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Biotransformation of Agricultural By-Products into Biovanillin through Solid-State Fermentation (SSF) and Optimization of Different Parameters Using Response Surface Methodology (RSM)

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Abstract: Vanillin is a flavorful and aromatic secondary metabolite found in vanilla plants. Natural vanillin, produced through processed vanilla beans accounts for scarcely 0.2% of industrial requirements. Vanillin produced via chemical methods and microbial fermentation fills the remaining gap. Among naturally available precursors for biovanillin synthesis, ferulic acid is widely used because of its structural similarity and abundant availability. Herein, various agricultural lignocellulosic by-products (sugarcane bagasse, wheat straw, rice straw, rice bran, and corn cob) were scrutinized for their ferulic acid content, and their biotransformation into biovanillin was examined by solid-state fermentation (SSF). Then, different physicochemical parameters, i.e., moisture content, pH, temperature, inoculum size, and incubation days, were optimized to achieve a high yield of biovanillin using central composite design (CCD) of response surface methodology (RSM). Among agricultural by-products tested, sugarcane bagasse produced 0.029 g/100 g of biovanillin using *Enterobacter hormaechei* through SSF. After optimization, the highest concentration of biovanillin (0.476 g/100 g) was achieved at a moisture content of 70%, temperature of 37.5 °C, pH 7.5, inoculum size of 4 mL and incubation time of 48 h. The F-value of 6.10 and *p-value* of 0.002 evidenced the ultimate significance of the model. The significance of the constructed model was supported by the 91.73% coefficient of determination (R^2), indicating that the effects of moisture, pH, and temperature were significant. HPLC and FTIR confirmed the sample identification and purity (was reported to be 98.3% pure). In conclusion, sugarcane bagasse appears to be a cost-effective substrate choice for large-scale biovanillin production.

Keywords: sugarcane bagasse; ferulic acid; biovanillin; *Enterobacter hormaechei*; solid-state fermentation; response surface methodology

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1. Introduction

Flavors have a significant influence on the taste and quality of food and beverages. Vanillin is one of the most commonly used flavoring agents in the food industry [1]. Vanillin ($C_8H_8O_3$) is a natural organoleptic substance of vanilla, which is obtained from the pods of the orchid *Vanilla planifolia*. It is a phenolic molecule classified as an aromatic aldehyde (3-methoxy-4-hydroxybenzaldehyde). Physiochemically, it is a white solid soluble in water with a molecular weight of 152.15 g/mol, a melting point of 80 °C to 83 °C, and a boiling point of 285 °C. It is widely used in the food, cosmetic, pharmaceutical, and medical industries as an essential aromatic molecule [2,3]

Vanilla is a natural blend comprising over 200 compounds from the cured pods of *Vanilla planifolia*, *Vanilla tahitiensis*, and *Vanilla pompona* [4,5]. Although about 110 species have been identified, only three species are important for trade and cultivation but *V. planifolia* is recognized for its flavor qualities, from where it is mainly planted and it is mainly collected from vanilla tropical orchids, which are usually harvested more than the other two varieties. There are three ways to obtain vanillin: the extraction of natural vanilla bean pods (the ripe vanilla fruit also called bean pods develop their odor characteristics in the process), the synthesis from chemical precursors, or biotechnological production by microbial fermentation. The *Food and Drug Administration* (FDA) designates vanilla extract as a solution containing at least 30% ethanol of a solution containing more than 25% water in one gallon of the final product [6].

In the global vanillin market, less than 0.5% of every 10,000 tons of vanillin is obtained from vanilla seeds [7]. The reason is that the production of vanilla pods to comply with global requirement is much lesser and limited in terms of soil and climate. Cultivation, pollination, harvesting, and drying require intensive work leading to changes in the price of natural vanillin; therefore, obtaining vanilla from natural plants is inadequate for the increasing market demand. Synthetic vanillin precursors are mainly lignin or fossil hydrocarbons, such as guaiacol, and the chemical process mainly involves the oxidation of sulphur in the wood lost during mashing. Although the price of synthetic vanilla is meager, consumer awareness of natural health products is increasing, and many companies are striving to explore new ways to produce natural flavors, including biovanillin. It can be produced via biotechnological methods, using lignin, ferulic acid, eugenol, or isoeugenol as natural precursors and microorganisms as hosts [8].

There is a prodigious interest in the use of natural foods and additives preferentially resulting from microorganisms instead of chemically synthesized counterparts [9]. Various bacterial species can metabolize ferulic acid as the sole carbon source to produce vanillin, vanillic acid, and protocatechuic acid. *Amycolatopsis*, *Streptomyces*, *Pseudomonas*, and *Delftia* are the most common microorganisms that can catalyze the conversion of ferulic acid to vanillin [10]. Ferulic acid is a phenyl propanoic acid that occurs naturally in plants and provides polysaccharides and lignin by measuring the stiffness of the cell walls. Vanillin is a temporary intermediate of ferulic acid catabolism in many bacteria and is rapidly converted to other compounds or used as a carbon and energy source. Agricultural wastes, such as all lignocellulosic, can be transformed into commercially viable products, including ethanol, glucose, and single-cell proteins. Ferulic acid is found in large quantities in the cell walls of many valuable agricultural crops, including wheat, maize, and sugar beet. Therefore, it can be used to produce value-added products such as vanillin. Solid-state fermentation (SSF) has attracted the attention of researchers in recent years that offer a higher yield and production efficiency and better product properties than submerged fermentation (SmF). In addition, capital and operating costs are lower due to cheap agricultural and agro-industrial waste as substrates [11].

Pakistan is an agricultural country where enormous amounts of crops are grown, resulting in considerable agricultural waste. In many parts of Pakistan, there are no facilities for waste disposal, leaving inefficient burning as the only option, resulting in increasing gaseous emissions and environmental pollution. The highest sugarcane is contributed by Punjab, accounting for about 650,000 ha. The yearly production of bagasse is around

12 million tons per year, with the majority being burned inadequately [12]. In this study, different ferulic acid-rich agricultural by-products (sugarcane bagasse, wheat straw, rice straw, rice bran, and corn cob) were utilized for biovanillin production using SSF. To the best of our knowledge, this is the first report on biovanillin production using agriculture by-products as a lignocellulosic substrate by SSF.

2. Materials and Methods

2.1. Raw Materials and Chemicals

The experimental work was carried out at the University of Veterinary and Animal Sciences, Institute of Biochemistry and Biotechnology in Lahore, Pakistan. Standard vanillin, ferulic acid, and 2-thiobarbituric acid were purchased from Sigma-Aldrich. All other chemicals were obtained from the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

2.2. Collection of Substrates

Agricultural waste sugarcane bagasse (*Saccharum officinarum* L.), rice straw (*Oryza sativa* L.), wheat straw (*Triticum aestivum* L.), rice bran (*Oryza sativa* L.), and corn cob (*Zea mays*) were collected from the local market of Lahore. The waste materials were rinsed, cut into smaller pieces, and oven-dried at 60 °C for 48 h. After that, the dried matter was ground into powder and sieved to attain a particle size of 1 mm. These solid wastes acted as a substrate for vanillin production via SSF.

2.3. Culture Maintenance and Inoculum Preparation

The culture of *Enterobacter hormaechei* (KT385666) was obtained from Government College University, Lahore. It was refreshed on sterilized nutrient agar plates and incubated at 30 °C for 24 h. A Loop full of culture was transferred to the sterilized nutrient broth and placed in an orbital shaker at 130 rpm, 30 °C for 24 h. It was maintained until the OD reached 0.6 at 600 nm, and used for fermentation experiments [13].

2.4. Extraction and Quantification of Ferulic Acid from Agricultural By-Products

Different agriculture wastes were dried at 60 °C in an oven for 48 h before the alkaline extraction of esterified ferulic acid (FA). The dried material was crushed into a powder and sieved through a mesh to achieve a particle size of 1 mm. A 5 g powder was accurately weighed and combined with 100 mL of a solution containing 2 M NaOH, 30% ethanol, and 15% ammonia in a 250 mL Erlenmeyer flask connected to a condenser. It was heated to 60 °C for 12 h before cooling to room temperature. To precipitate the hemicellulose, the pH was reduced to 2 using 2 M diluted HCl for solid–solid extraction. After filtering the solutions, 150 mL of ethyl acetate was added to the filtrates to extract the ferulic acid-rich fraction, and vortexed at 100 rpm for 15 min at room temperature to obtain a liquid–liquid extraction. The rotary vacuum evaporator evaporated the ethyl acetate, and the obtained ferulic acid fractions were concentrated [14,15].

Before the quantitative measurement of ferulic acid, the concentrated extract was diluted in a 2 mL combination of acetonitrile and water in the ratio of 1:1 [16]. The maximum absorbance of ferulic acid in the solution was observed at 310 nm. A standard curve of ferulic acid with a known concentration was used to determine the concentration of ferulic acid in the sugarcane bagasse samples [17].

2.5. Selection of Best Substrate for Biovanillin Production by SSF

The basal media contained 20 g glucose, 8 g yeast extract, 0.2 g K_2HPO_4, 0.5 g $MgSO_4$, and 1.3 mg $CaCl_2$ per liter. In a 250 mL Erlenmeyer flask, different substrates (sugarcane bagasse, wheat straw, rice straw, rice bran, and corn cob) were introduced. The substrate water ratio was established at 1:3 using the sterilized basal medium. Precisely 2 mL of inoculum was added [18]. For the biotransformation of ferulic acid into biovanillin, it was cultured in an incubator for 48 h at 30 °C and pH 7.0.

2.6. Optimization of Biovanillin for Maximum Production

Following the selection of the best substrate for biovanillin production, response surface methodology (Minitab version 17) was used to optimize cultural conditions for the maximal biovanillin production. Various optimization parameters for biovanillin were temperature 25–50 °C, moisture content 40–80%, inoculum size 1–5 mL, pH 5–10, moisture content 40–80%, and incubation time (8–72 h). Using response surface methods, a total of 32 experiments with various combinations of factors were conducted to study their individual and combined impacts on biovanillin synthesis.

2.7. Extraction of Biovanillin and Quantitative Estimation

To extract biovanillin, distilled water (25 mL) was added to the fermenting media and centrifuged for 20 min at 6000 rpm. The culture supernatant (50 µL) was obtained as a sample and added to 950 µL of 2-thiobarbituric acid solution (prepared by adding 500 µL of 24% HCl, 200 µL of 1% 2-thiobarbituric acid, and 250 µL of distilled water). Standard vanillin solutions (1 mL) of known concentrations (100–1000 µL/50 L) were cooled for 20 min at room temperature. A yellowish orange color developed in test tubes, and the optical density was measured at 434 nm. The OD values of standard vanillin solutions were determined, and the concentration of biovanillin in the sample was estimated [19].

2.8. Crystallization and Characterization of Biovanilin

Fermented media was centrifuged at 6000 rpm for 20 min to remove biomass. 6 N HCl was used to acidify the supernatant to pH 2. The extraction of biovanillin was carried out by adding three times the volume of ethyl acetate. A rotary vacuum evaporator evaporated the solvent, and the concentrated extract was recovered in water, which was further refrigerated at 4 °C to precipitate vanillin [1].

Biovanillin was identified by an FTIR spectrophotometer (Shimadzu/Prestige-21). HPLC (PerkinElmer ATLUS UPLC system with PDA detection) was performed for quantitative estimation. Pure vanillin procured from Sigma-Aldrich was used as a standard in both methods. As a stationary phase, the C18 5 µm (4.6 × 150 mm) column was used, while water: methanol (40:60) was used as mobile phase. The vanillin standard and sample (50 ppm) were injected at 10 µL volume, 1 mL/ min flow rate, 35 °C oven temperature, and detected at 280 nm [20].

2.9. Statistical Design

The parameters for the production of biovanillin were optimized by using central composite design (CCD) of response surface methodology (RSM). The 32 trials were generated containing five factors through Minitab 17 software to examine their individual and combined effects on biovanillin yield.

3. Results

3.1. Recovery of Ferulic Acid by Different Agricultural By-Products

From 100 g of sugarcane bagasse, rice straw, wheat straw, corn cob, and rice bran powder, 1.36 ± 0.65 g, 0.33 ± 1.2 g, 0.41 ± 1.4 g, 0.155 ± 0.80 g, and 0.00615 ± 1.22 g ferulic acid was recovered by liquid–liquid and solid–liquid extraction, respectively. Table 1 shows the ferulic acid content of various substrates. The literature reports 0.94 g/100 g ferulic acid in sugarcane bagasse [21], 0.4 8 g/100 g in wheat straw [22], 0.39 g/100 g in rice straw [23], 0.169 g/100 g in corn cob [24], and 0.0046 g/100 g ferulic acid in rice bran [25].

Table 1. Ferulic acid content of different agricultural by-products.

Sr. No.	Substrate	Ferulic Acid (g/100 g)
1	Sugarcane bagasse	0.94
2	Wheat straw	0.48
3	Rice straw	0.39
4	Corn cob	0.169
5	Rice bran	0.0046

3.2. Selection of The Best Biovanillin-Producing Substrate

For biovanilin production, different agricultural by-products, i.e., sugarcane bagasse (*Saccharum officinarum* L.), wheat straw (*Triticum aestivum* L.), rice straw (*Oryza sativa* L.), corn cob (*Zea mays*), and rice bran (*Oryza sativa* L.) in ratios 1:3 were fermented in fermentation medium as a source of ferulic acid using culture of *Enterobacter hormaechei* for 24 h. Each sample was tested independently for biovanillin production as fermentation was completed. Biovanillin was recorded in the highest concentration in sugarcane bagasse (0.029 g/100 g), as shown in Figure 1. The second highest production was obtained from wheat straw (0.0098 g/100 g), following rice straw (0.0092 g/100 g). Similarly, the solid-state bioprocessing of corn cob and rice bran provided 0.008 g/100 g and (0.0004 g/100 g of biovanillin, respectively. As sugarcane bagasse contains the highest ferulic acid (0.94 g/100 g), maximum biovanillin was produced through SSF. The ferulic acid content of other substrates was as follows: wheat straw (0.48 g/100 g), rice straw (0.39 g/100 g), corn cob (0.169 g/100 g), and rice bran (0.0046 g/100 g). Among all substrates, sugarcane bagasse was shortlisted and optimized for further analysis.

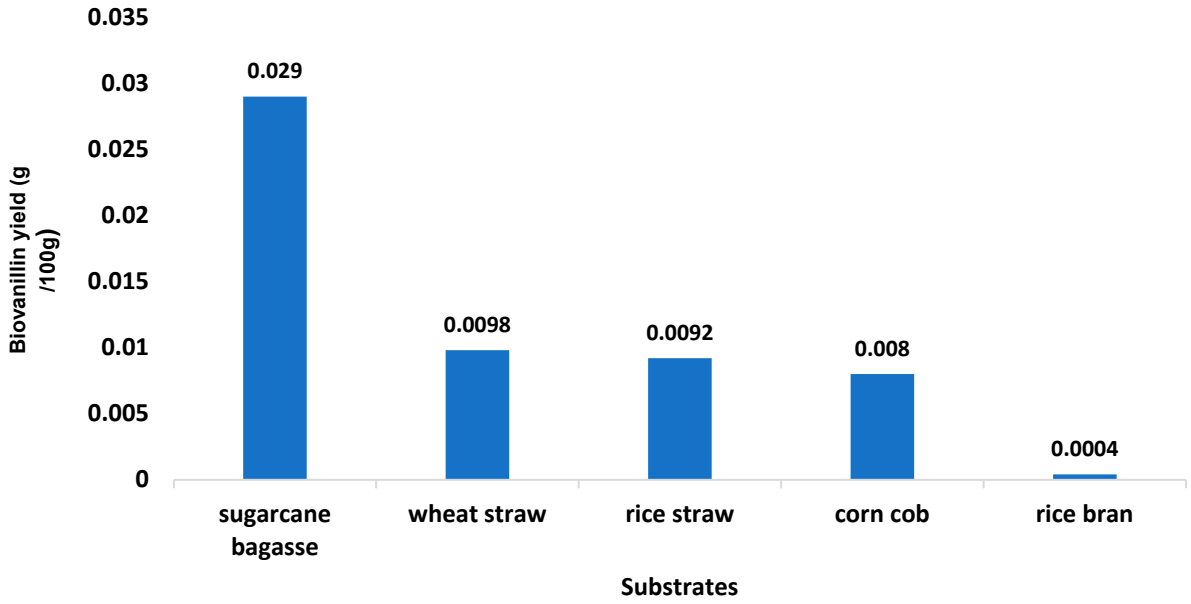


Figure 1. Screening of different substrates for biovanillin production.

3.3. Optimization of Process Parameters for Biovanillin Production through RSM

Sugarcane bagasse (*Saccharum officinarum* L.) was chosen as the best substrate for biovanillin production after screening different substrates. Using CCD (RSM) with sugarcane bagasse as a substrate, the biotransformation of ferulic acid into biovanillin was further enhanced. Table 2 shows the ranges of independent variables optimized for biovanillin production. Various fermentation parameters were considered independent, including inoculum size, incubation time, pH, temperature, and moisture content, whereas biovanillin production (g/100 g) was considered dependent. Inoculum size (1–5 mL), incubation time (8–72 h), pH (5–10), temperature (25–50 °C), and moisture content (40–80%) were the

fermentation parameters to be optimized through SSF. Thirty-two trials were generated with five different factors through Minitab software 17. The following conditions furnished the highest biovanillin production, i.e., moisture content (70%), incubation time (48 h), pH (7.5), temperature (37.5 °C), and inoculum volume (4 mL). The amount of biovanillin obtained at these conditions was (0.476 g/100 g), while moisture content (40%), incubation time (24 h), pH (10), temperature (50 °C), and inoculum volume (1 mL) gave the lowest yield, i.e., (0.249 g/100 g). Table 3 summarizes the observed and predicted values of response variables for biovanillin production. There was a remarkable agreement between the experimental and expected values. The significance of the proposed model is confirmed by the output of statistical findings (Table 4), with an F-value of 6.10 and a *p*-value of 0.002. The developed model's accuracy was validated by the coefficient of determination of 91.73%. The recommended model had an F-value of 6.10 and a *p*-value of 0.002 in the ANOVA table, indicating its significance. The R^2 (coefficient of determination) shows that the model is good and precise, with a value of 91.73%. A *p*-value of <0.05 indicates the significance of the impacts of independent factors. From the ANOVA table (Table 4), it is established that factor A (moisture content), factor B (pH), and factor D (temperature) had a significant effect on biovanillin production by SSF. In contrast, factors C (inoculum size) and D (incubation time) are insignificant. Interactive effects among various factors are shown in Table 4.

Table 2. Level of independent factors in the considered experiment.

Codes	Independent Parameters	Unit	Low Level	High Level
A	Moisture content	%	40	80
B	Inoculum size	mL	1	5
C	pH	-	5	10
D	Temperature	°C	25	50
E	Incubation time	hours	8	72

Table 3. Central composite design for the optimization of biovanillin using *Saccharum officinarum* L.

Sr. No.	Moisture Content (%)	pH	Inoculum Size (mL)	Temperature (°C)	Incubation Time (hours)	Vanillin Yield (g/100 g)	Predicted	Residuals
1	40	10	5	50	72	0.284	0.289	−0.005
2	60	7.5	3	37.5	24	0.347	0.348	−0.001
3	60	5	2	45	24	0.346	0.345	0.000
4	80	10	1	50	24	0.351	0.353	−0.003
5	60	7.5	2	37.5	48	0.344	0.346	−0.002
6	70	6.5	3	30	12	0.387	0.389	−0.002
7	50	7.5	3	25	72	0.347	0.351	−0.004
8	50	9	4	37.5	24	0.318	0.320	−0.002
9	60	7.5	3	37.5	12	0.345	0.345	0.000
10	80	5	1	50	48	0.387	0.391	−0.003
11	40	5	5	50	60	0.314	0.317	−0.003
12	40	10	5	30	48	0.308	0.311	−0.003
13	50	10	1	25	32	0.310	0.309	0.002
14	40	5	4	25	60	0.342	0.344	−0.002
15	40	10	1	50	24	0.249	0.245	0.004
16	60	7.5	3	45	60	0.342	0.347	−0.004
17	80	10	1	25	48	0.390	0.394	−0.004
18	80	6.5	5	50	48	0.404	0.414	−0.009
19	80	5	5	25	48	0.449	0.458	−0.009
20	40	7.5	3	50	72	0.286	0.289	−0.002
21	60	9	4	30	12	0.352	0.354	−0.002
22	80	5	5	50	32	0.411	0.419	−0.008
23	*** 70**	**7.5**	**4**	**37.5**	**48**	0.476	0.390	0.086
24	40	5	5	25	8	0.340	0.339	0.001
25	80	10	3	45	60	0.377	0.386	−0.008
26	60	7.5	3	25	72	0.372	0.378	−0.005
27	60	6.5	3	40	40	0.352	0.355	−0.003

Table 3. *Cont.*

Sr. No.	Moisture Content (%)	pH	Inoculum Size (mL)	Temperature (°C)	Incubation Time (hours)	Vanillin Yield (g/100 g)	Predicted	Residuals
28	40	5	1	50	50	0.285	0.282	0.003
29	80	5	1	25	25	0.418	0.420	−0.001
30	60	7.5	3	37.5	8	0.344	0.344	0.000
31	60	7.5	4	37.5	8	0.351	0.352	−0.001
32	80	10	5	25	12	0.411	0.418	−0.007

* The conditions for the highest yield of biovanillin have been indicated in bold letters.

Table 4. ANOVA study on CCD response parameters.

Source	DF	Adj SS	Adj MS	F-Value	*p*-Value
Model	20	0.068491	0.003425	6.10	0.002
Linear	0.047890	0.009578	17.07	0.000	
A	1	0.035115	0.035115	62.58	0.000
B	1	0.003483	0.003483	6.21	0.030
C	1	0.000854	0.000854	1.52	0.243
D	1	0.004401	0.004401	7.84	0.017
E	1	0.000454	0.000454	0.81	0.388
Square	5	0.001433	0.000287	0.51	0.763
A*A	1	0.000022	0.000022	0.04	0.846
B*B	1	0.000294	0.000294	0.52	0.485
C*C	1	0.000277	0.000277	0.49	0.497
D*D	1	0.000224	0.000224	0.40	0.540
E*E	1	0.000000	0.000000	0.00	0.999
2-Way Interaction	10	0.000497	0.000050	0.09	1.000
A*B	1	0.000176	0.000176	0.31	0.586
A*C	1	0.000120	0.000120	0.21	0.653
A*D	1	0.000011	0.000011	0.02	0.889
A*E	1	0.000001	0.000001	0.00	0.975
B*C	1	0.000012	0.000012	0.02	0.886
B*D	1	0.000012	0.000012	0.02	0.886
B*E	1	0.000034	0.000034	0.06	0.809
C*D	1	0.000129	0.000129	0.23	0.641
C*E	1	0.000178	0.000178	0.32	0.584
D*E	1	0.000047	0.000047	0.08	0.778
Error		11	0.006172	0.000561	
Total		31	0.074663		

The equation for the regression model is as follows:

$$\begin{aligned}\text{Vanillin (g/100 g)} &= 0.098 + 0.00004\,A + 0.0245\,B - 0.0285\,C + 0.0104\,D - 0.00097\,E \\ &+ 0.000012\,A*A - 0.00244\,B*B + 0.00502\,C*C - 0.000157\,D*D + 0.000000\,E*E \\ &+ 0.000097\,A*B + 0.000111\,A*C + 0.000005\,A*D + 0.000001\,A*E \\ &- 0.00031\,B*C - 0.000052\,B*D + 0.000050\,B*E - 0.000209\,C*D \\ &+ 0.000213\,C*E + 0.000009\,D*E\end{aligned}$$

Figure 2 depicted a graphical representation of observed and predicted biovanillin production data. The observed values were found to be nearly close to those expected. The contour plots in Figure 3 represent the correlations between numerous independent factors for biovanillin formation. These plots can be formed between any two independent variables, with the third variable's level held constant to achieve the best results. The color variations depict the stages of vanillin production between two independent components while maintaining a constant value for the third factor.

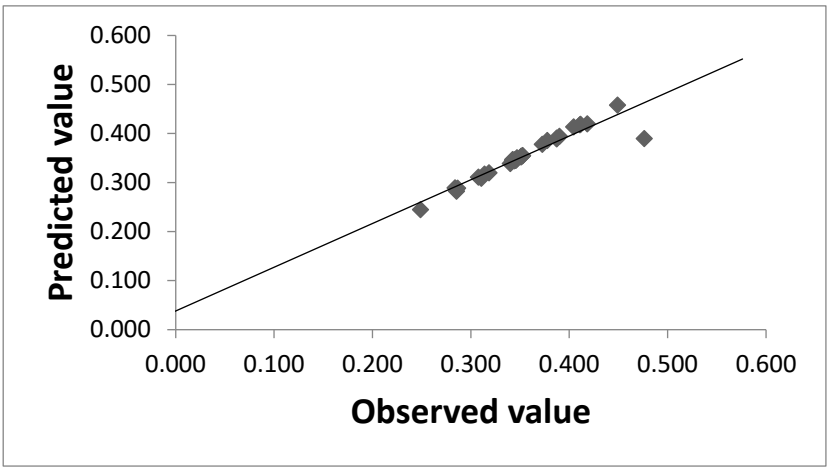


Figure 2. Biovanillin production graph comparing observed and predicted values.

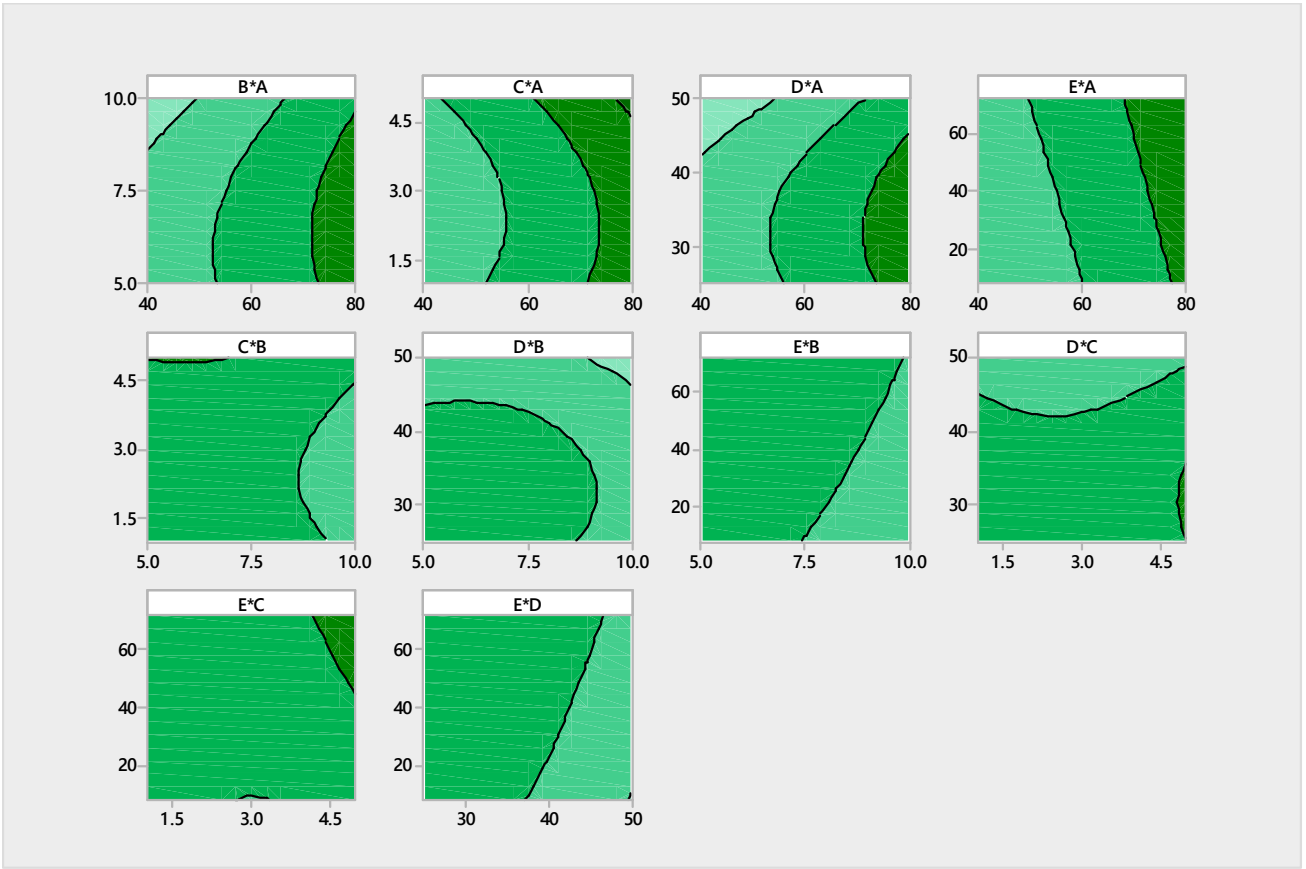


Figure 3. Contour plots for biovanillin production indicating the interactions between various factors (A: moisture content, B: inoculum size, C: pH, D: temperature, E: incubation time).

Figure 4 shows the desirability chart for biovanillin synthesis from sugarcane bagasse using *E. hormaechei* via SSF. It was determined that if the A parameter is 80, the B parameter is 6.71, the C parameter is 5, the D parameter is 31.8, and the E parameter is 72, the maximum biovanillin production expected will be 0.494 g/100 g. This result was very close compared to expected values, confirming the planned model's strong prediction and accuracy.

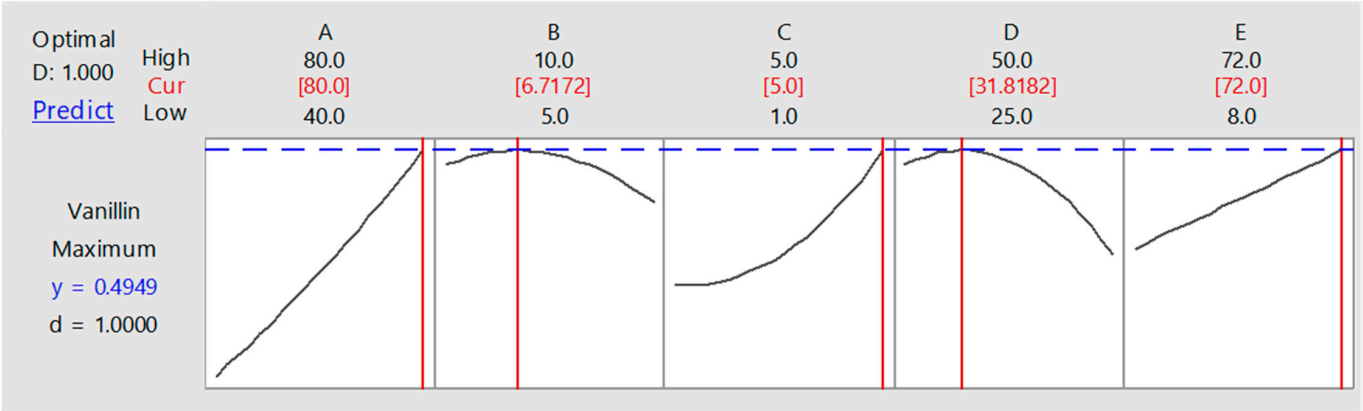


Figure 4. Desirability chart for biovanillin production using sugarcane bagasse via solid-state fermentation.

3.4. Identification and Quantitative Assessment of Biovanillin

The extracted biovanillin from sugarcane bagasse was characterized using Fourier transform infrared (FTIR) spectroscopy compared to standard vanillin. The FTIR spectrum of biovanillin produced via SSF by *E. hormaechei* exhibited identical peaks regarding the standard (Figure 5), indicating the presence of biovanillin in the fermented sample. In the FTIR spectrum of vanillin, the stretching vibration absorption of -OH was at 3250 cm^{-1}. The absorption of C–H stretching of the methyl group is at 2850 cm^{-1}. The peak at 1668 cm^{-1} corresponds to stretching vibrations of C=O of the aldehyde group, and the characteristic stretching vibration absorption of the benzene ring corresponds to three peaks at 1580, 1505, and 1500 cm^{-1}, respectively. The HPLC method shows 98.3% purity of biovanillin based on the sample and standard peaks shown in Figure 6.

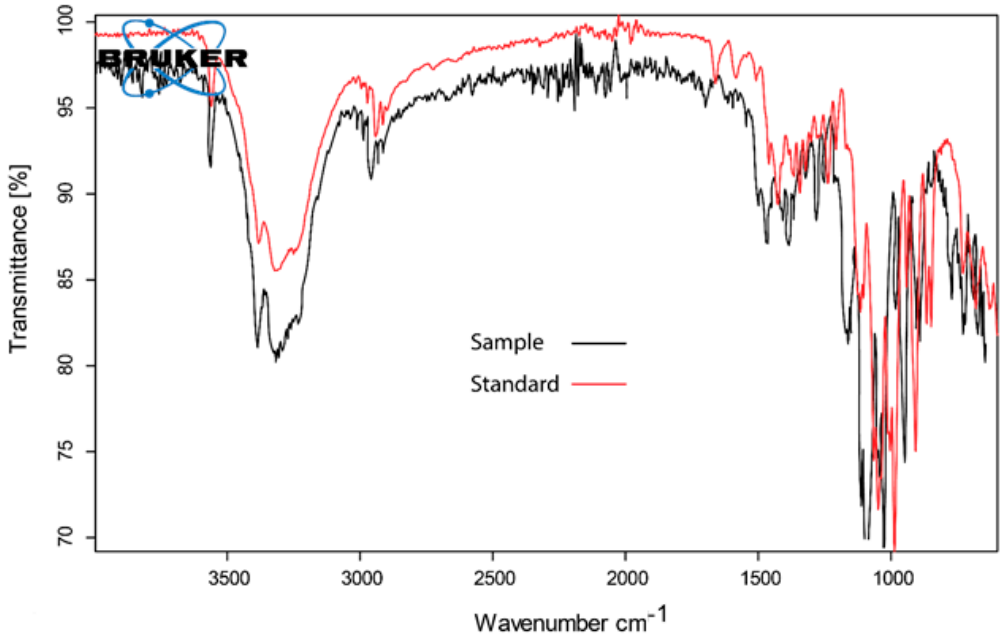
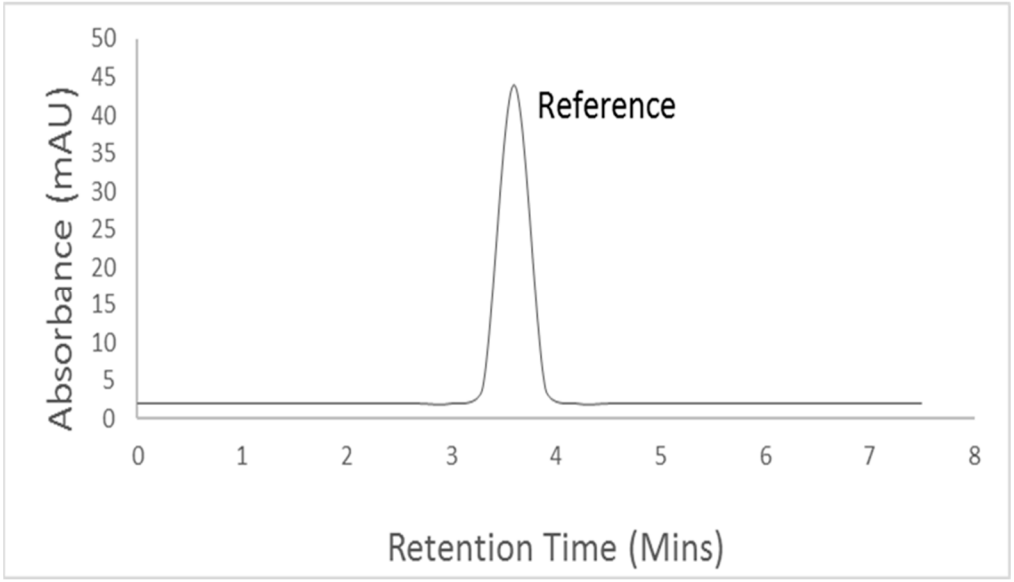
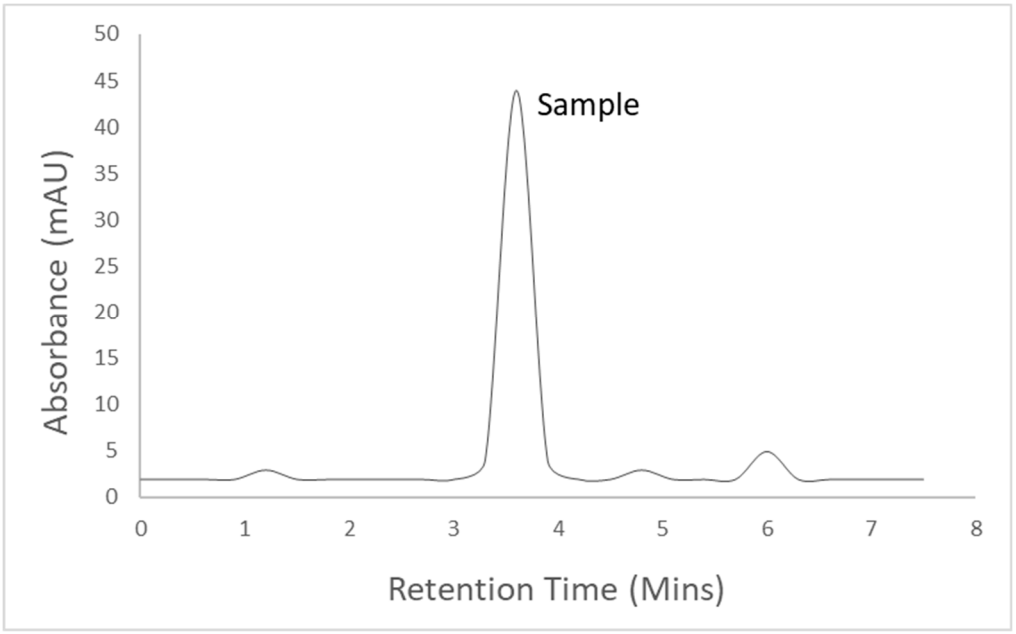


Figure 5. FTIR spectrum of biovanillin.



(**a**)



(**b**)

Figure 6. HPLC chromatogram of biovanillin (**a**) reference and (**b**) sample.

4. Discussion

Solid-state fermentation enables industrial processing wastes to be used as a substrate, thus minimizing pollution. Since it does not involve agitation, it can improve operational efficiency instead of submerged fermentation. Using agricultural waste in biotransformation processes provides a cost-effective alternative solution while assisting in reducing pollution caused by agro-waste residues, enhancing fermentation efficiency, and final product characteristics. In the current study, the maximum biovanillin yield (0.476 g/100 g) from sugarcane bagasse was obtained utilizing SSF at 70% moisture content, a 4 mL inoculum size, a 7.5 pH, a temperature of 37.5 °C, and a 48-h incubation time. Ferulic acid is found in abundance in agricultural waste ranging from 1 g/kg in corn kernels to 0.05 g/kg in processed wheat, 0.5 g/kg in whole wheat flour, and 1.5 g/kg in turmeric powder [26]. Sidestreams such as pomace, peels, and husks, which nevertheless contain flavor precursors, are invariably produced by the food sector. In keeping with the present trend, a Brazilian

patent filing described *Neurospora sitophila* conversion of cassava and malt bagasse to the fruity-smelling volatile ethyl hexanoate [27]. Sugarcane bagasse is generated during the extraction of sugarcane juice to manufacture sugar and sugar products. Even though it is biodegradable and has no negative effects on the environment, the amount of sugarcane bagasse generated causes severe environmental concerns. Bagasse is produced in 280 kg each ton of sugar produced [28], amounting to about 100 million tons per year [29]. Saccharification of sugarcane bagasse changes lignocellulosic sugars to simple sugars that many bacteria can use. Sugarcane bagasse is a lignocellulosic material that contains cellulose (38.59 ± 3.45%), hemicellulose (27.89 ± 2.68%), lignin (17.79 ± 0.62%), organic matter (1.61 ± 0.16%), extractives (1.11 ± 1.23%), and ashes (8.80 ± 0.02%) [30].

The maximum biovanillin yield (0.476 g/100 g) from sugarcane bagasse by *Enterobacter hormaechei* was obtained utilizing SSF at 70% moisture content, a 4 mL inoculum size, a 7.5 pH, a temperature of 37.5 °C, and a 48-h incubation time. Due to its toxic nature, when vanillin is left undisturbed in the solution for an extended period, its level begins to diminish. It is poisonous to cells at greater concentrations, so bacteria oxidize vanillin to produce vanillic acid, which is less hazardous. Therefore, the incubation period has a significant impact on biovanillin production [20]. A similar study observed a decrease in biovanillin concentration after a prolonged incubation period from 24 h. It was contradictory to the findings of [31] where *Enterobacter hormaechei* generated the maximum vanillin after 72 h of incubation and found that 6 g/L ferulic acids could be metabolized to 5.2 g/L vanillin with rice bran oil by-product. Our results also opposed [10]. The study used hydrolyzed lemongrass leaves, and *Phanerochaete chrysosporium* ATCC 24725 in a 2 L stirred bioreactor, and reported the maximum vanillin production (131 mg/kg) at pH 6.0, 35 °C, and an incubation time of 72 h. The extraction efficiency of SSF is less than that of submerged fermentation. It is because nutrients and microbes are not continually agitated in SSF, but the approach has the benefit of being a low-cost method since the ferulic acid found in sugarcane bagasse is used directly for production rather than after extraction. Soares et al. [32] reported production of fruity flavor through SSF using coffee husk by using *Ceratocystis fimbriata*.

Incubation temperature was optimized using a temperature range of 25 °C to 50 °C. The highest yield of (0.476 g/100 g) was obtained at 37.5 °C. Fermentation experiments at temperatures ranging from 25 °C to 50 °C were used to investigate the effect of incubation temperature. At 25 °C, the generation of biovanillin was lower (0.310 g/100 g). With increasing incubation temperature up to 35 °C, the strain's ability to convert ferulic acid into biovanillin increased. The highest level of biovanillin production (0.476 g/100 g) was recorded at 37.5 °C. As the temperature rose, the amount of biovanillin dropped. Because the bacterium is mesophilic, the enzymes denature at high temperatures, resulting in reduced biovanillin production [33]. The findings agreed with findings of another study on *P. acidilactici* synthesis of vanillin from rice bran [34]. The authors recorded the largest vanillin output of 1.269 g/L at 37 °C during the transformation of ferulic acid to biovanillin using a *Pediococcus* inoculum in RSM optimized medium consisting of rice bran 15% (*w*/*v*), peptone 0.5% (*w*/*v*), ammonium nitrate 0.1% (*w*/*v*), ferulic acid 0.005% (*w*/*v*), and magnesium sulphate 0.005% (*w*/*v*). However, results disagreed with [35] when *E. coli* JM109 cells expressing genes from *Pseudomonas fluorescens* BF13 were used to test the effect of three different incubation temperatures (22 °C, 30 °C, and 37 °C); they found results that were not similar to ours (the ferulic acid-degrader). In their study, an increase in the yield of biovanillin by lowering the temperature from 37 °C to 30 °C was observed. After a 3-h incubation with 0.5 mM ferulic acid, the maximum vanillin concentration of 0.35 mM was determined with a 70.4% molar production efficiency.

As few solid-phase fermentation studies have been conducted on biovanillin production, the results were compared with submerged fermentation. The greatest biovanillin production was recorded at a 4 mL inoculum size. Through RSM, inoculum size ranging from 1–5 mL was optimized. The highest production was obtained at an inoculum size of 4 mL. Further increase in inoculum size decreased the production. Inoculum size of

5 mL decreased the production to 0.284 g/100 g. Compared to smaller inoculum sizes, larger inoculum sizes result in faster consumption and nutrition exhaustion. The findings contradicted previous research by Saeed et al. [36], which found that bacteria with an inoculum size of 8% produce the most. Another study by Saeed et al. [20] found that bacteria with an inoculum size of 1 mL produce the highest yield. However, the findings matched another study by Saeed et al. [37]; the maximum yield was achieved using a 3 mL inoculum volume.

Another critical factor in SSF is the pH of the media. The effect of pH was observed by ranging the pH of the media from 5–10. The highest production was obtained at pH 7.5. An experiment by Yan et al. [38] used *Bacillus subtilis* to turn ferulic acid into vanillin and changed the baseline pH from 8 to 9.5 in fermentation tests. They discovered that raising the pH to 8.5 resulted in the highest vanillin production (0.64 g/L) and a 54.73% molar yield. In the one-step biotransformation of ferulic acid to vanillin utilizing *Pycnoporous cinnabarinus*, Tilay et al. [16] observed a maximum generation of vanillin with a molar yield of 42.16% at pH 6.5 when the pH of the fermented media ranged from 4 to 7. By changing the conversion efficiency of *Bacillus subtilis* from pH 5 to 7, Chen et al. [39] discovered that pH 9 was best for biovanillin synthesis from ferulic acid. Above this range, a rise in yield efficiency was observed, with a maximum increase of 78.33% at pH 9. In SSF, the moisture content is essential. The growth of microorganisms will be restricted if the moisture concentration is low. If the moisture content is high, the substrate will be filled with water, and oxygen will be ejected. The highest production was obtained at a moisture content of 70%. In a study carried out by Rashid et al. [40], the highest yield was found to be 42.86% (*w*/*v*), optimizing the moisture content in SSF. Similarly, in a study conducted by Nema at al. [41], an optimal total of 75% (*v*/*w*) of moisture content indicated maximum production by SSF.

5. Conclusions

Due to the high cost of ferulic acid, a good precursor for the synthesis of vanillin, the budget for fermentation operations has increased. Different agricultural by-products were tested for their ferulic acid content. Among them, sugarcane bagasse was chosen as the best alternative. The conditions for maximum biovanillin production were optimized using RSM, which generated different conditions involving five parameters, i.e., moisture content, pH, temperature, incubation time, and inoculum size. The highest production (0.476 g/100 g) was obtained at optimized conditions of 70% moisture content, temperature of 37.5 °C, pH 7.5, inoculum size of 4 mL, and incubation time of 48 h. HPLC and FTIR confirmed the purity of the sample. Using commercial ferulic acid at a large scale is costly; due to increasing biovanillin demand, current work would help the immense production of natural biovanillin using a cheaply available substrate.

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References

1. Chakraborty, D.; Selvam, A.; Kaur, B.; Wong, J.W.C.; Karthikeyan, O.P. Application of recombinant *Pediococcus acidilactici* BD16 (fcs+/ech+) for biotransformation of agrowaste to vanillin. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 5615–5626. [CrossRef] [PubMed]
2. Priefert, H.; Rabenhorst, J.; Steinbüchel, A. Biotechnological production of vanillin. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 296–314. [CrossRef] [PubMed]

3. Paul, V.; Rai, D.C.; Ramyaa Lakshmi, T.S.; Srivastava, S.K.; Tripathi, A.D. A comprehensive review on vanillin: Its microbial synthesis, isolation and recovery. *Food Biotechnol.* **2021**, *35*, 22–49. [CrossRef]
4. Ramachandra Rao, S.; Ravishankar, G.A. Vanilla flavour: Production by conventional and biotechnological routes. *J. Sci. Food Agric.* **2000**, *80*, 289–304. [CrossRef]
5. Arya, S.S.; Rookes, J.E.; Cahill, D.M.; Lenka, S.K. Vanillin: A review on the therapeutic prospects of a popular flavouring molecule. *Adv. Tradit. Med.* **2021**, *21*, 1–17. [CrossRef]
6. Waliszewski, K.N.; Pardio, V.T.; Ovando, S.L. A simple and rapid HPLC technique for vanillin determination in alcohol extract. *Food Chem.* **2007**, *101*, 1059–1062. [CrossRef]
7. Li, T.; Rosazza, J.P. Biocatalytic synthesis of vanillin. *Appl. Environ. Microbiol.* **2000**, *66*, 684–687. [CrossRef]
8. Gallage, N.J.; Møller, B.L. Vanillin–biotransformation and bioengineering of the most popular plant flavor and its de novo biosynthesis in the vanilla orchid. *Mol. Plant* **2015**, *8*, 40–57. [CrossRef]
9. Couto, S.R.; Sanromán, M.A. Application of solid-state fermentation to food industry—A review. *J. Food Eng.* **2006**, *76*, 291–302. [CrossRef]
10. Galadima, A.I.; Salleh, M.M.; Hussin, H.; Shiong, C.C.; Yahaya, A.; Mohamad, S.E.; Aziz, S.A.; Yusof, N.N.M.; Al-Junid, A.F.M. Improvement of biovanillin production with two-stage pH control strategy from lemongrass leaves hydrolysates using *Phanerochaete chrysosporium* ATCC 24725 in batch culture. *Biomass Conv. Biorefin.* **2020**, 1–10. [CrossRef]
11. Martins, S.; Mussatto, S.I.; Martínez-Avila, G.; Montañez-Saenz, J.; Aguilar, C.N.; Teixeira, J.A. Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. *Biotechnol. Adv.* **2011**, *29*, 365–373. [CrossRef] [PubMed]
12. Kanwal, S.; Chaudhry, N.; Munir, S.; Sana, H. Effect of torrefaction conditions on the physicochemical characterization of agricultural waste (sugarcane bagasse). *Waste Manag.* **2019**, *88*, 280–290. [CrossRef] [PubMed]
13. Ma, X.-k.; Daugulis, A.J. Effect of biotransformation conditions on vanillin production by *Amycolatopsis* sp. ATCC 39116 through an analysis of competing by-product formation. *Bioprocess Biosys. Eng.* **2014**, *37*, 891–899. [CrossRef] [PubMed]
14. Aarabi, A.; Mizani, M.; Honarvar, M.; Faghihian, H.; Gerami, A. Extraction of ferulic acid from sugar beet pulp by alkaline hydrolysis and organic solvent methods. *J. Food Meas. Charact.* **2016**, *10*, 42–47. [CrossRef]
15. Saeed, S.; Raza, S.Q.; Zafar, S.S.; Mujahid, H.; Irfan, M.; Mehmood, T. Microbial conversion of pomegranate peels to biovanillin using submerged fermentation and process optimization through statistical design. *Biomass Conver. Biorefin.* **2022**, 1–10. [CrossRef]
16. Tilay, A.; Bule, M.; Kishenkumar, J.; Annapure, U. Preparation of ferulic acid from agricultural wastes: Its improved extraction and purification. *J. Agric. Food Chem.* **2008**, *56*, 7644–7648. [CrossRef]
17. Barberousse, H.; Roiseux, O.; Robert, C.; Paquot, M.; Deroanne, C.; Blecker, C. Analytical methodologies for quantification of ferulic acid and its oligomers. *J. Sci. Food Agric.* **2008**, *88*, 1494–1511. [CrossRef]
18. Mehmood, T.; Ahmed, S.; Waseem, R.; Saeed, S.; Ahmed, W.; Irfan, M.; Ullah, A. Valorization of fruit peels into biovanillin and statistical optimization of process using *Enterobacter hormaechei* through solid-state fermentation. *Fermentation* **2022**, *8*, 40. [CrossRef]
19. Chakraborty, D.; Kaur, B.; Obulisamy, K.; Selvam, A.; Wong, J.W. Agrowaste to vanillin conversion by a natural *Pediococcus acidilactici* strain BD16. *Environ. Technol.* **2017**, *38*, 1823–1834. [CrossRef]
20. Saeed, S.; Baig, U.U.R.; Tayyab, M.; Altaf, I.; Irfan, M.; Raza, S.Q.; Nadeem, F.; Mehmood, T. Valorization of banana peels waste into biovanillin and optimization of process parameters using submerged fermentation. *Biocatal. Agric. Biotechnol.* **2021**, *36*, 102154. [CrossRef]
21. Ou, S.; Zhang, J.; Wang, Y.; Zhang, N. Production of feruloyl esterase from *Aspergillus niger* by solid-state fermentation on different carbon sources. *Enzym. Res.* **2011**, *2011*, 848939. [CrossRef] [PubMed]
22. Pan, G.X.; Bolton, J.L.; Leary, G.J. Determination of ferulic and p-coumaric acids in wheat straw and the amounts released by mild acid and alkaline peroxide treatment. *J. Agric. Food Chem.* **1998**, *46*, 5283–5288. [CrossRef]
23. Elzaawely, A.A.; Maswada, H.F.; El-Sayed, M.; Ahmed, M.E. Phenolic compounds and antioxidant activity of rice straw extract. *Int. Lett. Nat. Sci.* **2017**, *64*, 1–9. [CrossRef]
24. Lau, T.; Harbourne, N.; Oruña-Concha, M.J. Optimization of enzyme-assisted extraction of ferulic acid from sweet corn cob by response surface methodology. *J. Sci. Food Agric.* **2020**, *100*, 1479–1485. [CrossRef]
25. Webber, D.M.; Hettiarachchy, N.S.; Li, R.; Horax, R.; Theivendran, S. Phenolic profile and antioxidant activity of extracts prepared from fermented heat-stabilized defatted rice bran. *J. Food Sci.* **2014**, *79*, H2383–H2391. [CrossRef]
26. Rosazza, J.; Huang, Z.; Dostal, L.; Volm, T.; Rousseau, B. Biocatalytic transformations of ferulic acid: An abundant aromatic natural product. *J. Ind. Microbiol. Biotechnol.* **1995**, *15*, 457–471. [CrossRef]
27. Berger, R.G. Biotechnology as a source of natural volatile flavours. *Curr. Opin. Food Sci.* **2015**, *1*, 38–43. [CrossRef]
28. Cerqueira, D.A.; Rodrigues Filho, G.; da Silva Meireles, C. Optimization of sugarcane bagasse cellulose acetylation. *Carbohydr. Polym.* **2007**, *69*, 579–582. [CrossRef]
29. Cheng, J.; Zhu, M. A novel anaerobic co-culture system for bio-hydrogen production from sugarcane bagasse. *Bioresour. Technol.* **2013**, *144*, 623–631. [CrossRef]
30. Guilherme, A.d.A.; Dantas, P.; Soares, J.; Dos Santos, E.; Fernandes, F.; De Macedo, G. Pretreatments and enzymatic hydrolysis of sugarcane bagasse aiming at the enhancement of the yield of glucose and xylose. *Braz. J. Chem. Eng.* **2017**, *34*, 937–947. [CrossRef]
31. Mazhar, B.; Jahan, N.; Ali, N.M.; Andleeb, S.; Ali, S. Production of vanillin by a novel bacterium from waste residues of rice bran oil. *Punjab Univ. J. Zool.* **2017**, *32*, 137–142.

32. Soares, M.; Christen, P.; Pandey, A.; Soccol, C.R. Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation. *Process Biochem.* **2000**, *35*, 857–861. [CrossRef]
33. Gasson, M.J.; Kitamura, Y.; McLauchlan, W.R.; Narbad, A.; Parr, A.J.; Parsons, E.L.H.; Payne, J.; Rhodes, M.J.; Walton, N.J. Metabolism of ferulic acid to vanillin: A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. *J. Biol. Chem.* **1998**, *273*, 4163–4170. [CrossRef] [PubMed]
34. Kaur, B.; Chakraborty, D. Statistical media and process optimization for biotransformation of rice bran to vanillin using *Pediococcus acidilactici*. *Indian J. Exp. Biol.* **2013**, *51*, 935–943. [PubMed]
35. Barghini, P.; Di Gioia, D.; Fava, F.; Ruzzi, M. Vanillin production using metabolically engineered *Escherichia coli* under non-growing conditions. *Microb. Cell Fact.* **2007**, *6*, 13. [CrossRef]
36. Saeed, S.; Hashmi, A.S.; Tayyab, M.; Awan, A.R.; Anjum, A.A.; Firyal, S. Hyperproduction of alginate by mutated strain of *Azotobacter vinelandii* through submerged fermentation. *Pak. J. Zool.* **2016**, *48*, 1585–1589.
37. Saeed, S.; Mehmood, T.; Irfan, M. Statistical optimization of cultural parameters for the optimized production of alginic acid using apple (Malus domestica) peels through solid-state fermentation. *Biomass Conver. Biorefin.* **2020**, 1–9. [CrossRef]
38. Yan, L.; Chen, P.; Zhang, S.; Li, S.; Yan, X.; Wang, N.; Liang, N.; Li, H. Biotransformation of ferulic acid to vanillin in the packed bed-stirred fermenters. *Sci. Rep.* **2016**, *6*, 34644. [CrossRef]
39. Chen, P.; Yan, L.; Wu, Z.; Li, S.; Bai, Z.; Yan, X.; Wang, N.; Liang, N.; Li, H. A microbial transformation using *Bacillus subtilis* B7-S to produce natural vanillin from ferulic acid. *Sci. Rep.* **2016**, *6*, 20400. [CrossRef]
40. Rashid, J.; Samat, N.; Mohtar, W.; Yusoff, W. Optimization of temperature, moisture content and inoculum size in solid state fermentation to enhance mannanase production by *Aspergillus terreus* SUK-1 using RSM. *Pak. J. Biol. Sci.* **2011**, *14*, 533–539. [CrossRef]
41. Nema, A.; Patnala, S.H.; Mandari, V.; Kota, S.; Devarai, S.K. Production and optimization of lipase using *Aspergillus niger* MTCC 872 by solid-state fermentation. *Bull. Natl. Res. Cent.* **2019**, *43*, 82. [CrossRef]