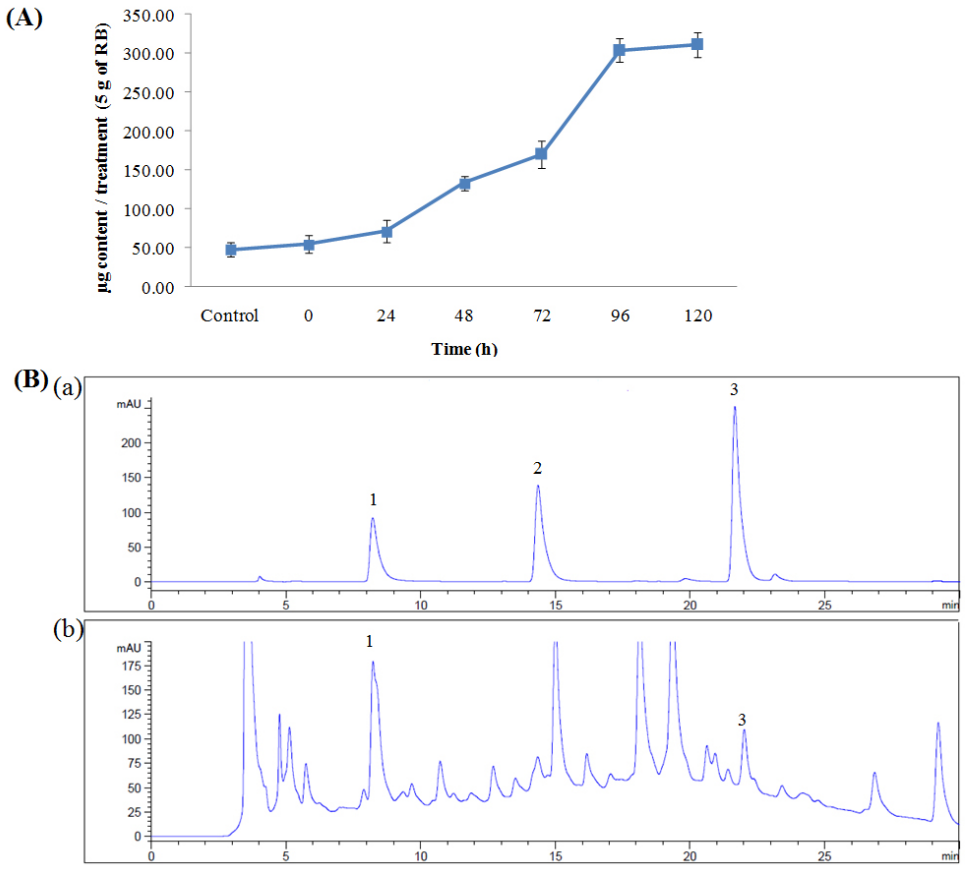


Fig. 3. (A) Concentration of ferulic acid during fermentation and values were represented as mean ± SD. (B) Representative HPLC-chromatogram of ferulic acid analysis. (a) Protocatechuic acid (1), vanillic acid (2) and ferulic acid (3) were used as standards. (b) Chromatogram of fermented sample, protocatechuic acid (1), and ferulic acid (3) was detected with several unknown peaks.

γ-oryzanol. The standard curve was generated using range 0.1-2.0 mg/ml of γ-oryzanol and plotted against peak area (R^2 = 0.9996).

Analysis of ferulic acid

The samples were filter sterilized through a 0.45 µm syringe filter prior to the HPLC analysis. Column specifications are as same as used for g-oryzanol analysis. Samples were eluted with gradient A (100% acetronitrile) and gradient B (1% of trifluoroacetic acid in water) mixture as followed: for 0 to 30 min with 90% to 70%, then 31 to 35 min with 70% to 90%, then 36 to 60 min with 90% of gradient A. The flow rate was fixed at 0.8 ml/min at 30 °C with UV detection system at 280 nm. Phenolic acids were identified by comparison of retention times and absorption spectrum with different standards of rice bran (photocatechuic, vanillic and ferulic acid). The ferulic acid standard curve was generated using concentration ranging from 0.003-0.5 mM of ferulic acid and were plotted against peak area (R^2= 0.9999).

Statistical analysis

Analysis of variance (ANOVA) was performed. Duncan's new multiple range test determined significant differences, at the 95% confidential level ($p < 0.05$) using statistical SPSS software version 17.0 (SPSS Inc, Chicago, U.S.A).

RESULTS AND DISCUSSION

About 21 *A. oryzae* strains were obtained from TISTR and health product research and development unit as detailed in materials and methods section. Screening of FAE production by *A. oryzae* was carried out by plate assay, and the ability of FAE production was detected based on the formation and size of the clear zone around fungal colonies. Among 21 strains, about 11 strains showed a positive result and 10 strains were found as non-FAE producers. *A. oryzae* HP 36 has been selected as potent FAE producer (3.20 ± 0.10 mm of clear zone) among the tested strains (Table. 1). All positive strains (n=11) were subjected to the kinetic evaluation of FAE production to find out the precious time required for maximum enzyme release and FAE activity was measured by spectrophotometric assay as detailed in materials and methods section. *A. oryzae* HP 36 produces ~ 2.16 ± 0.05 µmol/ h of FAE after 72 h of incubation. During later hours, the FAE production was declined (Fig: 1a). Each strain showed their maximum FAE activity with varied optimum incubation time, but *A. oryzae* HP 36 was the maximum FAE producer compared to other strains (Fig: 1b). Thus *A. oryzae* HP 36 has been selected for further fermentation of RB to produce FA.

Rice bran is the recognized as a better source of FA, because of high content of g-oryzanol (mixture of ferulic acid esters). RB was subjected to fermentation by *A. oryzae* HP at 30 °C for 5 days. As per the previous literatures, the degradation of ferulic acid esters and oryzanol by FAE leads to the formation of ferulic acid along with sterol and triterpenealcohol (Fig. 2). R group of ferulic acid esters and oryzanol are varied as detailed in Table. 2. Fermented products were collected after every 24 h of process and analyzed for the content of FA and residual γ-oryzanol.

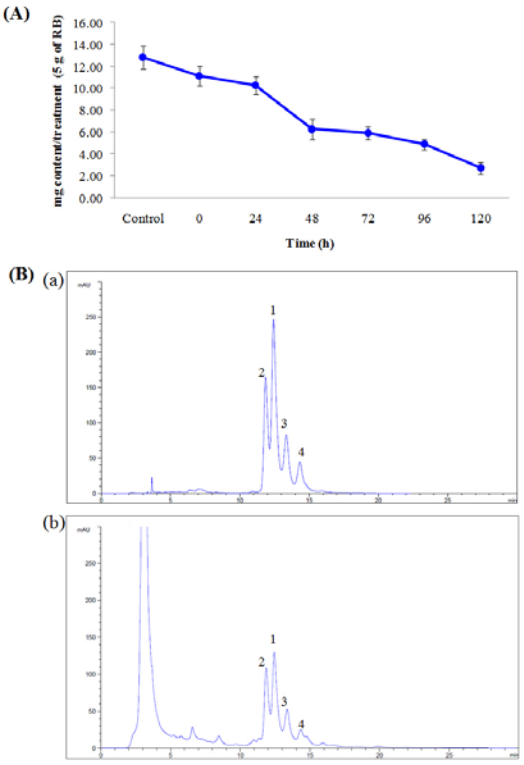


Fig. 4. (A) Depletion of γ- oryzanol concentration during fermentation and values were represented as mean ± SD. (B) Representative HPLC-chromatogram of γ-oryzanol analysis. (a) 24-methylenecycloartanyl ferulate (1), cycloartenyl ferulate (2), campesteryl ferulate (3) and β-Sitosteryl ferulate (4) were used as standards. (b) Chromatogram of fermented sample.

FA concentration was found as increased constantly during the fermentation process from 55.04 ± 11 to 311.18 ± 15.6 µg / 5 g of RB (Fig. 3A). HPLC analysis of FA content in fermented samples also supports the formation of FA during fermentation. The representative chromatogram with multiple peaks suggested that *A. oryzae* HP mediated fermentation triggers the degradation of RB residing compounds (Fig. 3B). Residual g-oryzanol content of the fermented samples were analyzed to justify the action of FAE on γ-oryzanol and the formation of FA. The concentration of γ-oryzanol was found as declined persistently during fermentation. Initial concentration of γ-oryzanol was 11.13 ± 0.89 mg/ 5 of RB, whereas after 5 days of fermentation depleted and the concentration was found as 2.71 ± 0.54 mg/ 5 of RB. Almost 5 fold reduction of γ-oryzanol content was recorded after *A. oryzae* treatment and HPLC chromatogram also clearly indicated the degradation of γ-oryzanol (Fig. 4).

Table 1. The ferulic acid esterase activity of *A. oryzae* strains by using agar diffusion technique. The efficiency and amount of FAE enzyme production depends on the size of clear zone formation.

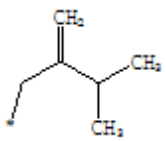
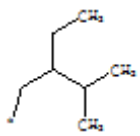
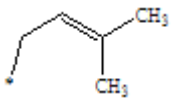
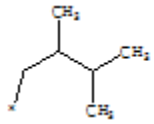
S. No.	Strains	Size of the clear zone (mm)*
1	*A. oryzae* TISTR 3014	2.77 ± 0.09
2	*A. oryzae* TISTR 3018	ND
3	*A. oryzae* TISTR 3019	2.62 ± 0.17
4	*A. oryzae* TISTR 3031	ND
5	*A. oryzae* TISTR 3040	3.15 ± 0.09
6	*A. oryzae* TISTR 3065	2.57 ± 0.08
7	*A. oryzae* TISTR 3131	ND
8	*A. oryzae* HP 7	ND
9	*A. oryzae* HP 11	3.17 ± 0.07
10	*A. oryzae* HP 14	2.91 ± 0.09
11	*A. oryzae* HP 19	2.12 ± 0.07
12	*A. oryzae* HP 22	ND
13	*A. oryzae* HP 36	3.20 ± 0.10
14	*A. oryzae* HP 42	ND
15	*A. oryzae* HP 49	2.37 ± 0.08
16	*A. oryzae* HP 52	2.62 ± 0.06
17	*A. oryzae* HP 57	ND
18	*A. oryzae* HP 62	ND
19	*A. oryzae* HP 67	ND
20	*A. oryzae* HP 73	3.16 ± 0.05
21	*A. oryzae* HP 82	ND

* Inhibition zone was measured from the margin of fungal colony (not included diameter of fungal colony); ND means the clear zone was not detected.

Sequence similarity based screening of FAE coding genes in *A. oryzae* end up with the identification of putative genes, XP_001818628 (rAoFaeB) and XP_001819091 (rAoFaeC), which were cloned in *Pichia pastoris* and characterized for its optimum temperature, pH, thermostability, and substrate specificity. rAoFaeB and rAoFaeC are active on ±-naphthylacetate, ±-naphthylbutyrate, α-naphthylcaproate, and α-naphthylcaprylate, but, inactive on acyl-chain substrates with more than ten carbon atoms. These recombinant enzymes can effectively releases the FA from wheat arabinoxylan with the combination of xylanase[23]. *A. oryzae* contains 13 FAEs, called feruloylome. About three predicted FAE coding genes, A.O.2, A.O.8, and A.O.10, were characterized by cloning and expression in *P. pastoris*.

Pharmacophore models were developed for those enzymes according to substrate specificity and common features[25]. A novel type-A feruloyl esterase coding gene of *A. oryzae* was cloned and expressed in *P. pastoris*. About 37 kDa

Table 2. Chemical structures of the four major substitution components of γ-oryzanol

Compound	R1 substitution	Compound	R2 substitution
24-Methylenecycloartanylferulate		β-Sitosterylferulate	
Cycloartenylferulate		Campesterylferulate	

protein showed optimal de-esterification at 50 °C, pH of 4-6 and inert to metal ions but influenced by Cu^{2+}. About 47.8 % of FA in wheat bran was released by recombinant enzyme[24]. In the present study fermentation was carried out at 30 °C and achieved notable amount of FA formation and further detailed standardization of temperature, pH and co-factor will provide the in-depth knowledge on FAE of *A. oryzae* HP.

CONCLUSION

A. oryzae HP 36 mediated conversation of γ-oryzanol to FA was demonstrated in this study. This is the primary study, portrait the efficiency of ferulic acid esterase of *A. oryzae* in naive condition and use of RB as substrate for FA production. Further, thorough characterization of enzyme FAE and standardization of fermentation condition are under progress.

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