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Abstract

Sweet corn cob (SCC), an agricultural by-product of the corn processing industry, contains more than 80% insoluble-bound ferulic acid (FA). Extraction of these bound phenolics can be achieved through chemical or enzymatic hydrolysis, however the shift towards greener chemistry has raised awareness towards the use of enzymatic hydrolysis. In the present study, the ability of ferulic acid esterase (FAE) and xylanase (XY) to catalyse the hydrolysis of FA from SCC was investigated. Response surface methodology (RSM) based on a five-level, four-factor central composite rotatable design (CCRD) was used to establish the optimum conditions for enzymatic hydrolysis of FA from SCC. SCC was treated with the combination of FAE and XY at various concentrations (FAE: 0.00 to 0.04 U/g; XY: 0.00 to 18093.5 U/g), temperatures (45 to 65°C) and pH (pH 4.5 to 6.5). RESULTS: The optimum extraction conditions predicted by the model were: FAE concentration of 0.02U/g, XY concentration of 3475.3 U/g, extraction pH of 4.5 and extraction temperature of 45°C. CONCLUSION: Under these conditions, the experimental yield of FA was 1.69 ± 0.02g kg^{-1} of SCC, which is in agreement with the value predicted by the model.

Keywords

Sweet corn cob; response surface methodology; ferulic acid; ferulic acid esterase; xylanase.

Highlight

- RSM was used to optimise the extraction of ferulic acid from SCC.
- Optimum extraction conditions were FAE concentration at 0.02U/g, XY concentration at 3475.4 U/g, pH 4.5 and 45°C.

- The yield of ferulic acid was 1.69 ± 0.02g kg^{-1} of SCC at optimum extraction conditions.

1.0 INTRODUCTION

The annual world production of corn is about 520 teragram with most of the corn being used for animal feed or human consumption (64 and 19% of global production, respectively) (1, 2). Sweet corn cobs (SCC) are an agricultural by-product of the corn-processing industry. Zheng, Choo (3) reported that the average yield of corn cob is about 14% of grain yield, which accounts for up to 16% of the total corn stover in a field. Utilization of corncob as animal beddings (4), biological substrate for the production of furfural (5), carbon adsorbents (6) and forage protein (7) have been widely studied. In addition, research on corn cob as a source of ferulic acid (FA) has notably increased in recent years (8). FA has been reported to exhibits a wide range of therapeutic effects against various diseases including diabetes, cancer, neurodegenerative and cardiovascular diseases (9). In addition, FA can also prevent oxidation and has been approved to be used as food additives in beverages, food and cosmetic in Japan (10). Based on previous findings using alkaline hydrolysis, 97% of FA in corn cob was present in insoluble bound form. The insoluble bound FA is covalently bound to the polysaccharide components of plants through ester linkages and these crosslinks significantly limit the degradation of cell wall by rumen microorganism, thus limiting the digestibility by ruminants (11).

Extraction of FA has been carried out via alkaline (12, 13), acidic (14), pressurised solvents (15), ultrasonic (16), supercritical CO_2 (17), microwave-assisted (18) and enzymatic (19) extraction. Commonly, the release of FA from SCC has been carried out using alkali

hydrolysis (13, 20, 21). The yield of FA from SCC was reported to be 3.06g kg^{-1} (22), 7.65g kg^{-1} (23) and 1171mg L^{-1} (20), respectively. However, this conventional method of hydrolysis has several disadvantages including usage of large amount of solvent and subsequent solvent disposal problems (24), leading to an increase in environmental pollution. In this context, enzymatic hydrolysis has drawn great interest due to its lower environmental impact as the use of chemicals is negligible and requires low energy (25). Recently, Pérez-Rodríguez, Torrado Agrasar (26) investigated the utilization of high hydrostatic pressure along with feruloly esterase on the release of FA from corn cob. Ferulic acid esterase (FAE) was reported to break the ester linkage between FA and the attached sugar, thus releasing the FA from the complex cell wall (11). However, a specific cell wall degrading enzyme such as xylanase could be used to further improve the extraction by solubilizing part of the cell wall structure and forming low molecular weight ferulolyted compounds, to allow FAE to act on these low molecular weight ferulolyted compounds releasing the FA (27).

However, the main drawback of using enzymatic hydrolysis is the low hydrolysis rate as compared to chemical hydrolysis. To overcome this, several physicochemical factors such as incubation temperature, incubation time, enzyme concentration and pH need to be considered prior to enzymatic hydrolysis (28). The conventional optimization involves changing one independent variable at a time while keeping the rest of the factors constant. However, this conventional experimental design does not include interaction among the variables and therefore is often incapable of detecting the optimum conditions (29). In order to overcome this problem, response surface methodology (RSM) can be used to carry out optimization studies (30). RSM is less laborious and time-consuming than conventional

optimisation methods as it reduces the number of experimental trials needed to evaluate the effect of multiple parameters and their interaction (28). Previously, RSM has been used to optimize the extraction of FA from various agricultural waste including paddy straw (2) , rice bran, maize bran, wheat straw, wheat bran, sugar cane bagasse, orange peels, pomegranate peels and pineapple peels (31).

Previous studies on the alkali hydrolysis of SCC showed that it contains 3.06g kg^{-1} of total FA (13). Hence, this research aimed to investigate the effect of extraction parameters (enzyme concentration of FAE and XY, pH, and temperature) on the yield of FA from sweet corn cob. RSM optimisation by central composite rotatable design (CCRD) was used for model fitting and to predict the optimum condition for the extraction FA from sweet corn cob.

2.0 EXPERIMENTAL

2.1 Materials

Sweet corn cob (SCC) used in this study was harvested in Senegal in December 2015 and was kindly provided by Barfoots of Botley Company Ltd (West Sussex, United Kingdom). Ferulic acid esterase (FAE) by *Clostridium thermocellum* and endo-1,4-β-xylanase (XY) by *Trichoderma viride* were purchased from Prozomix Limited (Northumberland, United Kingdom) and Megazyme International Ireland Limited (Bray, Ireland). All other chemicals used in this experiment were of analytical grade.

2.2 Sample preparation

The corn kernels were removed manually from the cob and discarded. The sweet corn cobs were then chopped into 5cm pieces in length, and frozen in a blast freezer (-18°C) for an hour

and then freeze dried (Christ Gamma 2-16, Martin Christ Gefriertrocknungsanlagen, Germany) until constant weight was achieved. The dried samples were finely ground in a mill (Apex Comminuting Mill, Sherborne, Dorset, UK), sieved through a 150 mesh screen (particle size <0.1mm), thoroughly mixed and stored in the freezer (-80°C) until further analysis.

2.3 Enzyme activity test

Enzyme activity assays were performed at 45°C in sodium phosphate buffer at pH 4.5. One unit of enzyme was defined as the amount of enzyme used to release 1μmol of product per minute. FAE was assayed with methyl ferulate as the substrate as previously described by Kroon, Williamson (32). The amount of FA that was released was analysed by using HPLC as describe in Section 2.6. The activity of *Trichorderma viride* xylanase was assayed using beech wood arabinoxylan (1mg/mL) as the substrate. Xylanase activity was determined by measuring the release of reducing sugar by 3,5-Dinitrosalicylic acid (DNS) reagent (33), and was expressed as xylose equivalent. Briefly, 4mL of DNS reagent was added to 1mL of test sample and placed in boiling water for 5 minutes. The reaction was terminated by placing tubes in a boiling water bath for 10 minutes and the absorbance was read at 540nm.

2.4 Preliminary work: determination of independent variables and their levels

Preliminary experiments were conducted to select a suitable range of enzyme concentration, temperature, pH and time of FAE and XY for the design of the experimental RSM run. First, the concentration of FAE was determined by hydrolysing freeze dried SCC (5%) using various concentrations of FAE (0.02, 0.05, 0.19U/g of SCC) for 4 hours at 37°C and pH 6.5 (optimum temperature and pH from manufacturer). FAE concentration at 0.02U/g

of SCC showed the highest amount of FA (Supplementary material, Figure S1). Then, hydrolysis of FA from freeze dried SCC (0.1g) using FAE (0.02U/g) was carried out at different pH (pH 4, 5, 6, 7) and temperature (20, 35, 40, 50, 60°C) for an hour to obtain the optimum pH and temperature of FAE. The concentration of enzyme used in this research is lower than the amount reported by Pérez-Rodríguez, Torrado Agrasar (26) (0.044U of FAE per gram of dry milled corn cob). The content of FA was then quantified using HPLC (Section 2.6). Similarly, the end product of XY hydrolysis (878.9 U/g of SCC) at different pH and temperature, xylose, was analysed using the DNS method as describe in Section 2.3.

Adopting the best working temperature (55°C) and pH (5.5) for both FAE and XY, the combination of FAE:XY at different ratios (1:0, 1:1, 1:10, 1:100, 1:1000, 1:10000, 2:0, 2:1, 2:10, 2:100, 2:1000 and 2:10000 U/U) were used to determine the best concentration for the maximum release of FA from SCC. The combination of FAE:XY at 1:10000 was found to release the maximum amount of FA (Supplementary material, Figure S2). Finally, FAE at 0.02U/g with XY at (9048.5U/g) was used to hydrolyse 0.1g of SCC at 55°C and pH 5.5 at various extraction time (1 to 24 hours) to determine the best extraction time for the release of FA from SCC. Based on the results, the three levels (lower, middle, upper) of each variable were determined and selected for RSM. All experiments were carried out in duplicate (n=2) unless otherwise mentioned.

2.5 Enzymatic hydrolysis

Five grams of freeze dried SCC powder were defatted in a Soxhlet apparatus with hexane for six hours before the hydrolysis. For each experiment, a mixture of defatted SCC (0.1g) with

varying amount of phosphate citrate buffer, FAE (0.00 to 0.04U/g) and XY enzymes (0.00 to 18093.50 U/g) was used as shown in **Table 1**. The mixtures were stirred in a shaking water bath at different reaction temperatures (45 to 65°C), for three hours. The pH of the mixtures was varied from pH 4.5 to 6.5. The range of enzymes, pH and incubation temperature were determined based on the preliminary experiments. After the reaction was completed, the enzyme was inactivated by placing the mixture in a water bath at 90°C for five minutes. The suspension was centrifuged at 18,000 xg for ten minutes and the supernatant was collected. FA in the supernatant was extracted 6 times using diethyl ether at a supernatant-to-solvent ratio of 1:1 and was evaporated to dryness. The extract containing the FA was then re-dissolved in methanol prior to HPLC analysis.

2.6 HPLC analysis of FA

The quantification of FA was carried out according to the method as described in Lau, Harbourne (13). Briefly, the analysis was performed with HP Agilent 1050 liquid chromatography equipped with a DAD detector, with a Zorbax SB-C18 column (2.1 x 15mm, 1.8 micron). The mobile phase used was (A) formic acid/HPLC water (0.1:100 v/v) and (B) formic acid/acetonitrile (0.1/100 v/v). Solvent B was increased to 25% (0- 25 min), followed by 90% B for 30 minutes and then a final wash of 100% B for 10 minutes. The injection volume was 5µL with a flow rate of 0.2mL/min. Detection at 280nm was used for the quantification of FA using an external calibration curve (concentration from 0.01 to 0.2g kg^{-1} of FA; R_2 = 0.9998).

2.7 Experimental design

RSM was used to determine the optimum conditions for the enzymatic hydrolysis of FA from SCC powder. After determining the preliminary range of the extraction variables, a five-level-four-factor central composite rotatable design (CCRD) with 31 experiments was employed in this study (**Table 1**). The experimental design and statistical analysis were performed using Minitab® software 17.1.0. The variables optimised were concentration of FAE (X_1), concentration of XY (X_2), pH (X_3) and temperature (X_4). The design consisted of sixteen factorial points, eight axial points and seven replicates of the centre point. The 31 experiments were randomised and the response (yield of FA) was recorded in **Table 2**. Data from the CCRD were analysed by multiple regression to fit the quadratic polynomial model. The analysis of variance and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. *p*-values of less than 0.05 were considered to be statistically significant.

Verification and validation of the model were conducted by running three additional confirmation experiments using the optimum conditions generated by the RSM. The experimental and predicted values were compared and tested for statistical differences.

3.0 RESULTS AND DISCUSSION

3.1 Preliminary determination of temperature, pH, FAE and XY ranges in RSM

The efficiency of FAE and XY is indicated by the increasing yield of FA and xylose, respectively. For FAE, the extraction yield of FA increased with the increase in pH value (pH 4 to pH 6.5) and reached a maximum concentration (0.28 ± 0.00mg FA/g) at pH 6 (Figure 1A). Furthermore the amount of FA extracted by FAE increased as extraction temperature increased

from 20 to 50^0C (Figure 1B), reaching the maximum yield (0.20 ± 0.03 mg FA/g) at 50^0C. Previously, Topakas, Vafiadi (34) reported that microbial FAEs have a wide range of temperature and pH dependences, with optimal activities occurring between 30 to 60°C and pH 4-8 . Therefore, the FAE used in this experiment was working within its optimal temperature and pH range.

The yield of xylose by XY increased as the pH increased from pH 3 to pH 5, and decreased as the pH increased further to pH 7.5 (Figure 1A). In addition, the yield of xylose increased as temperature increased from 20°C to 60°C, with a maximum yield of 13.61 ± 0.13mg XE/g (Figure 1B). The yield of xylose decreased rapidly as temperature increased to 70°C. Results from this research are in agreement with Polizeli, Rizzatti (35), where they reported that the peak activity of endoxylanases generally falls between 40 and 80°C and between pH 4.0 and 6.5. Iyer and Ananthanarayan (36) reported that several phenomenon are known to promote changes of the activity and spatial configuration of an enzyme such as pH, ionic strength, temperature, autolysis or chemical agents. They further reported that these physical denaturants can disrupt the hydrogen bond in the enzyme and results in aggregation or formation of highly disordered structure. Therefore, it was crucial to determine the best working pH and temperature for both FAE and XY used in the experiment.

Figure 1C shows that the yield of FA was markedly affected during the first 6 hours of hydrolysis. An increase in incubation time for up to 24 hours did not increase the yield of FA. No significant difference was found between 3 hours (1.05 ± 0.00mg FA/g) and 6 hours (1.02 ± 0.03mg FA/g) of extraction, which might be due to the accumulation of products inhibiting enzyme activity or the depletion of the substrates. In a study conducted by Frieden and Walter

(37) on product inhibition of enzyme, it was reported that the products of almost all enzyme-catalysed reactions may act as suppressants when present in high enough concentrations relative to the enzyme and substrate. Consequently, a period of 3 hours was chosen and used throughout the experiments.

Therefore, when taking into consideration the best working temperature and pH conditions for both FAE and XY, 55°C and pH 5.5 were chosen as the middle point for RSM, along with 50°C and pH 5 for the lower point, and 60°C and pH 5 for the high point (Table 1).

3.2 Statistical Analysis and the model fitting

In this study, there were a total of 31 runs for optimizing the four individual parameters in the CCRD. The yield of FA along with the experimental conditions are shown in **Table 2**. Results showed that the yield of FA ranged from 0.00 to 1.45g kg^{-1} FA. The maximum amount of FA (1.45g kg^{-1}) was found in conditions of X_1=0.0285 U/g, X_2=4526U/g, X_3=5, X_4 = 50°C. The results were fitted with a second order polynomial equation:

$$\text{mg FA} = 3.53 + 65.1\,X_1 - 0.0002\,X_2 + 0.32\,X_3 - 0.082\,X_4 - 1838\,X_1^2 - 0.00X_2^2 - 0.0625\,X_3^2 + 0.000351\,X_4^2 - 0.001\,X_1X_2 + 3.34\,X_1X_3 + 0.173\,X_1X_4 + 0.000032\,X_2X_3 + 0.000003\,X_2X_4 - 0.001\,X_3X_4$$

The statistical significance of the regression model was evaluated by the *p*-value and F-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 3. The determination coefficient (R^2=0.893) indicates that the model was adequate for prediction within the range of experimental variables. Table 3 showed that the linear coefficient (X_1) and the quadratic coefficient (X_1^2 and X_2^2) were found significant at

p<0.001. The linear coefficient (X_2 and X_4) and interaction coefficient (X_2X_3) were significant at p<0.01 and p<0.05. The other term coefficients (X_3, X_3^2, X_4^2, X_1X_3, X_1X_4, X_2X_4 and X_3X_4) were not statistically significant (p>0.05). Three-dimensional and contour plots were used to predict the relationships between the dependent and independent variables (Figure 2 and 3).

3.3 Effect of FAE, XY, pH and temperature on the yield of FA

The effect of FAE (X_1) concentration, XY (X_2) concentration, pH (X_3), temperature (X_4) and their interactions on the extraction efficiency of FA from SCC are reported in Table 3. The yield of FA was positively correlated to the linear effect of FAE concentration (p≤0.001), XY concentration (p≤0.01) and temperature (p≤0.01). Concentration of FAE was highly significant (p<0.001) in the release of FA from SCC. Similarly, concentration of XY (X_2) was found to be significant in this study. The presence of XY was reported to contribute towards the degradation of arabinoxylan, and thus enhance the release of FA. These endoxylanases attack the arabinoxylan backbone in an irregular manner, causing a decrease in the degree of polymerisation of the substrate and thus liberating the xylose, xylobiose and oligomers while retaining their configuration (38). This is in agreement with Yu, Maenz (11) who also reported the release of FA from oat hulls using combinations of FAE and XY.

The effect of various parameters (pH, temperature and concentration of FAE and XY) and their interaction on the yield of FA is illustrated in the response surface plot. To visualise the effect of independent parameters and their interaction, three dimensional (Figure 2) and contour plot (Figure 3) were used to show the effects of two factors on the response at a time while keeping the other two factors at level zero. The three dimensional and contour plots in

Figure 2 and 3a, which show the yield of FA as a function of FAE and XY concentration at a fixed extraction pH (pH5.5) and temperature (50°C), indicated that the extraction yield of FA increased as the concentration of FAE increased from 0.00 to 0.03U/g, followed by a decrease in the extraction yield of FA at FAE concentrations higher than 0.03U/g. Similarly, the yield of FA increased as the concentration of XY increased to 11,000U/g, and decreased as concentration of XY increased.

The three dimensional response and the contour plots at varying FAE concentration over a range of pH at fixed XY concentration and temperature are presented in Figure 2 and 3b. It can be observed that the yield of FA increased as FAE increased to 0.08U/g, however, as pH increased from pH 6.0 to 6.5, the yield of FA decreased. In Figure 2 and 3c, the three dimensional response surface and the contour plots were developed for the extraction yield of FA with varying pH and XY concentration at a fixed FAE concentration and temperature. The plots indicated that the maximum extraction yield of FA can be achieved when the concentration of XY increased to 10,000U, and decreased at higher concentration of XY. It also showed that yield of FA decreased as pH increased from pH 6.0 to 6.5.

Figure 2 and 3d showed the three dimensional response surface plot and contour plot at varying temperature and FAE concentration at fixed extraction conditions of pH 5.5 and XY concentration. It can be seen that increasing FAE concentration increases the yield of FA, however, as temperature increases, the yield of FA decreases. It can be observed that the yield of FA by XY decreases as temperature increases above 45^0C (Figure 2 and 3e). However, no interactions were found between temperature and pH at fixed amount of FAE and XY concentration (Figure 2 and 3f).

3.4 Verification of predictive model

The accuracy of the model equation for predicting the optimum response value was carried out under the following condition: FAE concentration (0.02U/g), XY concentration (3472 U/g), pH (4.5) and temperature (45°C). This set of optimum conditions was determined by the RSM optimization (Table 4) and was used to validate the experimental and predicted yields of the responses using the model equation. A mean value of 1.69 ± 0.02g kg^{-1} (n=3) was obtained from the experiment. This further validates the RSM model, showing that the model was adequate for the optimization of FA extraction from SCC.

Pérez-Rodríguez, Torrado Agrasar (26) reported that the enzymatic hydrolysis of corn cob using Ultraflo®, in combination with thermal pre-treatment released a higher amount of FA (226mg/L) than the raw sample (177 mg/L). As compared to the FA content obtained by alkali hydrolysis (3.06g kg^{-1} of SCC, Chapter 2), enzymatic hydrolysis only extracted about half of the amount of FA in SCC (1.69g kg^{-1} of SCC). This result was lower than oat hull (69%) (11) and wheat bran (95%) (27) but higher than that from maize bran (0.6%) (39) and barley spent grain (30%) (40). The discrepancy in the release of FA might be due to the complexity of the cell wall material (lignification), and also the physical and steric factors caused by branching of the arabinoxylan backbone (11). Faulds, Kroon (39) reported that the highly branched xylose in the side chain of heteroxylan backbone of maize bran may hinder the action of endoxylanases, thus FAE can only act on those easily accessible regions. Therefore, less-substituted xylan substrate such as barley spent grain and wheat bran are better substrates for the release of FA by FAE, as compared to more substituted substrates such as maize bran (40). Furthermore, wheat bran containing nonlignified cell walls is more susceptible to enzymatic

degradation Yu, Maenz (11) as compared to the highly ligninfied corn cob (41) that is less susceptible to the esterase.

4.0 CONCLUSION

RSM is a useful tool in the optimization of the enzymatic hydrolysis of FA from sweet corn cob. The concentration of FAE, XY and temperature markedly affects the extraction efficiency of FA from sweet corn cob and thus, optimization of these parameters is crucial to obtain the maximum yield of FA. Under the optimum condition, the yield of FA (1.69 ± 0.02g kg^{-1}) agreed closely with the predicted yield obtained from the model. Enzymatic hydrolysis offers several advantages including less time required (3 hours as compared to 6 hours in alkali hydrolysis), no need for a chemical solvent and is product specific. However, enzymatic hydrolysis of SCC with the combination of FAE and XY does not release a high amount of FA as compared to alkali hydrolysis. Therefore, combination of novel technologies with enzymatic hydrolysis may be further explored to increase the yield of extraction for FA in SCC.

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6.0 Conflict of Interest

The authors declared that they have no conflict of interest.

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Figure Caption

Figure 1: Effect of pH, temperature and time on enzymatic hydrolysis of ferulic acid (FA) from sweet corn cob using ferulic acid esterase (FAE) and xylanase (XY). Different letters showed significant difference ($p<0.05$) between treatments.

Figure 2: Response surface (3-D) showing the effect of FAE (X_1), XY concentration (X_2), pH (X_3) and temperature (X_4) on yield of ferulic acid (FA).

Figure 3: Contour plots showing the effect of FAE (X_1), XY concentration (X_2), pH (X_3) and temperature (X_4) on yield of ferulic acid(FA).

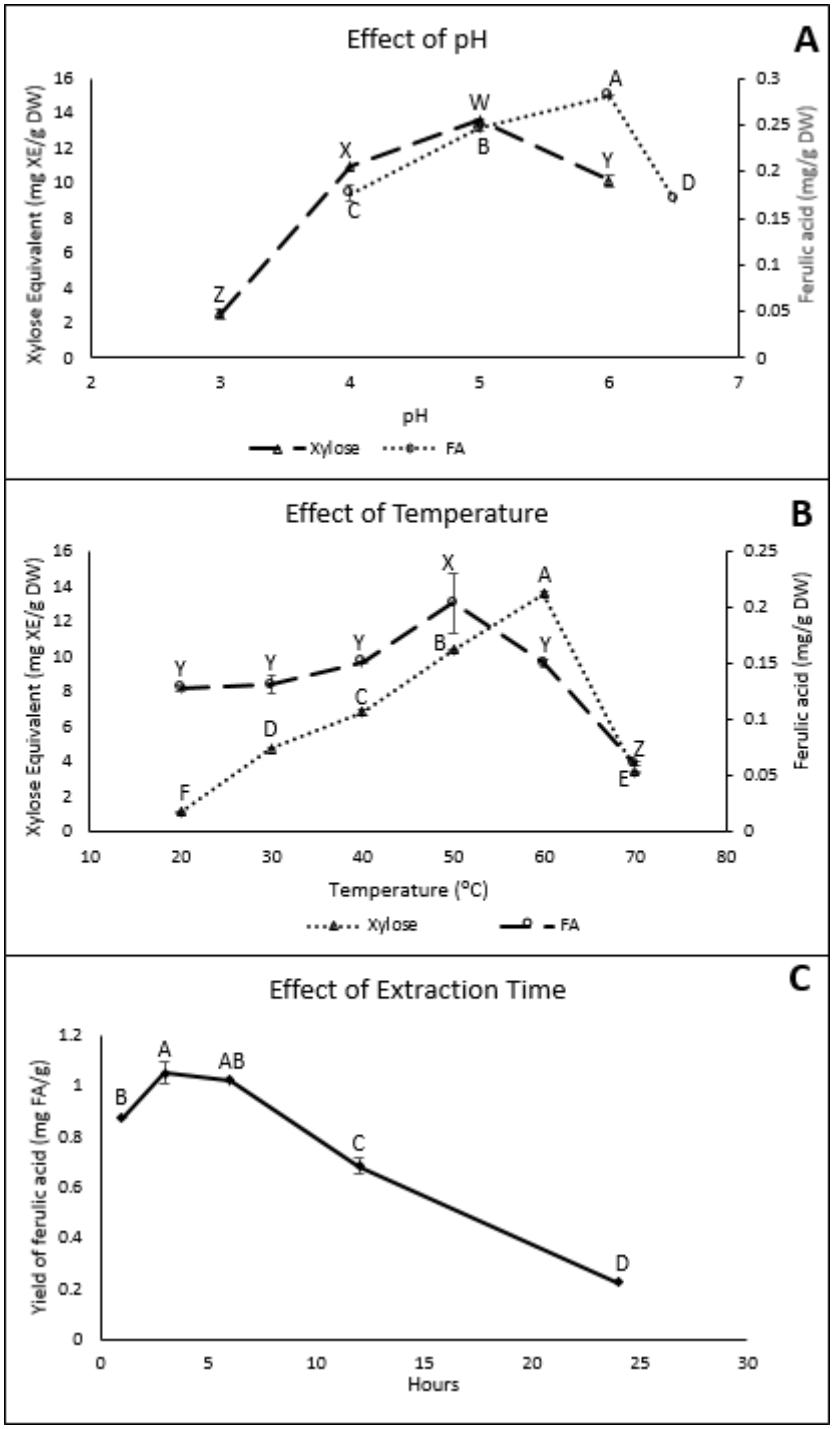

Figure 1

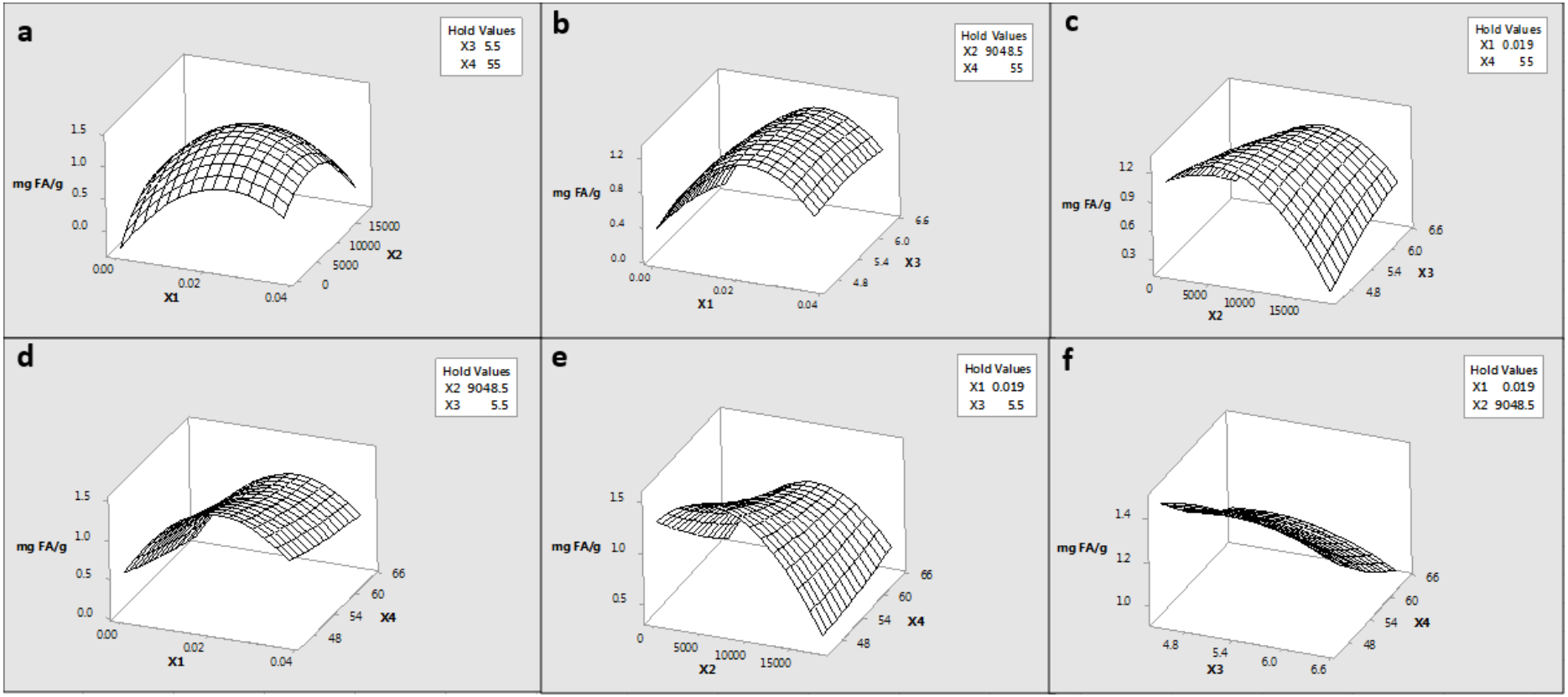

Figure 2

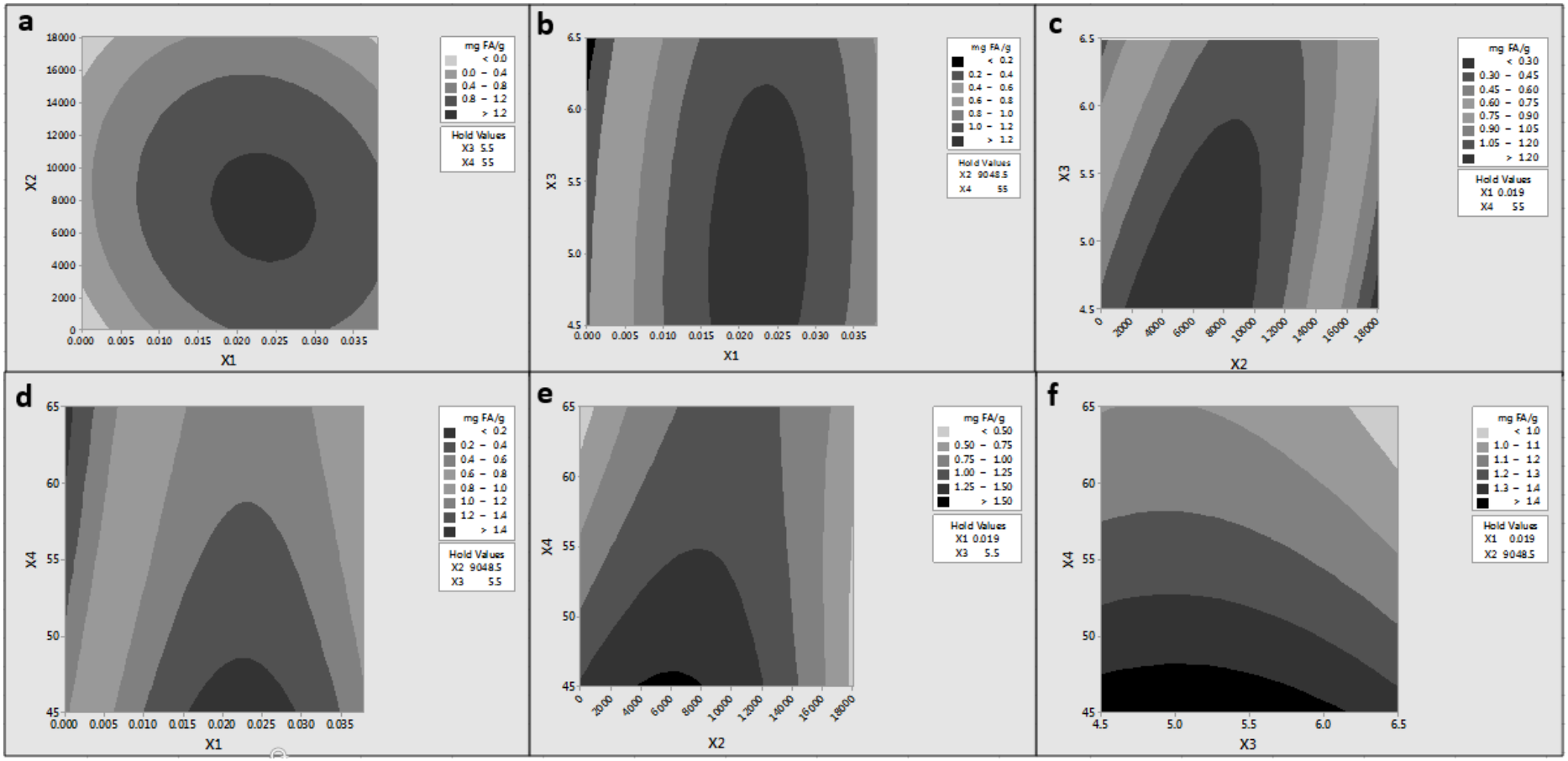

Figure 3

Table 1: Variables and their levels for central composite rotatable design

Variables	Levels				
	-α	**-1**	**0**	**+1**	**+α**
Ferulic acid esterase concentration/X_1 (U/g)	0.00	0.01	0.02	0.03	0.04
Xylanase concentration/ X_2 (U/g)	0.00	4526.00	9048.20	13571.00	18093.50
pH/ X_3	4.5	5.0	5.5	6.0	6.5
Temperature/ X_4 (°C)	45	50	55	60	65

Table 2: Central composite rotatable design and response values for the yield of ferulic acid (g kg^{-1})

Standard Order	**Concentration of Ferulic acid esterase/X_1 (U/g)**	**Concentration of Xylanase/X_2 (U/g)**	**Extraction pH/X_3**	**Extraction Temperature/X_4 (^oC)**	**Ferulic acid yield (g kg^{-1})**	
					Experimental[a]	**Predicted**
1	0.01	4526.00	5.00	50.00	1.20	1.14
2	0.03	4526.00	5.00	50.00	1.45	1.42
3	0.01	13571.00	5.00	50.00	0.85	0.78
4	0.03	13571.00	5.00	50.00	0.84	0.92
5	0.01	4526.00	6.00	50.00	1.02	0.94
6	0.03	4526.00	6.00	50.00	1.23	1.28
7	0.01	13571.00	6.00	50.00	0.85	0.83
8	0.03	13571.00	6.00	50.00	0.98	1.02

9	0.01	4526.00	5.00	60.00	0.91	0.83
10	0.03	4526.00	5.00	60.00	1.11	1.15
11	0.01	13571.00	5.00	60.00	0.73	0.73
12	0.03	13571.00	5.00	60.00	0.81	0.90
13	0.01	4526.00	6.00	60.00	0.62	0.57
14	0.03	4526.00	6.00	60.00	0.93	0.95
15	0.01	13571.00	6.00	60.00	0.79	0.73
16	0.03	13571.00	6.00	60.00	0.91	0.95
17	0.00	9048.50	5.50	55.00	0.00	0.46
18	0.04	9048.50	5.50	55.00	1.00	0.97
19	0.02	0.00	5.50	55.00	0.61	0.83
20	0.02	18093.50	5.50	55.00	0.52	0.48
21	0.02	9048.50	4.50	55.00	1.17	1.25

22	0.02	9048.50	6.50	55.00	1.03	1.10
23	0.02	9048.50	5.50	45.00	1.37	1.43
24	0.02	9048.50	5.50	65.00	1.03	1.05
25	0.02	9048.50	5.50	55.00	1.28	1.24
26	0.02	9048.50	5.50	55.00	1.19	1.24
27	0.02	9048.50	5.50	55.00	1.20	1.24
28	0.02	9048.50	5.50	55.00	1.27	1.24
29	0.02	9048.50	5.50	55.00	1.14	1.24
30	0.02	9048.50	5.50	55.00	1.28	1.24
31	0.02	9048.50	5.50	55.00	1.30	1.24

[a] Averages of duplicated determination (n=2) from experiments.

Table 3: Estimated regression model of relationship between response variables (yield of ferulic acid) and independent variables ferulic acid esterase (X_1), xylanase (X_2), pH (X_3), *and* temperature (X_4).

Factor	**Sum of Square**	**Mean Square**	**F-ratio**	***p*-value**
X_1	0.45	0.45	25.36	*
X_1^2	0.79	0.79	44.17	*
X_2	0.15	0.15	8.37	**
X_2^2	0.64	0.64	35.83	*
X_3	0.03	0.03	1.57	NS
X_3^2	0.01	0.01	0.39	NS
X_4	0.22	0.22	12.50	**
X_4^2	0.00	0.00	0.12	NS
X_1X_2	0.03	0.03	1.53	NS
X_1X_3	0.00	0.00	0.23	NS
X_1X_4	0.00	0.00	0.06	NS
X_2X_3	0.08	0.08	4.65	***
X_2X_4	0.07	0.07	3.80	NS
X_3X_4	0.00	0.00	0.01	NS

*Significance at $p \leq 0.001$

**Significance at $p \leq 0.01$

***Significance at p≤0.05

NS = not significant

Table 4: Predicted and experimental values of the responses at optimum condition

	FAE Concentration (U/g)	**XY Concentration (U/g)**	**pH**	**Temperature (°C)**	**Yield of ferulic acid (g kg^{-1})**
Predicted	0.02	3475.32	4.50	45.00	1.70
Experimental[a]	0.02	3475.32	4.50	45.00	1.69 ± 0.02

[a] Means ± standard deviation of triplicate determinations (n=3) from experiments.