MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Rifaiea *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 19 (2023) 1119-1130

RESEARCH ARTICLE

Production of Vanillin from Pumpkin Peels via Microbiological Fermentation using *Aspergillus niger*

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Abstract Vanilla is the main natural flavouring agent used in industries such as pharmaceuticals, food, flavouring, and fragrance, in which vanillin is the major component. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a secondary metabolite of plants and the major organoleptic aroma component of natural vanilla. The vanillin compound can be produced using the following routes: direct vanilla bean extraction, chemical synthesis, and biotechnological processes (bio-vanilla production). Nowadays, the chemical synthesis method used for vanillin production has been rejected by the United States and European legislation, while plant-derived vanillin is expensive. The current study demonstrates vanillin production from pumpkin peels (*Cucurbita moschata*) by *Aspergillus niger* via one-step fermentation approach. This study implements different concentrations of sodium hydroxide (1.0 M and 2.0 M) during alkaline hydrolysis pretreatment and different feeding volumes of hydrolysates during the biotransformation processes of ferulic acid into vanillin, classified as small feeding volumes (SFV) and large feeding volumes (LFV). Detection and quantification analysis were carried out using high performance liquid chromatography (HPLC), resulting in vanillin yield of 0.49 mg/L (1.0 M SFV), 0.5 mg/L (1.0 M LFV), 0.33 mg/L (2.0 M SFV), 0.59 mg/L (2.0 M LFV). Analysis with ultraviolet-visible (UV-VIS) spectrophotometry using thiobarbituric acid as reagent was carried out as well, resulting in vanillin yield of 2.76 µg/ml (1.0 M SFV), 3.78 µg/ml (1.0 M LFV), 2.68 µg/ml (2.0 M SFV), 3.05 µg/ml (2.0 M LFV). In conclusion, pumpkin peels can be considered a great source of ferulic acid and *Aspergillus niger* was reported as an efficient fungus in converting ferulic acid to vanillic acid, which will then be transformed into vanillin.

Keywords: Vanillin, ferulic acid, fermentation, pumpkin peels, alkaline hydrolysis.

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Received: 22 Sept. 2023

Accepted: 28 Nov. 2023

Introduction

About 250 components are comprised together making up the wondrous flavour of vanilla, and vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most significant organoleptic components of the vanilla flavour, depending on its abundant presence in the vanilla pod [5]. The physicochemical properties of vanillin can be described as a form of white crystalline powder with a sweet vanilla-like smell [8]. It is mostly used as a flavour enhancer, enriching the taste and aroma of food or beverage it is added to. The compound vanillin is also commonly recognised as the alternative form or imitation of the vanilla compound due to the similarities of flavour and aromatic properties. The type or source of vanillin significantly impacts how it is utilised. While synthetic vanillin is extensively used in the polymer sector as well as in the manufacturing of products such as detergents, balms and perfumes, the natural and identical-natural vanillin is only utilised in consumables, for instance food, beverages, confectionery, and pharmaceuticals [7].

Vanillin can be obtained through three methods; artificially produced through chemical synthesis using the process of nitrobenzene oxidation, direct extraction from vanilla beans, or via bioconversion of precursors using microbiological fermentation [2, 4, 6]. In other words, to attain vanillin yield generated from carbon sources, two artificial approaches can be utilised. When comparing the cost of synthetic vanillin to vanillin made from existing vanilla beans, the price of synthetic vanillin is reported to be around a hundred times lower. The high cost of vanillin obtained from vanilla is linked to the demanding requirements of the plant itself, as well as the completed product. This is primarily due to the agro-climatic conditions, growing, pollination, harvesting, and hardening required during the process of vanilla bean plantation.

Throughout the years, the agricultural sector has experienced a growth in horticultural crop production during harvesting seasons simultaneous to the rising demands of consumers towards these crops, specifically fruits and vegetables. Therefore, in order to fulfil the consumer needs for the consumption of fruits and vegetables, a significant increase in waste in the industry can be detected. This is because processing procedures of agricultural waste that are carried out by the industry greatly contribute to the disposal of various undesired parts such as peels, seeds and stalks. As mentioned in a study by Nyong *et al*. [11], pumpkin fruits are one of the large quantities that make up agricultural waste, alongside cocoa, palm bunches, plantains, and bananas, where these wastes are often discarded and burnt. Pumpkin peels are a fantastic alternative source of lignin content, a component of plant cells, which can be optimised to yield vanillin through the process of microbiological fermentation. Fermentation in vanillin production is a form of one-step bioconversion of the extracted ferulic acid into an intermediate compound known as vanillic acid. Further fermentation, results in the conversion of vanillic acid intermediates into the desired end compound, vanillin. Therefore, this research proposes the idea that the greater the ferulic acid content that is fed to and consumed by the microbiological fermenter during fermentation, the greater the vanillin yield that can be obtained.

Hence, optimising these plant-based wastes to the fullest, will contribute to the production of various valuable phytochemical components. For instance, besides the pumpkin flesh, other parts of the pumpkin are mostly considered waste and undesired materials, which can potentially contribute to the industry as an alternative source in the production of vanillin compounds. As pumpkins are easy to grow, are widely available in local markets, and can be purchased at reasonable prices, this study creates a purpose for using pumpkin peels, which in most times are simply discarded or thrown away, after the pumpkin flesh is obtained. By utilising pumpkin peels as primary samples in the production of vanillin, the amounts of pumpkin biowaste can be lessened in the future days ahead.

This study also aims to bring about the importance and relevance of fungi in biodegradation of both ferulic acid and vanillic acid, to obtain the product of vanillin through biotransformation processes, known as fermentation. The technique used for this study applies the concept of bioconversion using fungi, which is a subject that has received significantly less research and is not as extensively investigated in comparison to bioconversions involving bacterial fermenters despite fungi being associated with advantages such as having useful enzymatic activity and significant resistance towards toxicity [12]. The method discussed mainly throughout this research is known as the one-step fermentation process, where the vanillin compound, a major component found in vanilla, is obtained as the desired product.

In this research particularly, the fungus recognised as *Aspergillus niger* will be utilised to achieve the desired vanillin yield through processes of fermentation, which was carried out with different feeding volumes of hydrolysate solutions made from two varying solvent concentrations. The hydrolysate solutions are the main supplier of ferulic acid compounds, also known as common vanillin precursors. Therefore, by using a single microorganism, and presenting differences in the feeding volumes and solvent concentrations as crucial variables in this study, the yield of vanillin from pumpkin peel samples obtained will be observed and discussed. Quantification analysis using high-performance liquid chromatography (HPLC) and ultraviolet-visible (UV-VIS) spectrophotometry with thiobarbituric acid reagent for the presence of ferulic acid, vanillic acid and vanillin compounds will be carried out to provide the statistical data needed for this research.

Materials and Methods

Chemicals involved are Sodium hydroxide, Hydrochloric acid, Calcium carbonate, Ethyl acetate, Acetic acid HPLC Grade (HiPerSolv CHROMANORM), Methanol HPLC Grade (HiPerSolv CHROMANORM), Ferulic acid reference standard (Sigma-Aldrich, Switzerland), Vanillic acid reference standard (Sigma-Aldrich, Switzerland), Vanillin reference standard (Sigma-Aldrich, Switzerland), and 2-thiobarbituric acid.

Pumpkin fruits, locally known as Labu Manis or scientifically as *Cucurbita moschata*, were purchased from AEON Big Shopping Centre, a local supermarket located at Shah Alam, Selangor. These pumpkins are spherical in shape, appear green when young and gradually turns yellowish orange as they ripen. These pumpkins were then prepared thoroughly as samples used in obtaining vanillin, particularly during the earlier stages of the process. For this research, six pumpkin fruits were peeled, and the skins were put through moisture removal in oven dryers, a grounding process to transform it into powder form and pretreatments such as alkaline hydrolysis using sodium hydroxide to obtain ferulic acid.

Preparation of Pumpkin Peel Samples

Six medium sized pumpkin fruits with an average weight of 5.3 kg were thoroughly peeled and chopped into smaller sized pieces with a rough thickness of 0.3 mm each. Pumpkin peels obtained were weighed and washed with distilled water. The clean peels were then placed onto a metal tray covered with aluminum foil before undergoing the process of oven drying. Through this process, a significant amount of moisture from the pumpkin peels were removed using a hot air oven, where they were dried at an increased temperature of 105 ˚C for 12 – 14 hours [16, 15]. This drying process was performed until the remaining moisture content ranged between 3 – 5%.

Chemical Pretreatment of Pumpkin Peels

The next step after moisture removal using the oven drying method is the chemical pretreatment, or specifically, the process of alkaline hydrolysis using sodium hydroxide. Before the pretreatment, the dried pumpkin peels were ground into a bright yellow powdered state using an electrical grinder that acts as a source of ferulic acid, which then was weighed to a total of 165 g. In the pretreatment stage, with a ratio of 1:30, the hydrolysis process was carried out by introducing 5 g of the ground form of dried pumpkin peels to 150 ml aqueous solutions of sodium hydroxide at two different concentrations; 1.0 M and 2.0 M. This was carried out safely in a fume hood to any undesired incidents when preparing the solutions. Triplicate samples were made for each concentration of sodium hydroxide in 250 ml Erlenmeyer flasks. The pumpkin peel powder was made sure to be mixed well with the sodium hydroxide solutions, producing a dark coloured liquor.

All sample solutions were heated to a constant temperature of 120 ˚C for one hour in an autoclave [16, 15, 8]. As sodium hydroxide was introduced to the pumpkin peels, swelling occurred as a result of lignin degradation. The hydrolysate solutions obtained from the alkaline hydrolysis process was observed and can be described as a form of black liquor, which was present with soluble lignin content and other phenolic compounds from pumpkin peels. All liquid hydrolysates then underwent filtration processes using colander filters with nylon cloth of 400 mesh. Filtrations are done to ensure an efficient separation was made of the black liquor from the residues that were present in it [16].

Pumpkin peel residues that were accumulated in the nylon filters were washed three times with deionised water to remove any excess salt of sodium hydroxide that were remaining on the filtered residues [3]. Next, pH adjustments were carried out towards the collected black liquor through careful acidification, where concentrated solution of 10% hydrochloric acid was carefully added to the hydrolysate solutions until they reach pH 2. Centrifugation of the liquid solution was done at room temperature for 10 minutes at 3000 rpm. This ensures the recovery of the soluble lignin content, which has precipitated out from the black liquor. Then, the pH of the supernatant was carefully adjusted until it reached a neutral state of pH 5 – 6 using calcium carbonate.

Extraction of Ferulic Acid

Through liquid-liquid extraction methods, ethyl acetate was used as the primary solvent in the ferulic acid extraction [14, 16, 15]. This particular step in the production of vanillin ensures a complete extraction of ferulic acid, a precursor compound of vanillin, from the liquor is carried out. In 50 mL falcon tubes, ethyl acetate was added to the neutralised hydrolysate liquor at an equal amount. It was shaken vigorously at room temperature to ensure the recovery of ferulic acid from the black liquor. The solutions were centrifuged at room temperature for 10 minutes at 3000 rpm, forming two separated layers of the solvent from the hydrolysate solution. After the centrifugation process, a water bath was heated to a boiling temperature of 77 ˚C, and ethyl acetate solvent (upper layer) was removed as it evaporated from the hydrolysate samples (bottom layer) through vacuum by a rotary evaporator into a round flask. The whole process was done until ethyl acetate solvent was completely absent from all the hydrolysate samples. The ferulic acid concentrated hydrolysate solutions were recovered into flasks. Meanwhile the accumulated ethyl acetate solvent was discarded.

Microbial Fermentation of Ferulic Acid Using *Aspergillus Niger*

The fungal culture of *Aspergillus niger* were obtained from the Microbiology Laboratory of Universiti Sains

Islam Malaysia, Nilai, Negeri Sembilan (Malaysia). The culture was grown on potato dextrose agar (PDA) at room temperature.

Preparation of spore suspension: *Aspergillus niger* growing in Petri dishes containing potato dextrose agar (HiMedia, India) at temperatures of 30°C. The spores of *Aspergillus niger* culture were rinsed twice with 15 mL of sterile deionised water and scraped carefully using a sterilised glass scraper to isolate the fungal mycelia from the agar, further releasing the spores from the fungus. The spore suspension was strained with a nylon filter after being diluted with sterilised water. Then, this mixture of spore suspension was centrifuged at 3000 rpm for 10 mins using a microcentrifuge (Beckman Coulter, Germany), where 1 mL of it will be inoculated to the basal medium.

Preparation of basal medium: Once the spores were harvested after one week of growth and the spore suspension was made. About 1 mL of spore suspension (at concentration of 10^6– 10^7 cfu mL−1) was inoculated into 100 mL basal medium, which consisted of the carbon source for *Aspergillus niger* which is maltose (20 g/L), ammonium sulphate (1.8 g/L), yeast extract (5 g/L), magnesium sulphate (0.5 g/L), dipotassium hydrogen phosphate (0.2 g/L) and calcium chloride (0.132 g/L). The basal medium was adjusted to pH 5.5 and then autoclaved for one hour.

Fermentations were done in 250 mL Erlenmeyer flasks in a shaking incubator with an agitation of 110 rpm at 30°C. For small feeding mode samples, 1 mL of neutralised hydrolysate solution were fed to *Aspergillus niger* from day three until day six, and fermentation was stopped at day seven. Meanwhile, large feeding mode samples were fed with 10 mL of hydrolysate solutions on day three and day four, and fermentation was stopped at day five. Before feeding, 4 mL of medium was removed for SFV samples and 20 mL of medium was removed for LFV samples. After the fermentation period ends, the fermented samples were filtered to separate the cell pellets from the fermentation medium. All experiments were done in triplicates. Ferulic acid, vanillic acid and vanillin content were quantified using HPLC analysis and UV-VIS spectrophotometer.

Sample Analysis Using High-Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out to quantify the presence of ferulic acid, vanillic acid, and vanillin content from both fermented and unfermented samples (neutralised hydrolysate solutions). The analysis using HPLC (Agilent Technologies, California, USA) was equipped with a UV detector at wavelength 280 nm. A 250 mm × 4.6 mm HPLC column Zorbax Eclipse C18 (Agilent Technologies, California, USA) with 5 µm particle size was used, and samples were eluted using two solutions of mobile phases: 1% acetic acid solution (solvent A) and methanol (solvent B). The mobile phase gradient was fixed at the ratio of 80:20 (A: B) from 0 to 24 min, 60:40 (A: B) from 24 to 27 min, 20:80 (A: B) from 27 to 36 min, and 80:20 (A: B) from 36 to 40 min.

The analysis was conducted with a flow rate fixed at 1 mL min−1 and an injection volume of 10 µL. In preparing the mobile phase, filtration was carried out for both 0.1% acetic acid and methanol solutions using a 47 mm nylon membrane with a pore size of 0.45 µm pores and degassed for 30 minutes. All the standards (ferulic acid, vanillic acid and vanillin) and samples were centrifuged at room temperature for 30 minutes at 5000 rpm, and then filtered through 0.22 µm nylon membrane into 1.5 mL HPLC vials before the HPLC analysis. Vanillic acid, vanillin and ferulic acid purchased from Sigma Aldrich, Switzerland were used as the reference standards. All experiments were conducted in triplicates. The results were expressed as mean ± standard deviation. The significant differences between the means were determined through one-way ANOVA at a confidence interval of 95% using Minitab Statistical Software version 19 (Minitab LLC, USA)

Sample Analysis Using Ultraviolet-Visible (UV-Vis) Spectrophotometry with Thiobarbituric Acid

For this study, UV-VIS analysis was carried out to identify the presence of ferulic acid, vanillic acid, and vanillin contents in the fermented samples. First and foremost, 10 ml of ferulic acid, vanillic acid and vanillin standard solutions were prepared by adding 5 mL of 24% HCl, 2 mL of 1% thiobarbituric acid and 0.5 mL standard solution in distilled water. To prepare the fermented samples, 1.5 ml of fermented media was centrifuged using 2 ml falcon tubes for 30 min at 7000 rpm. Then, 50 µL of the culture supernatant was obtained as a sample and added using a micropipette into test tubes containing 950 µL of 2-thiobarbituric acid solution (prepared with 500 µL of 24% HCl, 200 µL of 1% 2-thiobarbituric acid, and 250 µL of distilled water). Both the sample and standard solutions prepared were mixed well and heated in a water bath at 55 °C for one hour and cooled down at room temperature for 20 minutes.

The absorbance of unknown vanillin and the standard vanillin solution was measured at 434 nm using a UV-Vis spectrophotometer. The OD values of standard vanillin solutions were determined, and the concentration of vanillin content in the samples was calculated from the standard calibration graph obtained. All experiments were conducted in triplicates. The results were expressed as mean ± standard deviation. The significant differences between the means were determined through one-way ANOVA at 95% confidence interval using Minitab Statistical Software version 19 (Minitab LLC, USA).

Results and Discussion

Quantification of Ferulic Acid, Vanillic Acid and Vanillin Contents Using HPLC

HPLC analysis was carried out to quantify the presence of ferulic acid in both fermented and unfermented samples. This analysis was also used to quantify vanillic acid and vanillin content in the fermented samples. Standard solutions for ferulic acid, vanillic acid, and vanillin of 5, 10, 30, 50 and 80 ppm were prepared, and calibration curves for each standard concentration was plotted. These solutions were assessed and used as references to be compared with the HPLC results of fermented and unfermented samples, in terms of the retention time (min). As assessed using the HPLC analysis, standard vanillic acid, vanillin and ferulic acid solutions were recorded with a retention time of 14.89 min, 20.23 min and 40.32 min respectively (Figure 1). The peak areas were used to calculate the concentrations of ferulic acid, vanillic acid and vanillin contents using the linear equation of y = mx.

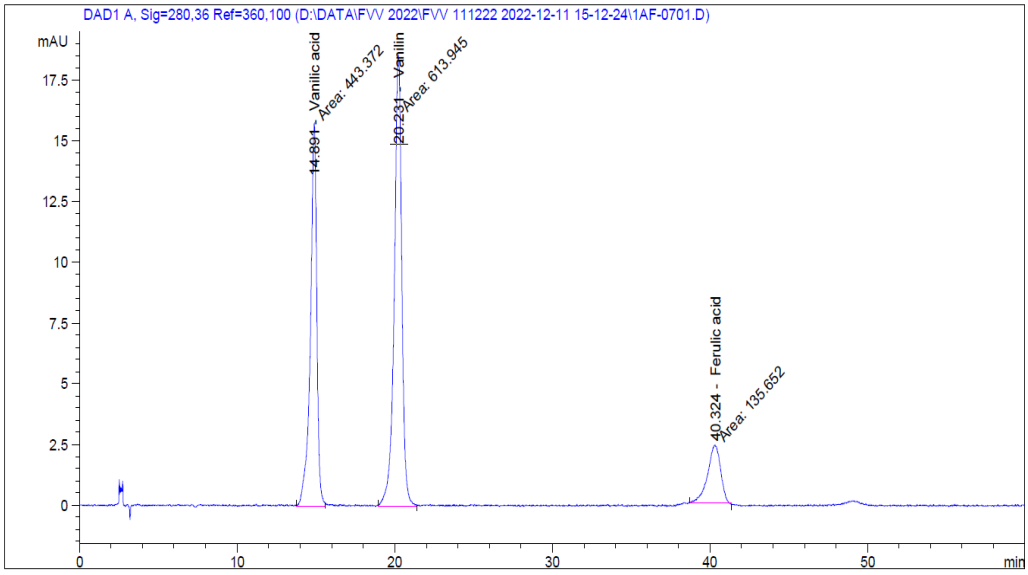

Figure 1. HPLC Chromatogram of Vanillic Acid, Vanillin and Ferulic Acid (80 ppm)

Statistical analysis was performed on Minitab software version 19 using one-way analysis of variance (ANOVA), resulted in p-values of 0.030 for ferulic acid content, 0.712 for vanillic acid content and 0.005 for vanillin content in the tested samples. At a confidence level of 95%, the results of the one-way ANOVA test can be interpreted as not statistically significant for vanillic acid content as the p-value was higher than $\alpha = 0.05$ (p-value > 0.05). Meanwhile, it was considered statistically significant for ferulic acid and vanillin contents, as their p-values were observed to be lower than $\alpha = 0.05$ (p-value < 0.05).

Table 1. Mean Concentrations of Ferulic Acid, Vanillic Acid and Vanillin (mg/L)

	Samples	Ferulic Acid	Vanillic Acid	Vanillin
1.0 M	SFV	n/d	3.77 ± 2.96	0.49 ± 0.048
	LFV	4.13 ± 1.3	1.80 ± 1.77	0.5 ± 0.025
	Hydrolysate	5.21 ± 2.73		
2.0 M	SFV	n/d	1.95 ± 2.09	0.33 ± 0.057
	LFV	4.26 ± 3.78	2.15 ± 2.28	0.59 ± 0.094
	Hydrolysate	30.1 ± 24.7		

n/d not detected
SFV small feeding volume
LFV large feeding volume

Table 1 shows the yield obtained for fermented samples classified as SFV and LFV, and for non-fermented samples classified as hydrolysate. The presence of ferulic acid content was reported as undetected for SFV samples from both concentrations of 1.0 M and 2.0 M. The absence of ferulic acid in small feeding mode samples justifies the total consumption of ferulic acid by *Aspergillus niger* throughout the fermentation period, converting it into vanillic acid intermediates and further into vanillin compounds. These findings can be supported by a study by Tang and Hassan [16], where the ferulic acid was not detected in the SFV samples fed with liquors made from pineapple peels and pineapple crown leaves. The previous study also mentioned similar values for ferulic acid used for feeding and ferulic acid consumed in units of mg/L [16]. This further proves the justifications made regarding the data mentioned in Table 1, where all ferulic acid introduced to SFV samples were fully consumed or degraded by the microbial fermenter.

Whereas ferulic acid content in LFV samples in 1.0 M and 2.0 M was reported with concentrations of 4.13 mg/L and 4.26 mg/L respectively, considering the amount of ferulic acid concentrated hydrolysate solutions were given in much greater volumes during the fermentation period. Hence, the detected concentrations of ferulic acid can be justified as the remaining ferulic acid compounds, which have not been consumed by *Aspergillus niger* and was not yet converted into vanillic acid intermediates. Furthermore, the detection of vanillic acid content in all fermented samples plays a huge role in providing support regarding the degradation activity of ferulic acid by *Aspergillus niger* did in fact take place during fermentation periods. According to the study by Motedayen *et al*. [9], among 10 different strain of fungi that was utilised in the bioconversion of ferulic acid into vanillic acid, two strains of *Aspergillus niger*, which were *Aspergillus niger* K8 and *Aspergillus niger* ATCC 200345 produced the two highest yields of vanillic acid concentration.

The presence of ferulic acid was analyzed not only for fermented samples but also for non-fermented samples, which are the black liquor samples, or hydrolysate solutions. The presence of ferulic acid in hydrolysate solutions with concentrations of 1.0 M and 2.0 M were recorded to be 5.21 mg/L and 30.1 mg/L respectively, with a significant difference of 24.89 mg/L. The 2.0 M hydrolysate solution contained a greater concentration of extracted ferulic acid as compared to the 1.0 M hydrolysate solution. This particular result was further discussed in the subtopic focusing on the effects of chemical pretreatment via alkaline hydrolysis on ferulic acid extraction.

In addition to that, the presence of vanillin compounds was also identified in all the fermented samples. It was observed that for each concentration of fermented samples, the samples supplied with large feeding volumes contained a higher concentration of vanillin compared to samples provided with small feeding volumes. Previous research by Tang and Hassan [16] reported that the production of greater vanillin concentration and molar yield was detected in large feeding mode samples compared to small feeding mode samples [16]. From here, it can be understood that more vanillin content can be produced, when greater volumes of ferulic acid containing hydrolysate solutions were fed to the fermented media.

Detection of Ferulic Acid and Vanillin Compounds Using UV-Vis Spectrophotometer with Thiobarbituric Acid

Analysis using a UV-VIS spectrophotometer was carried out to identify the presence of vanillin compounds in fermented samples. 2-thiobarbituric acid was used as the reagent for this analysis. The reaction between the thiobarbituric acid solution with the standard vanillin solutions was observed to produce a light, yellow coloured solution. Solutions with a pale, yellow colour were noticed in the fermented samples, thereby colour-wise, confirming the presence of the vanillin compound.

The absorbance of the spectrophotometric analysis for standard vanillin solutions were determined over a calibration of concentrations of 3, 5 and 8 µg/ml. The regression equation obtained through the plotted graph was $y = 0.1012x - 0.1825$ with a correlation coefficient (r^2) value of 0.9737. Based on the graphical data, the concentration of vanillin content in fermented broth samples were interpreted using the linear regression equation and tabulated accordingly in units of µg/ml.

Table 2. Mean Concentrations of Vanillin Content (µg/ml).

Concentration of Solution	Feeding Modes	Mean Concentrations (µg/ml)
1.0 M	SFV	2.764 ± 0.328
	LFV	3.783 ± 0.250
2.0 M	SFV	2.677 ± 0.215
	LFV	3.048 ± 0.271

SFV small feeding volume
LFV large feeding volume

By using the Minitab software version 19, statistical analysis using the one-way analysis of variance (ANOVA) resulted in a p-value of 0.004, where it can be interpreted to be statistically significant at 95% confidence level, as the p-value was smaller than $\alpha = 0.05$ (p-value < 0.05). For samples of concentration 1.0 M, *Aspergillus niger* produced 2.76 µg/ml of vanillin in the SFV samples and 3.78 µg/ml of vanillin in the LFV samples (Table 2). Whereas in samples of concentration 2.0 M, 2.68 µg/ml of vanillin was obtained from SFV samples and 3.05 µg/ml of vanillin in LFV samples (Table 2). From here, it was also observed that the mean concentration of vanillin content in samples with small feeding modes for both concentrations had smaller mean values compared to the samples with large feeding modes. This indicates that the greater the feeding volume of ferulic acid concentrated hydrolysate solution to the fungal broth culture during the fermentation period, the greater the amount of ferulic acid that can be converted into vanillin, hence increasing the amount of vanillin produced.

This analysis provides a form of additional confirmation of the presence of desired vanillin compound in the fermented samples. However, a difference was recorded when vanillin concentrations between the fermented samples from 1.0 M and 2.0 M were observed further. The SFV and LFV samples in concentration 1.0 M were detected with a greater vanillin concentration than SFV and LFV samples in concentration 2.0 M. This finding is considered inversely correlated with the findings regarding ferulic acid concentration, where the 1.0 M concentration samples obtained lower ferulic acid content compared to 2.0 M concentration samples.

Effects of Chemical Pretreatment Via Alkaline Hydrolysis on Ferulic Acid Extraction

Generally, a pretreatment is used to decrease the cellulose crystallinity, eliminate lignin and hemicellulose, and promote porosity in the materials [10]. Physical, physico-chemical, chemical and biological methods can all be used in the pretreatment process. For this research, alkaline hydrolysis was chosen as the chemical pretreatment for pumpkin peel samples. Sodium hydrolysis solutions were prepared at 1.0 M and 2.0 M concentrations, with triplicate samples for each concentration. Using sodium hydroxide solution as the solvent, ferulic acid extraction was successfully obtained from the pumpkin peel samples as hydrolysate solutions. As shown in Figure 2, the 1.0 M hydrolysate samples obtained a significantly lower ferulic acid concentration (5.21 mg/L) than the 2.0 M hydrolysate samples (30.1 mg/L).

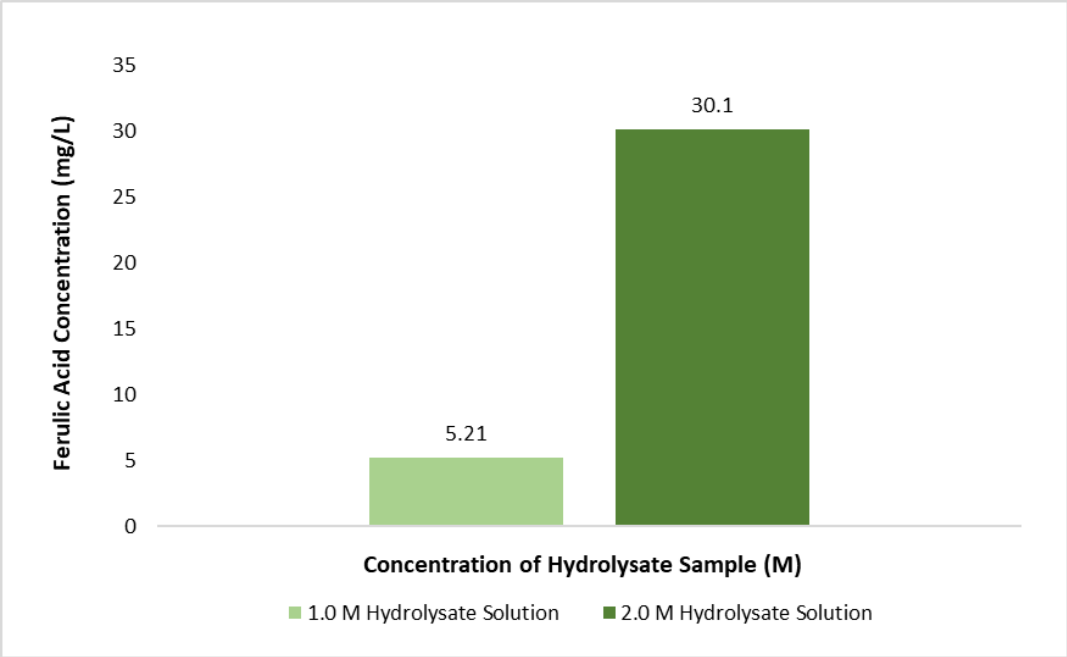

Figure 2. Mean Concentration of Ferulic Acid Content (mg/L)

As mentioned by previous studies, ferulic acid may be released from the cell wall of plant cells through the hydrolysis of ester bonds by using strong alkali or cell wall hydrolysing enzymes [13]. From here, the use of sodium hydroxide was a major contributor to the efficiency of ferulic acid extraction, as it is classified as a strong base. Strong bases are known to completely ionise in water and produce high concentrations of hydroxide ions in the solution. It has also been acknowledged in a study by Aarabi *et al*. [1], that ferulic acid release is significantly influenced by alkali concentrations and the duration of hydrolysis, where treatments with mild conditions often produce limited solubilisation and that treatments with harsh conditions can lead to product degradation. This shows that the 2.0 M solution of sodium hydroxide can produce higher concentrations of ferulic acid than the 1.0 M solution. Therefore, the greater the concentration of solvent used, the greater the ferulic acid content that is extracted.

Table 3. pH Values of Pumpkin Peel Hydrolysate Solutions.

Sample Concentration	Initial pH	pH After Acidification with HCl	pH After Added with $CaCO_3$
1.0 M	12.70	2.35	5.24
2.0 M	12.79	2.33	5.31

HCl hydrochloric acid
$CaCO_3$ calcium carbonate

Ph Adjustments to Hydrolysate Solutions

After pumpkin peels were treated with sodium hydroxide solutions at concentrations of 1.0 M and 2.0 M, filtration activities were carried out using nylon cloth filters and pH adjustments were made to the black liquor using acidification methods. Concentrated hydrochloric acid was introduced to the filtered black liquor until an acidic state of pH 2 was achieved (Table 3). According to Tang and Hassan [16], samples were acidified to ensure the recovery of soluble lignin from the black liquor. As the pH value neared pH 2, a precipitation was seen to form at the bottom of the 250 mL Erlenmayer flask. However, filtration was already carried out to eliminate residual sediments of the pumpkin peel after alkaline hydrolysis.

Centrifugation was done to remove the formed sediments in the hydrolysate. Then the supernatant was collected and neutralised using calcium carbonate until pH 5 – 6 was reached (Table 3). The acidified soluble lignin was contained in the collected supernatant, which underwent liquid-liquid extraction to release ferulic acid compounds. The addition of calcium carbonate to the supernatant was required for the ferulic acid bioconversion because vanillin production improves when it takes place in environments that are less acidic to neutral [15].

Effects of Single Fungal Implementation and One-Step Bioconversion Process on Vanillin Synthesis

In the development of this research, the one-step technique for fermentation was applied and only one microorganism was utilised in the production of vanillin, which was *Aspergillus niger*. The results for this research displayed in Figure 3 show the concentration of ferulic acid, vanillic acid and vanillin for all fermented samples. The yield obtained for ferulic acid content present in the samples had decreased compared to the start of the fermentation process, which is due to the consumption of ferulic acid by *Aspergillus niger* as the main carbon source for its growth. The act of consuming ferulic acid by this fungus, converts the ferulic acid into an intermediate compound, vanillic acid. According to a recent study by Tang and Hassan [16], the ferulic acid metabolic route by *Pseudomonas fluorescens* involves the formation of vanillic acid as the product due to the enzymatic activity of vanillin dehydrogenase in the bioconversion of vanillin. Therefore, this action was thought to be the cause of vanillic acid accumulation during the ferulic acid fermentation by *Aspergillus niger* since it followed a similar ferulic acid metabolic pathway to *Pseudomonas fluorescens* [16].

As displayed in Figure 3, vanillic acid was detected in all fermented samples (SFV 1.0 M, LFV 1.0 M, SFV 2.0 M and LFV 2.0 M), where the results prove the ability of *Aspergillus niger* as a microorganism that can produce vanillic acid. Based on a research discussion by Motedayen *et al*. [9], observations were made as the bioconversion process progressed and the concentration of ferulic acid was mentioned to have decreased. Meanwhile, the concentration of vanillic acid increased simultaneously within the first 36 hours. This proves how *Aspergillus niger* utilises the ferulic acid introduced in the fermented media, thus supporting the findings obtained in this research. Motedayen *et al*. [9] also reported that since vanillin and coniferyl alcohol were not seen during *Aspergillus niger* K8's biotransformation of ferulic acid, it may be inferred from the findings that the reaction involves the breakdown of a propenoic chain to vanillic acid. *Aspergillus niger* C28B25 strain was also used to demonstrate a comparable pathway for the breakdown of ferulic acid, yielding 57% vanillic acid [9].

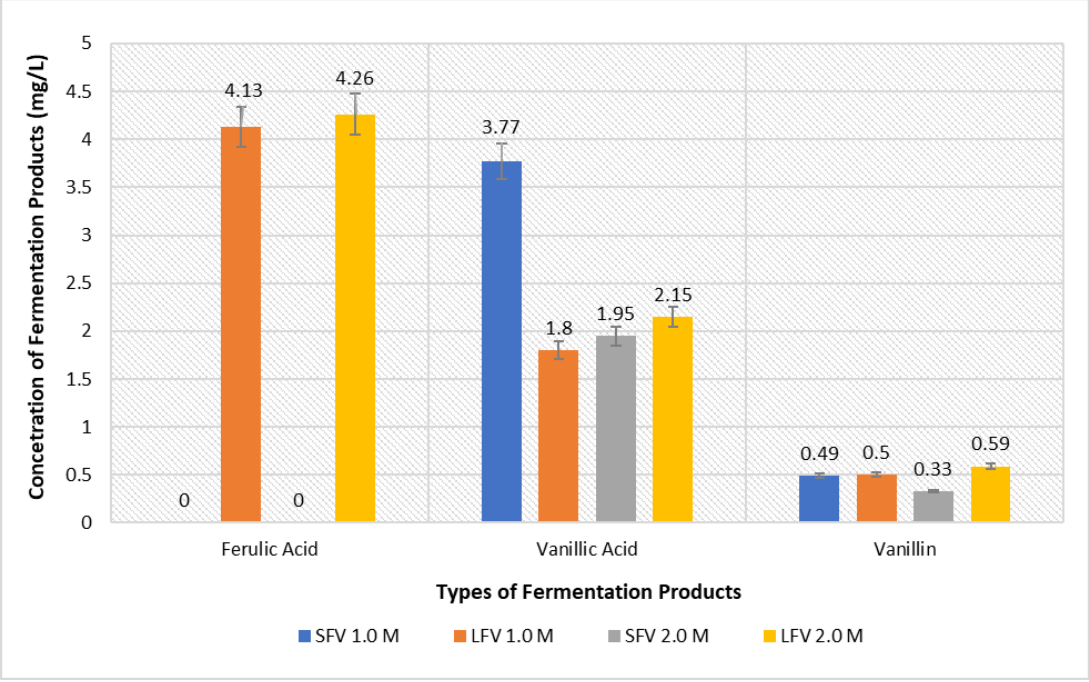

Figure 3. Concentrations of Ferulic Acid, Vanillic Acid and Vanillin Content (mg/L)

In the process of obtaining vanillin from vanillic acid intermediates, Figure 3 displays the concentrations of vanillin content that were detected, and it was observed that all fermented samples (SFV 1.0 M, LFV 1.0 M, SFV 2.0 M and LFV 2.0 M) were present with vanillin compounds. However, the results of vanillin concentration, though detected, were considered significantly low. In relation to this, despite the abundance of previous studies, such as those by Lesage-Meessen *et al*. [6], Motedayen *et al*. [9], Rejani and Radhakrishnan [14], Thibault *et al*. [17] and Zheng *et al*. [18], which had researched about the synthesis of vanillin using microbiological conversions involving various strains of *Aspergillus niger*, they had only utilised this fungus primarily to convert ferulic acid into vanillic acid, and had utilised another microorganism for the conversion of vanillic acid into vanillin.

The reasons why vanillin content was detected to be significantly scarce was justified in the discussion made by one previous study from Thibault *et al*. [17], which stated that the yield of vanillin can be reduced by three significant divergent pathways. By-passing the route to vanillin, the first pathway produced ferulic acid oligopolymers through a laccase activity. The remaining two methods included breaking the propanoic chain in ferulic acid to produce vanillic acid. Among these two pathways, in one case, vanillin was utilised to convert vanillic acid into vanillyl alcohol. Meanwhile in the other instance, methoxyhydroquinone was produced by the decarboxylation of vanillic acid.

Therefore, in order to make up for the lack of vanillin yield in the one-step bioconversion technique involving only *Aspergillus niger*, a suitable approach to better facilitate the manufacture of vanillin from ferulic acid precursor was looked into. Thibault *et al*. [17] reported that, a novel two-step technique was developed, aiming to enhance the production of vanillin from ferulic acid, which involves the combination of two filamentous fungi that have complementing transformational abilities. Therefore, in other words, this newly developed approach is the technique now known as the two-step bioconversion. This shows that, to produce a significant amount of vanillin from vanillic acid intermediates, *Aspergillus niger* is not capable of doing it all by itself. Instead, it must be paired with another microorganism to complete the vanillin production process, which in some studies it is paired with *Phanerochaete chrysosporium* [9, 14] or with *Pycnoporus cinnabarinus* [6, 17, 18].

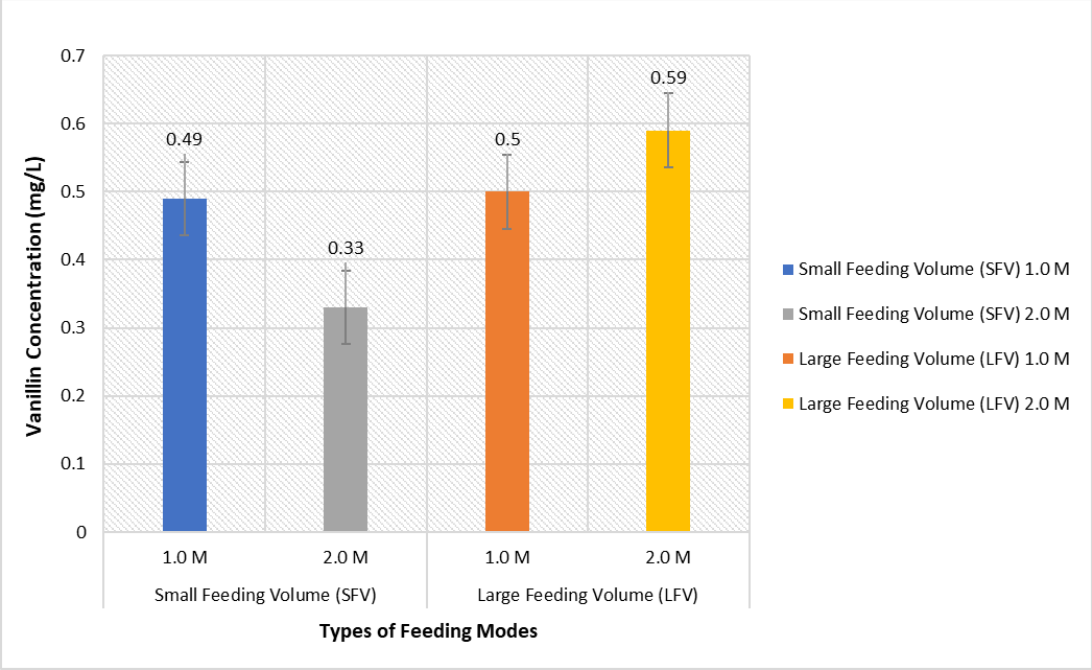

Figure 4. Content of Vanillin in Samples of Different Feeding Modes (mg/L)

Effects of Different Feeding Modes on Vanillin Production

In this study, two different feeding modes were applied in a fed-batch system, where the hydrolysate solutions of both 1.0 M and 2.0 M were introduced to the broth culture of *Aspergillus niger*. One mode was classified as small feeding volume (SFV) which was given 1 mL of ferulic acid hydrolysate (1.0 M

and 2.0 M) during days three, four, five, and six. In contrast, the second mode was classified as large feeding volume (LFV), which was given 10 mL of ferulic acid hydrolysate (1.0 M and 2.0 M) during days three and four.

From Figure 4, the graphs display the yield of vanillin produced in different feeding modes implemented during fermentation processes. Though not much, the differences between the two feeding modes can still be discerned. *Aspergillus niger* broth fed with larger volumes of ferulic acid hydrolysate produced a slightly higher vanillin yield than the ones fed with smaller volumes of hydrolysate. These findings were supported by a recent study which used similar feeding modes but only for LFV samples and not SFV samples. As Tang and Hassan [16] reported, vanillic acid and vanillin yields were influenced by the interaction between the ferulic acid content present in feeding liquors and the feeding modes, which resulted in LFV samples yielding higher vanillin content compared to SFV samples. As seen in Figure 4, both 1.0 M and 2.0 M concentrations of LFV samples had obtained a vanillin yield of 0.50 mg/L and 0.59 mg/L, meanwhile the 1.0 M and 2.0 M concentrations of SFV samples obtained only 0.49 mg/L and 0.33 mg/L of vanillin yield. Results of the LFV samples show a higher vanillin content than the SFV samples, though not much difference is present, however, proceeds to confirm the statement reported by Tang and Hassan [16] mentioned previously.

From here, a molar conversion (%) can be calculated from the yield concentration (mg/L) of vanillin obtained from the fermentation, where it can be used to represent the percentage yield (%) of vanillin produced from the ferulic acid consumption through fermentation by *Aspergillus niger.* As calculated by Tang and Hassan [16], molar conversions of vanillin yield from larger feeding volumes showed greater percentage (6.3% from pineapple peel samples and 8.9% from pineapple crown leave samples) as the yield concentration was also high, meanwhile smaller feeding volumes showed a much lower percentage (n/d from pineapple peel samples and 0.5% from pineapple crown leave samples) as the yield concentration was considerably lower. Therefore, comparisons made between the results by Tang and Hassan [16] and by this study may contribute to further proving that larger feeding volumes (LFV) do significantly improve the produce of vanillin from ferulic acid consumption through fermentation process by *Aspergillus niger*, as compared to smaller feeding volumes (SFV).

Conclusions

In this study, entitled 'Production of Vanillin from Pumpkin Peels Via Microbiological Fermentation Using *Aspergillus Niger*', observations were made regarding the effects of two varying concentrations of sodium hydroxide solution (1.0 M and 2.0 M) and two different feeding volumes (LFV and SFV) towards the production of vanillin through the one-step approach for bioconversion processes. The first variable implemented in this study which is the sodium hydroxide solvent concentration used for pretreatment, where it focused on the extraction of ferulic acid from lignin in pumpkin peels. Results showed remarkable differences between ferulic acid content in 1.0 M and 2.0 M hydrolysate solutions. Pumpkin peels pretreated with 2.0 M sodium hydroxide produced significant amount of ferulic acid content (about six times greater) compared to pumpkin peels pretreated with 1.0 M sodium hydroxide solution.

For the second variable studied which is the feeding modes that were introduced to the fermentation broth, the vanillin yield was successfully detected and quantified in all fermented samples of LFV and SFV, despite the concentration of vanillin being low. Nonetheless, data produced regarding the vanillin yield were satisfactory and reasonable comparisons were able to be deduced, which indicated that large feeding volumes of ferulic acid hydrolysate given during fermentation using *Aspergillus niger* had produced greater concentrations of vanillin content when compared to small feeding volumes samples.

Lastly, *Aspergillus niger* is well-known for its capability to degrade ferulic acid and converting it into vanillic acid. Based on the results obtained through this study, a high production of vanillin from ferulic acid precursor can be ensured by utilizing 2.0 M NaOH as the solvent for the pretreatment of pumpkin peel. It is also recommended that vanillin production should involve the implementation of the one-step fermentation by using *Aspergillus niger* accompanied by large feeding volumes of hydrolysate solution.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

This work is part of a research project supported by the Ministry of Higher Education, Malaysia, and Universiti Sains Islam Malaysia (USIM).

References

[1] Aarabi, A., Mizani, M., Honarvar, M., Faghihian, H., & Gerami, A. (2015). Extraction of ferulic acid from sugar beet pulp by alkaline hydrolysis and organic solvent methods. *Journal of Food Measurement and Characterization, 10*(1), 42-47. Retrieved from https://doi.org/10.1007/s11694-015-9274-z.

[2] Brunati, M., Marinelli, F., Bertolini, C., Gandolfi, R., Daffonchio, D. & Molinari, F. (2004). Biotransformations of cinnamic and ferulic acid with actinomycetes. *Enzyme and Microbial Technology*, *34*(1), 3-9. Retrieved from https://doi.org/10.1016/j.enzmictec.2003.04.001.

[3] Gundupalli, M.P., Kajiura, H., Ishimizu, T. & Bhattacharyya, D. (2020). Alkaline hydrolysis of coconut pith: process optimization, enzymatic saccharification, and nitrobenzene oxidation of Kraft lignin. *Biomass Conversion and Biorefinery.* Retrieved from https://doi.org/10.1007/s13399-020-00890-z.

[4] Karmakar, B., Vohra, R.M., Nandanwar, H., Sharma, P., Gupta, K.G. & Sobti, R.C. (2000). Rapid degradation of ferulic acid via 4-vinylguaiacol and vanillin by a newly isolated strain of *Bacillus coagulans*. *Journal of Biotechnology*, *80*(3), 195-202. Retrieved from https://doi.org/10.1016/S0168-1656(00)00248-0.

[5] Khoyratty, S., Kodja, H. & Verpoorte, R. (2018). Vanilla flavour production methods: A review. *Industrial Crops and Products*, *125*, 433-442. Retrieved from https://doi.org/10.1016/j.indcrop.2018.09.028.

[6] Lesage-Meessen, L., Delattre, M., Haon, M., Thibault, J.F., Ceccaldi, B.C., Brunerie, P. & Asther, M. (1996). A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. *Journal of Biotechnology*, *50*, 107-113. Retrieved from https://doi.org/10.1016/0168-1656(96)01552-0.

[7] Martău, G. A., Călinoiu, L.-F., & Vodnar, D. C. (2021). Bio-vanillin: Towards a sustainable industrial production. *Trends in Food Science & Technology*, *109*, 579-592. Retrieved from https://doi.org/10.1016/j.tifs.2021.01.059.

[8] Mehmood, T., Ahmed, S., Waseem, R., Saeed, S., Ahmed, W., Irfan, M. & Ullah, A. (2022). Valorization of Fruit Peels into Biovanillin and Statistical Optimization of Process Using *Enterobacter hormaechei* through Solid-State Fermentation. *Fermentation, 8*(2), 40. Retrieved from https://doi.org/10.3390/fermentation8020040.

[9] Motedayen, N., Maznah, I. & Nazarpour, F. (2013). Bioconversion of ferulic acid to vanillin by combined action of *Aspergillus niger* K8 and *Phanerochaete crysosporium* ATCC 24725. *African Journal of Biotechnology*, *12*(47), 6618-6624. Retrieved from https://doi.org/10.5897/AJB2013.12416.

[10] Noor Syahierah, M. S., Zulkali, M. M. D. & Ku Syahidah, K. I. (2008). Ferulic acid from lignocellulosic biomass: review. *Proceedings of Malaysian Universities Conferences on Engineering and Technology (MUCET 2008),* 1-8. Retrieved from http://dspace.unimap.edu.my/123456789/2620.

[11] Nyong, B. E., Obo Okokon, I., Precious Okokon, I. & Jones, B. B. (2021). Physico-chemical composition of telfairia occidentalis (fluted pumpkin fruit) pulp. *Journal of Research in Pharmaceutical Science, 7*(6), 2347-2995. Retrieved from https://www.researchgate.net/publication/352994313.

[12] Patil, P. D. & Yadav, G. D. (2018). Comparative studies of white-rot fungal strains (*Trametes hirsuta* MTCC-1171 and *Phanerochaete chrysosporium* NCIM-1106) for effective degradation and bioconversion of ferulic acid. *ACS Omega*, *3*(11), 14858-14868. Retrieved from https://doi.org/10.1021/acsomega.8b01614.

[13] Paul, V., Rai, D. C., Ramyaa Lakshmi T. S, Srivastava, S. K. & Tripathi, A. D. (2021) A comprehensive review on vanillin: its microbial synthesis, isolation and recovery. *Food Biotechnology, 35*(1), 22-49. Retrieved from https://doi.org/10.1080/08905436.2020.1869039.

[14] Rejani, C. T. & Radhakrishnan, S. (2022). Microbial conversion of vanillin from ferulic acid extracted from raw coir pith. *Natural Product Research, 36*(4), 901-908. Retrieved from https://doi.org/10.1080/14786419.2020.1849194.

[15] Saeed, S., Baig, U. U. R., Tayyab, M., Altaf, I., Irfan, M., Raza, S. Q., Nadeem, F. & Mehmood, T. (2021). Valorization of banana peels waste into biovanillin and optimization of process parameters using submerged fermentation. *Biocatalysis and Agricultural Biotechnology, 36*, 102154. Retrieved from https://doi.org/10.1016/j.bcab.2021.102154.

[16] Tang, P. L. & Hassan, O. (2020). Bioconversion of ferulic acid attained from pineapple peels and pineapple crown leaves into vanillic acid and vanillin by *Aspergillus niger* I-1472. *BMC Chemistry*, 14(7). Retrieved from https://doi.org/10.1186/s13065-020-0663-y.

[17] Thibault, J-F., Asther, M., Ceccaldi, B. C., Couteau, D., Delattre, M., Duarte, J. C., Faulds, C., Heldt-Hansen, H-P., Kroon, P., Lesage-Meessen, L., Micard, V., Renard, C. M. G. C., Tuohy, M., Van Hulle, S. & Williamson, G. (1998). Fungal bioconversion of agricultural by-products to vanillin. *LWT- Food Science and Technology, 31*(6), 530-536. Retrieved from https://doi.org/10.1006/fstl.1998.0411.

[18] Zheng, L., Zheng, P., Sun, Z., Bai, Y., Wang, J. & Guo, X. (2007). Production of vanillin from waste residue of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus*. *Bioresource Technology*, *98*(5), 1115-1119. Retrieved from https://doi.org/10.1016/j.biortech.2006.03.028.