JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Preparation of Ferulic Acid from Agricultural Wastes: Its Improved Extraction and Purification

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Ferulic acid (FA) is a phenolic antioxidant present in plants, which is widely used in the food and cosmetic industry. In the present study, various agricultural wastes such as maize bran, rice bran, wheat bran, wheat straw, sugar cane baggasse, pineapple peels, orange peels, and pomegranate peels were screened for the presence of esterified FA (EFA). Among the sources screened, maize bran was found to contain the highest amount of EFA. Pineapple peels, orange peels, and pomegranate peels were also found to contain traces of EFA. Alkaline extraction of EFA from maize bran was carried out using 2 M NaOH. Response surface methodology (RSM) was used for optimization of EFA extraction, which resulted in a 1.3-fold increase as compared to the unoptimized conventional extraction technique. FA was analyzed by means of high-performance liquid chromatography (HPLC). Purification was carried out by adsorption chromatography using Amberlite XAD-16 followed by preparative high-performance thin-layer chromatography (HPTLC). The recovery of Amberlite XAD-16 purified FA was up to 57.97% with HPLC purity 50.89%. The fold purity achieved was 1.35. After preparative HPTLC, the maximum HPLC purity obtained was 95.35% along with an increase in fold purity up to 2.53.

KEYWORDS: Ferulic acid (FA); extraction; optimization; response surface methodology (RSM); purification; high-performance thin-layer chromatography (HPTLC)

INTRODUCTION

Ferulic acid (FA) is the most abundant hydroxycinnamic acid found in plant cell walls. It is covalently linked by ester linkages to polysaccharides (*1*) and ether or ester bonds to lignin (*2*) in plant cell walls. In nature, FA is present in various plants such as citrus fruits (*3*), wheat (*4*), spinach (*5*), sugar beet (*6, 7*), cereals (*1*), sugar cane bagasse (*8, 9*), neem (*10*), pineapple (*11, 12*), and so forth. It has many important applications in the field of medicine such as an antioxidant activity (*13*), cholesterol-lowering activity (*14*), prevention against thrombosis and atherosclerosis (*15*), antimicrobial and anti-inflammatory activity (*16*), and anticancer effect (*17*). It is known for beneficial effects such as protective action in coronary diseases and in increasing sperm viability (*18*). Currently, it is widely used in food as a preservative because of its antioxidant and antimicrobial actions, as a cross-linking agent (*19, 20*), and as an antioxidant in cosmetic industries (*21*). The preservative property of FA in food is due to its ability of acting as an inhibitor of fatty acid peroxidation and UV-absorber (*21*), and hence, it is used as a photoprotective constituent in many skin lotions and sunscreens (*22*). The structure of FA is shown in **Figure 1**.

Extraction is a technique for isolation of plant constituents and often considered to be the bottleneck of most analytical procedures. There are two ways for preparation of FA. The first way is chemical synthesis involving the condensation reaction of vanillin with malonic acid catalyzed by piperidine (*23*). Though this method produces ferulic acid a mixture of trans- and cis-isomers with a high yield of FA, it takes as long as three weeks to complete the reaction. The other way is extraction from natural resources where FA is one of the most abundant phenolic acids in plants. The extraction of FA using various methods such as enzymatic (*24*), alkaline (*4, 25−27*), and acidic extraction (*8, 28*) had been performed earlier. Different techniques are employed for extraction, which help in releasing the various constitutive monomers from the cell wall polysaccharides and can be used for further specific application. The extraction method may affect the yield and profile of phenolic acids released because these exist in esterified forms in plant cell walls (*26*). Alkaline treatment essentially involves hydrolytic cleavage of ester linkages between lignin and plant polysaccharides thereby releasing phenolic acids (*29*). In this study, only esterified FA (EFA) was released by alkaline treatment and the etherified FA leftovers as it is in residue. FA extraction from natural matrices is complicated due to their diversity and sensitivity to oxidation and hydrolysis. Selection of extraction methods to be used often depends on protective measures employed for phenolic compounds (*30*) and to some level on the nature of the matrix means fruit, seed, leaf, or stem (*8*).

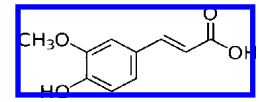

Figure 1. Structure of FA.

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10.1021/jf801536t CCC: $40.75
Published on Web 08/16/2008

The conventional one factor at-a-time optimization method provides information of only one parameter at a time in which other parameters are kept constant. The statistical procedure makes available an alternative methodology to optimize a particular process by considering mutual interactions among the variables and gives an estimate of the combined effect of variables selected for study on the final result. Response surface methodology (RSM) employed in this study is based on the fundamental principles of statistics, randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is also known as full factorial central composite design (CCD). Previously, RSM was successfully employed for the extraction of *Z*-ligustilide, *Z*-butylidenephthalide, and FA in *Angelica sinensis* (*31*).

In the present study, alkaline extraction of EFA from various agricultural wastes such as maize bran, rice bran, wheat bran, wheat straw, sugar cane baggasse, pineapple peels, orange peels, and pomegranate peels was explored. Extraction efficiency of EFA in maize bran was highest in all agricultural wastes from primary studies and was further improved by using a statistical approach called RSM. The purification of extract obtained after RSM optimized protocol was performed using three polymeric adsorbents viz. polyvinyl polypyrrolidone (PVPP), Amberlite XAD-4, and Amberlite XAD-16. An attempt had been made to improve the purity of FA obtained after adsorption chromatography by high-performance thin-layer chromatography (HPTLC).

MATERIALS AND METHODS

Materials and Chemicals. Maize bran, sugar cane baggasse (SCB), rice bran, wheat bran, wheat straw, pineapple peels, orange peels, and pomegranate peels were obtained from a local market. Standard FA was procured from M/S Himedia Ltd., Mumbai, India. Ethyl acetate, sodium hydroxide, sodium dihydrogen phosphate, hydrochloric acid, sodium hydrogen sulfite, acetonitrile HPLC grade, acetic acid HPLC grade, Amberlite XAD-16, Amberlite XAD-4, PVPP, and ethanol were purchased from M/S, s. d. Fine-Chem. Ltd., Mumbai, India. Silica gel 60 F_{254} plates were obtained from Merck (Darmstadt, Germany). Pure water filtered through 0.2 μ was prepared using a Sartorious arium 611UV system.

Alkali Extraction of EFA. All the samples were dried in a universal oven with forced convection at 40 °C for 12 h prior to extraction. The dried samples were ground in a mill, and the powder of each sample was collected for the study. In this study, a simple extraction method was employed as compared to previously used lengthy, cumbersome, and uneconomical methods including enzymatic and alkaline extraction (*32, 33*).

Accurately weighed 2 g of sample was taken into an Erlenmeyer flask (250 mL) and saponified with NaOH (2 M, 60 mL) for 24 h at room temperature on a rotary shaker at 180 rpm. To prevent degradation and/or oxidation of FA, 0.001 g of sodium hydrogen sulfite was added to the solution. The alkaline hydrolysate was centrifuged, the supernatant was acidified (pH < 2) with dilute hydrochloric acid (2 M), and the released phenolic acids were extracted using ethyl acetate (60 mL, thrice). All organic fractions were collected and concentrated (up to 2−3 mL) by rotary vacuum evaporator (Buchi Rotavapor R-124, Switzerland). The concentrated extract was dissolved in a 2 mL acetonitrile/water (1:1) mixture prior to the quantitative and qualitative analysis of FA.

Identification Method for FA. The qualitative analysis of FA extracts along with standard FA were performed on precoated 10 cm × 20 cm (0.25 mm thick) silica gel 60 F_{254} plates. The samples prepared in acetonitrile/water (1:1) were applied on HPTLC sheets by a dosimeter. The mobile phase used for this was benzene/dioxane/acetic acid in the proportion of 85:15:1 (*34*). Plates were developed at room temperature in a vertical separating chamber to the height of approximately 12 cm from the start. The chamber was previously saturated with the appropriate mobile phase (saturation time was 1 h). After drying, the plates were visualized in UV light (254 nm) and compared with standard FA.

Quantitative Analysis of FA. The quantitative analysis of all selected sources was performed on a Jasko high-performance liquid chromatograhpy (HPLC) system, fitted with a reverse phase C_{18} column Waters Spherisorb 5 μm ODS_2 (4.6 mm × 250 mm), guard column (50 mm × 4.6 mm ID), Jasko PU-1580 Intelligent HPLC pump, and UV detector (Jasko UV-1575). The elution of FA was carried out isocratically at a 1.0 mL/min flow rate using a mobile phase composition of acetonitrile/water (20:80) and 1% acetic acid. The injection volume was 20 μL, and analysis of FA was monitored at 320 nm. The concentrated samples were diluted suitably with the mobile phase. The standard curve of FA was prepared in the range 25−250 μg/mL.

Optimization of FA Extraction by Statistical Method. The design variables selected for statistical optimization study were NaOH molarity, extraction temperature, and extraction time. The effect of these variables on extraction was evaluated for total FA content as a response. Optimal conditions for the extraction of phenolic compounds from maize bran dependent on evaluated variables were obtained using the predictive equations of RSM.

Purification of FA. Purification of FA was studied by using three matrices viz. polyvinyl polypyrrolidone (PVPP), Amberlite XAD-4, and Amberlite XAD-16. These three matrices were screened to find the static and dynamic binding capacity of these matrices for FA. Further studies for purification of FA were carried out with Amberlite XAD-16.

Elution Chromatogram of FA. Primarily, Amberlite XAD-16 resin was washed extensively with methanol/water (1:1) three times and packed in a 1 cm diameter column. Then, the column was equilibrated with dilute hydrochloric acid solution of pH 2.0. The FA extract was passed with a volumetric flow rate of 1.0 mL/min through the equilibrated column until the exhaustion point was reached. The saturated column was washed with dilute hydrochloric acid solution to remove unabsorbed FA. Elution of adsorbed FA was carried out using an ethanolic solution of ammonium hydroxide (0.1%) with a volumetric flow rate of 1.0 mL/min (*35*). The eluted fractions were analyzed for FA content. The percent recovery of FA was calculated by the following equation:

$$\% \text{ recovery} = \frac{\text{total ferulic acid eluted}}{\text{total ferulic acid loaded}} \times 100 \qquad (1)$$

Purification by Preparative HPTLC. The fractions showing maximum FA content after elution from Amberlite XAD-16 were pooled. A preparative HPTLC was developed on precoated 10 cm × 20 cm (0.25 mm thick) silica gel 60 F_{254} plates. Pooled sample was applied on HPTLC sheets by a dosimeter. The plate was developed at room temperature in a vertical separating chamber to the height of approximately 12 cm from the start. The chamber was previously saturated with the appropriate mobile phase benzene/dioxane/acetic acid in the proportion of 85:15:1. After drying, visualization was performed in UV light (254 nm). The sample was compared against the standard FA. The plate showing a FA band was then scraped into a test tube and dissolved in 2 mL of acetonitrile. The solution was filtered through a 0.2 μ filter and analyzed by HPLC for final purity. Regeneration was accomplished with a solvent such as acetone or ethanol. Adsorbent was always stored in methanol, to prevent bacterial growth.

RESULTS AND DISCUSSION

Screening of Various Sources. In the present study, the EFA released from different agricultural waste during saponification by 2 M NaOH was analyzed. In spite of relatively low delignification yield by NaOH, it was selected in this study because of its selectivity property in the release of phenolic compounds such as *p*-coumaric acid and ferulic acid (*36*). The alkaline hydrolysis of maize bran produced 1.487% w/w EFA resulting from separation of the lignin−polysaccharide conjugate by breaking of the α-ester bond present between them. It was reported earlier that the content of total FA was up to 3.1% in maize bran (*25*). A small amount of EFA was also detected in SCB (0.893% w/w), rice bran (0.972% w/w), rice husk (1.022% w/w), wheat bran (0.346% w/w), and wheat straw (0.867% w/w) extracts. Among the various other sources screened such as pomegranate peels, pineapple peels, and orange peels were found

Table 1. Experimental Ranges and Levels of the Independent Test Variables

		coded levels				
independent variable	symbol	−1.68	−1	0	+1	+1.68
NaOH (M)	*A*	0.64	2.00	4.00	6.00	7.36
temp (°C)	*B*	21.6	25.0	30.0	35.0	38.0
time (h)	*C*	10.56	16.00	24.00	32.00	37.44

to contain traces of EFA about 0.192, 0.018, 0.021% w/w, respectively. To the best of our knowledge, this has not been reported in the literature. The yields obtained using the present method was slightly higher for samples of wheat and rice bran. The reported phenolic acids content in different wheat bran fractions was up to 0.2% w/w FA (*4*) and in rice bran was up to 0.9% w/w (*37*). Some observed up to 1.1% w/w of FA in wheat straw, but in the case of our results, it was to some extent low (*2*).

As maize bran was found to be a maximum FA containing source, further investigations were made with it. Each extract of all the screened sources containing FA are qualitatively confirmed by comparing with standard FA by the TLC method (results not shown).

Optimization by Statistical Method. RSM was used for optimization of FA extraction. In the alkaline extraction, the alkali concentration and hydrolysis duration are recognized as being important factors in the release of phenolic compounds (*38*), since mild treatment generally leads to partial solubilization and severe conditions may produce degraded product (*39*). As well as, increase in the yield of EFA by alkaline extraction was observed earlier in dewaxed SCB with elevated temperature (*8*); hence, three factors selected for this part of the study were temperature, NaOH concentration, and extraction time. The ranges and the levels of the variables investigated in this study are given in **Table 1**. Coding of the variables was done according to the following equation:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i} \quad \text{and} \quad i = 1, 2, 3, \ldots, k \tag{2}$$

where x_i is a dimensionless value of an independent variable, X_i is a real value of an independent variable, X_{cp} is a real value of an independent variable at the center point, and ΔX_i is the step change of a real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i. Each factor in the design was studied at five different levels (−1.68, −1, 0, 1, 1.68). All variables were taken at a central coded value considered as zero. In general, RSM is constructed in such a way that $2^f + 2f + 1$ experiments are required where f represents the number of factors to be studied. Therefore, a three-factor RSM requires 15 experimental points, each of which being a result of different experimental conditions. Five additional experiments were carried out at the center point to estimate the overall error; the total number of experiments thus amounted to 20. The experimental conditions for the RSM and concentration of FA are presented in **Table 2**. The experiments were performed in random order to avoid systematic error. By applying multiple regression analysis on the experimental data, the results of the RSM were fitted with a second-order polynomial equation. Thus, a mathematical regression model for total FA fitted in the coded factors was given as the following:

Table 2. Response Surface Methodology Matrix of Three Test Variables in Coded and Natural Units along with the Observed Responses

				FA (% w/w)	
run	NaOH	temp	time	actual	predicted
1	−1	−1	−1	1.01	1.01
2	1	−1	−1	0.53	0.68
3	−1	1	−1	0.70	0.77
4	1	1	−1	0.55	0.53
5	−1	−1	1	0.99	1.06
6	1	−1	1	1.36	1.34
7	−1	1	1	0.18	0.09
8	1	1	1	0.42	0.47
9	−1.68	0	0	0.49	0.49
10	1.68	0	0	0.59	0.52
11	0	−1.68	0	1.91	1.82
12	0	1.68	0	0.87	0.89
13	0	0	−1.68	0.49	0.39
14	0	0	1.68	0.35	0.38
15−20	0	0	0	0.85	0.82

Table 3. Regression Results from the Data of RSM Experiments

factor	*F* value	*P* value
model	44.78	<0.0001
A	0.24	0.6321
B	160.16	<0.0001
C	0.024	0.8812
A^2	26.57	0.0004
B^2	79.17	<0.0001
C^2	51.19	<0.0001
AB	0.68	0.4273
AC	28.69	0.0003
BC	40.09	<0.0001

$$\text{ferulic acid} = 0.82 + 0.011A - 0.28B - 3.368E - 0.03C - 0.11A^2 + 0.19B^2 - 0.15C^2 + 0.024AB + 0.15AC - 0.18B \tag{3}$$

where A, B, and C were the coded values of the test variables NaOH, temperature, and time respectively. The significance of each coefficient was determined by Student's *t*-test and *P* values, which were listed in **Table 3**.

The model *F* value of 44.78 implies that the model is significant. There is only a 0.01% chance that a model *F* value this large could occur due to noise. A *P* value less than 0.05 indicates that model terms are significant. In this case, B, A^2, B^2, C^2, AC, and BC are significant model terms. Values greater than 0.1000 indicate that the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. Since the quadratic response surface is calculated in $(f + 1)$ dimensions, where f is the number of factors in the RSM, the quadratic response surface for the three factors involved generates a four-dimensional response surface, which can be readily visualized in a three-dimensional (3D) response surface. The response model was mapped against two experimental factors while the third was held constant at its optimum. This 3D response function is depicted in **Figure 2**. From the central point of the contour plot or from the bump of the 3D plot, the optimal composition of medium components was identified. The optimal values of the three factors were NaOH 4M, temperature 21.6 °C, and time 24 h. After optimization by RSM, extraction efficiency was increased from 1.486 to 1.91% w/w (1.3-fold) as compared to unoptimized method of extraction.

Purification of FA. After optimization of the method for extraction of FA, the studies were carried out to increase the purity of the ferulic acid from plant extract. The different resins

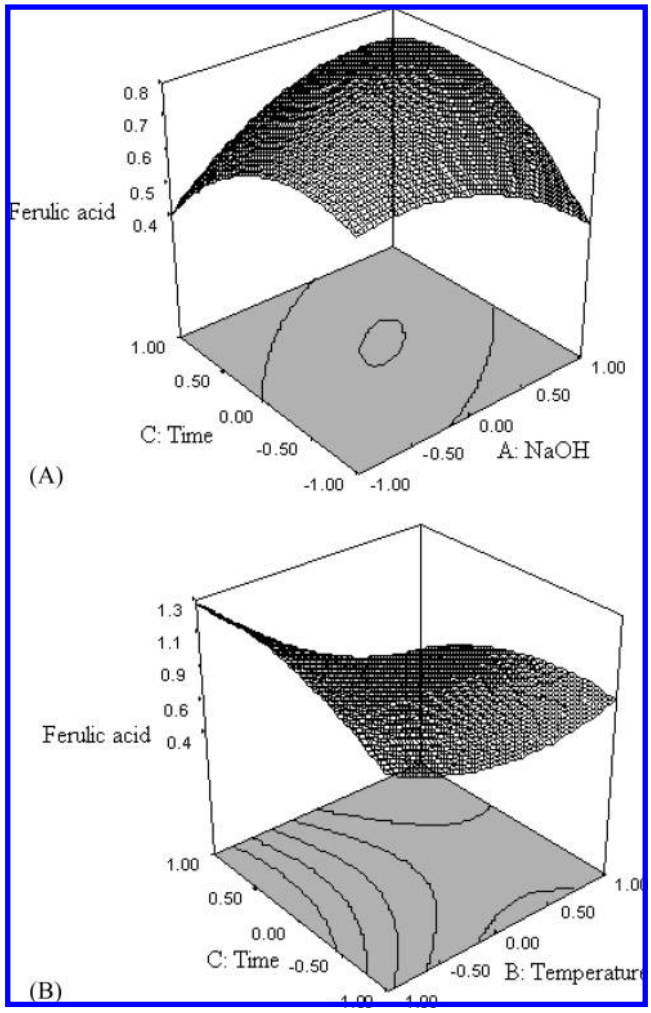

Figure 2. Response surface for concentration response function of FA: (**A**) extraction time (min, *C*) vs NaOH molarity (M, *A*) with temperature (°C, *B*) held at its optimum; (**B**) extraction time (min, *C*) vs temperature (°C, *B*) with NaOH molarity (M, *A*) held at its optimum.

(PVPP, Amberlite XAD-4, and Amberlite XAD-16) reported to be used in purification of phenolic compounds were checked for their capacity to purify FA with minimal impurities and maximum recovery. The preliminary static experiments carried out with these resins showed good adsorption efficiency and specificity for FA. Adsorption on PVPP involves hydrogen binding with phenolic and carboxyl groups on FA (*40*). Similarly, adsorption on Amberlite or styrene-divinylbenzene copolymers involves hydrophobic interaction with the aromatic ring of FA (*41*). All the chosen adsorbents were screened for maximum static binding capacity. Amberlite XAD-16 showed maximum static binding capacity for FA (data not shown); hence, it was selected for further purification study. It has been observed that Amberlite XAD-16 is the superior resin among different polystyrene matrixes used for purification of ferulic acid (*35*). The selectivity of FA was found more toward Amberlite XAD-16.

Elution Chromatogram of FA. The elution profile of FA from Amberlite XAD-16 is shown in **Figure 3**. Most of the FA was released from the fraction numbers 2−11. The combined fractions of FA (4−9) give 50.89% HPLC purity along with 57.97% recovery, and the fold purity was increased to 1.35. With ferulic acid, there are other phenolic acids present in the

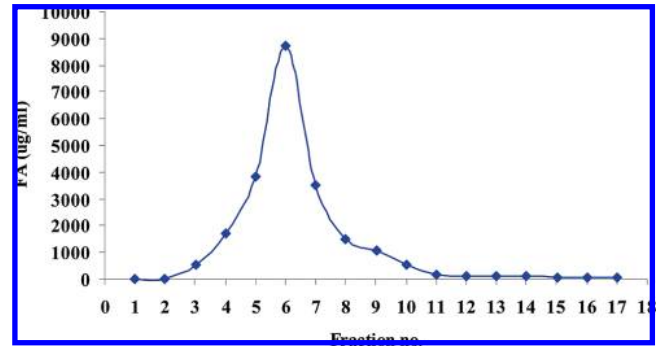

Figure 3. Elution chromatogram of FA.

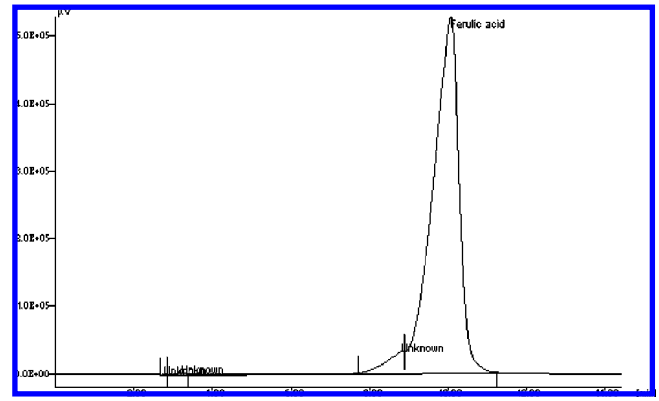

Figure 4. Chromatogram of HPTLC purified FA.

aliquots collected after column chromatography, which decreases the overall percent HPLC purity of FA. To further purify FA, preparative HPTLC was carried out.

Purification by Preparative HPTLC. In the FA collected after column chromatography when purified by preparative HPTLC, the percent HPLC purity of the FA increased up to 95.35%, and the fold purity increased up to 2.53. The increase in fold purity was due to improved resolution and separation by the thin-layer chromatographic method. The thin-layer chromatography was previously used for separation of FA from alkali-soluble phenolics produced using grass cell materials (*42*). **Figure 4** shows the HPLC chromatogram of preparative HPTLC purified FA.

Conclusion. Among the sources screened, maize bran was found to contain the highest amount of FA. By using a univariate approach coupled with RSM, extraction parameters, such as NaOH molarity, temperature, and time, for extraction of FA in maize bran were optimized. The maximum FA extraction was observed with optimized conditions such as NaOH 4M, temperature 21.6 °C, and time 24 h. Interaction between time and NaOH was most significant. Optimization by RSM gave 1.3-fold increase in FA extraction. Amberlite XAD-16 was found to be the best matrix for purification of FA with improved fold purity. Further purification of FA using preparative HPTLC could be the best choice for improving the fold purity. Thus, from the current study, it can be concluded that agricultural wastes such as maize bran can be a promising source of FA. Increasing demands for antioxidants such as FA require efficient extraction and purification techniques. It was observed that other sources such as pineapple peels, orange peels, and pomegranate peels contained traces of FA, which could be further investigated for better recovery.

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Received for review March 8, 2008. Revised manuscript received July 2, 2008. Accepted July 8, 2008. Authors are grateful to the Department of Biotechnology, Ministry of Science and Technology, India, for providing financial assistance during the course of this research.

JF801536T