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## Production of Vanillin from Pineapple Peels Using Alkaline Hydrolysis and Microbial Fermentation

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**Abstract**— Vanillin is one of the most commonly utilized aromatic flavoring chemicals in the food and cosmetics industries. It is derived from natural sources, making it more expensive than synthetic vanillin, and it constitutes less than one percent of the annual market demand. Pineapple peel stands out as a valuable source for extracting ferulic acid, which in turn is utilized in the synthesis of vanillin. As a result, researchers are exploring alternative methods for producing vanillin, such as biotechnological production from ferulic acid. In this study, the capability of pineapple peels as a substrate for the microbial fermentation of ferulic acid by *Aspergillus niger* to produce vanillin in a single step was investigated. The biotransformation of ferulic acid from pineapple peel by alkaline hydrolysis was optimized using different concentrations of NaOH. Further, the detection and quantification of vanillin and ferulic acid were carried out using High-Performance Liquid Chromatography and thiobarbituric acid (TBA) method. Through HPLC analysis, the amount of vanillin concentration produced from the supernatant culture was  $1.47 \pm 0.24$   $\mu\text{g/ml}$  from 1.0 M NaOH concentration and  $2.83 \pm 0.44$   $\mu\text{g/ml}$  from 2.0 M NaOH concentration. From this study,  $57.09 \pm 1.84$   $\mu\text{g/ml}$  and  $83.84 \pm 4.01$   $\mu\text{g/ml}$  of ferulic acid were produced from the 1.0 M NaOH and 2.0M NaOH, respectively. In addition, using the TBA technique, vanillin concentrations were calculated, resulting in  $12.92 \pm 0.54$   $\mu\text{g/ml}$  and  $15.38 \pm 0.77$   $\mu\text{g/ml}$  from 1.0 M and 2.0 M NaOH concentrations, respectively. Briefly, the pineapple peel has been discovered as a good source for vanillin production using *Aspergillus niger* in the fermentation method.

**Keywords**— Vanillin, *Aspergillus niger*, pineapple peel, ferulic acid, biotechnological route.

### I. INTRODUCTION

The pineapple produced in Malaysia is one of the highest qualities. The Ministry of Agriculture in Malaysia has selected the pineapple processing sector as a high-ability industry. One of the largest global sources of lignocellulosic wastes and residue is pineapple peels [1]. The countries of Thailand, China, Brazil, Colombia, Kenya, Nigeria, and India

are all key contributors to the global pineapple supply. The pineapple industry plays a vital part in the growth of Malaysia's society and economy despite its small size in comparison to the export supply of agricultural industries such as palm oil [2]. In addition, in 2015, Malaysia produced about 412,720 tons of pineapple products, making it the 18th largest pineapple manufacturer in the world. As part of the eleventh Malaysia Plan, the country's pineapple plantation

will be enhanced in the next years, leading to an uptick in production among the world's leading pineapple exporters by 2020.

Vanilla flavours derived from vanilla pods primarily consist of vanillin as their primary component [3]. Most of the time, it is employed in confections, sweets, and baked goods as a flavouring agent. The microbiological transformation of a variety of substrates like ferulic acid (FA) into vanillin is the most common method for producing vanillin [2], and FA is recognized as the most efficient precursor to produce vanillin [4]. In addition, it could be acquired via various processes, including biotransformation, chemical synthesis, and hydrolysis, amongst others. Besides, biotransformation is one the most promising sustainable resources, as it can supply the expanding demand for healthful and pure vanillin without damaging the environment. There have already been many attempts to modify vanillin biologically by utilizing the natural resources available, including eugenol, phenolics, lignin, sugars, FA, and waste. According to [5], it is expected that the global production of bio vanillin will expand by 7.4 percent between the years 2017 to 2025, showing a higher opportunity for the production of bio vanillin from agricultural wastes such as pineapple peels.

In 2018, about 335,488 tons of pineapple, along with 137,550 tons of peel waste, were produced in Malaysia [6]. During pineapple processing, more than fifty percent of the fruit is discarded as waste, encompassing various parts of the fruit, including crowns, peels, pits, and stems. As a result, the volume of this waste aligns with pineapple production, and the expansion of the pineapple processing industry has led to increased disposal of additional agricultural by-products. This poses a significant environmental challenge because only a portion of the pineapple residues is crushed and repurposed as animal feed. While it's typically expected that most factories would have their own waste management systems, there may still be instances where waste is sent to landfills or managed inadequately, contributing to environmental concerns such as anaerobic digestion caused by microbial degradation [2]. Consequently, the sewage system could face difficulties due to the waste's high moisture and sugar content, making it susceptible to microbial degradation.

In the meantime, the worldwide demand for natural vanillin production is extremely strong, but due to its high price and poor output, the market demand cannot be met. As reported by [7], the price of synthetic vanillin is about \$15 per kilogram, whereas the price of natural vanillin varies from \$1,200 to \$4,000 per kilogram. Even though it is lower in price, most of the synthetic vanillin contains carcinogenic or racemic compounds that are hazardous to humans and may lead to serious health issues. In addition, since racemic mixes exist in the manufacturing of synthetic vanillin, natural vanillin is preferred [8]. Thus, this has contributed to the research of alternate ways for the production of vanillin, such as biotechnological synthesis from fungi and bacteria. Hence, the aim of this research is to study the production of vanillin from pineapple peels by *Aspergillus niger*.

The research will aid in achieving the largest production of vanillin from pineapple peel using the *Aspergillus niger* fermentation process. In addition, the study contributes to the

development of an additional way for manufacturing vanillin, which suggests an ecological approach to the manufacturing process through the use of inexpensive, abundant, safe, and renewable biomass feedstock (pineapple peels), microbial conversion, and biotechnology pathways.

## II. LITERATURE REVIEW

*Pineapple:* Pineapple, scientifically known as *Ananas comosus* L. Merryl, is a popular tropical fruit that is widely consumed across the globe due to its high market value, delicious taste, and several health benefits [9]. The production of pineapples has significantly increased, rising from 4 million tons in 1960 to 16 million tons in 2005. This substantial growth highlights the pineapple's significant presence in the global fruit market. There is a strong demand for both fresh pineapples and pineapple products, including pineapple pulp, juice, and canned pineapple, which can be marketed all around the world [10]. Aside from that, according to [11], pineapple is quite possibly one of the most widely cultivated fruits, with the majority coming from Malaysia, India, Brazil, Thailand, and the Philippines.

In Southeast Asia, the Philippines exported the highest number of pineapples, followed by Malaysia and Thailand in 2018. Malaysia is part of a new group of pineapple-producing countries. Malaysia exports approximately 20,000 metric tons of fresh pineapples annually [12]. In 2016, the global pineapple production was estimated at 24.78 million metric tons with Costa Rica (2930.66 metric tons), Brazil (2694.56 metric tons), Philippines (2612.47 metric tons), India (1964 metric tons), Thailand (1811.59 metric tons, and Nigeria (1591.28 metric tons) as the top five pineapple producers in the world [13]. Pineapple has long been one of the most popular of the non-citrus tropical and subtropical fruits, largely because of its attractive flavour and refreshing sugar-acid balance.

*Pineapple waste:* The development of the pineapple processing industry has resulted in a rise in the number of agricultural by-products that are discarded, including the pineapple core, crown leaves, and strips. Approximately 75 percent of pineapples are thrown away in canneries [1]. As reported by [14], since the global demand for pineapples is increasing, the oversupply of pineapple residue and its removal methods have emerged as a critical problem. Through various thermochemical, physicochemical, and biochemical processes (anaerobic digestion and microbial fermentation), these leftovers can be converted into valuable products and used to produce energy as well as bioproducts. However, these pineapple remnants or trash are often disposed of due to antiquated technology and the lack of awareness of most local companies and farmers regarding the potential economic uses for pineapple waste [12].

The open burning of these wastes was once a common practice in the past for the management of waste, despite the fact that this practice contributes to the pollution of the air [15]. During the preparation, transportation, and storage of pineapples, around eighty percent of the pineapple's component parts, including the leaves, crowns, cores, and peels, are discarded [13]. These wastes contain high concentrations of carbohydrate, moisture, albumins, vitamins,

and lipids, all of which are highly prone to breakdown by bacteria and hence contribute to environmental issues [16]. Therefore, proper waste disposal techniques must be implemented to avoid the release of environmentally hazardous pollutants [17]. Creating value-added items from pineapple waste is one of the sustainable methods [18]. According to [14], it is prudent to convert pineapple waste into useful products based on its chemical compositions and functional capabilities, such as creating vanillin from FA in pineapples.

**Properties in pineapple peels:** Pectin, cellulose, lignin, and hemicellulose make up the majority of pineapple peel, whereas cellulose accounts for 20 to 25 percent of the dry weight. Cellulose is a big crystalline homopolymer composed of anhydrous-glucopyranose units connected via a beta 1-4 glycosidic linkage hydrogen bond. Pineapple is constituted mostly of water and carbohydrates, both of which are excellent sources of sugars, minerals, vitamins, organic acids, and fibre [19]. Several investigations have determined that dried pineapple is mainly composed of lignin (6.87 percent), cellulose (23.67 percent), pectin (5.71 percent), and hemicellulose (15.61 percent) [20]. According to [13], pineapple is used to manufacture a variety of products, including jam, fruit pulps, and canned juice. Unfortunately, only 20 percent of the whole pineapple natural product is juice; the remaining pieces, including the core, crown, base, and peeled skin, are discarded, contributing to environmental deterioration. These residues have a high concentration of hemicellulose, cellulose, and other complex carbohydrates, but a low protein content. Pineapple is mostly composed of pectin, cellulose, and hemicellulose, which are all important fiber components [21].

Furthermore, pineapple fiber contains hemicellulose (19 percent), lignin (5–15 percent), pectin (1–5 percent), and cellulose (79–83 percent). Pineapple fiber is an essential natural fiber with superior flexural rigidity and tensile strength. Due to its high cellulose content, pineapple fiber is composed of multicellular, hydrophilic lignocellulosic fibers [19]. FA is a chemical with considerable potential for use in the food and cosmetics industries since it can be converted to vanillin [10].

**Vanillin:** Vanillin is the primary chemical responsible for the vanilla flavor that is extracted from vanilla beans (4-hydroxy-3-methoxybenzaldehyde) (Figure. 2.1). It is a common flavoring compound that is ubiquitously used in industries such as cuisine, confectionery, fragrances, and medicines [3]. It is widely acknowledged that vanillin is one of the most important flavors in the entire globe [22]. According to [23], vanillin can be generated from various compounds, such as isoeugenol, tyrosine, glucose, and capsaicin, by the utilization of a wide variety of genetically engineered microbes, bacteria, fungi, and plant cells. Vanillin is demanded on a global scale of more than 15,000 tons, while only 2,000 tons are extracted from vanilla beans [8].

Vanillin can be broken down into a few different categories, including natural, chemical, biotechnological, and natural identical. The current annual global consumption of vanilla beans, vanilla extract, and vanillin is around 18,000,000 kg [10]. However, only around 0.25 percent of

commonly consumed vanillin originates from vanilla pods. This is owing to the sluggish growth of vanilla orchids and the lower content of vanillin in vanilla beans (approximately 2 percent by dry weight of cured vanilla beans) [23]. Besides, vanillin extraction from vanilla beans is a time-consuming and costly process [24], similar to the cultivation of vanilla beans itself.

**Fungi:** *Aspergillus niger* ATCC 9142 is one of the first microorganisms known to convert isoeugenol to vanillin with an efficiency of only 10% since the vanillin produced degrades into vanillic acid and vanillyl [25]. Other than that, *A. niger* is also broadly utilized in various industrial processes, from food products to the medication industry [26]. Besides, filamentous fungi such as *A.niger* and *A.flavus* have been shown to be able to transform eugenol into vanillin [27]. In addition, *A. niger* produces a wide range of biocatalysts that degrade plant polysaccharides, including cellulose and pectin [28]. These biocatalysts are vital in converting the natural carbon sources of these fungi into smaller molecules that are taken up into the cell.

As reported [29], *A. niger* has been officially approved for enzyme production in the food industry in France. *Aspergillus niger* is then able to grow and colonize on a variety of substrates due to its mycelial fragment's mode and black in color (Figure. 2.4). The colour can change over time with growth stages. Young colonies may initially appear white or light-coloured and gradually darken as they mature. The pigmentation or coloration of *Aspergillus niger* has been observed to have minimal influence on the efficiency of vanillin extraction. Vanillin extraction is predominantly determined by factors such as the strain's inherent enzymatic capacity, metabolic pathways, precursor availability, and the applied extraction methodologies rather than the visual characteristics of colony colour. As shown by [30], *Aspergillus* species were examined for their ability to bioconvert eugenol to vanillin by one-step conversion, and it is shown that *A. niger* and *A.flavus* were discovered to have the ability to convert eugenol to vanillin.

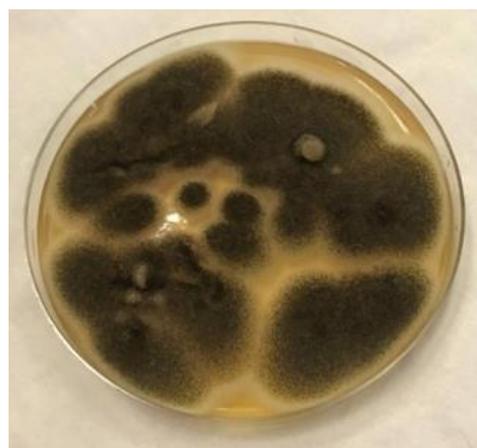


Figure 1 shows *Aspergillus niger* after 5 days.

**Precursor in Vanillin Production: Ferulic Acid:** Bioconversion is the transformation of potential precursors (FA, vanillic acid, and eugenol) catalysed by fungi or enzymes [31]. It is found in common agricultural waste

leftovers such as cereal bran and sugar beet pulp and was therefore selected as the raw material for fungi to turn into vanillin. Due to its chemical resemblance with vanillin, FA is an ideal precursor for vanillin, which inspired many scientists to develop a biotechnological approach by converting it into vanillin. Enzymatic treatment of raw materials liberated FA, which was then biotransformed into vanillin by two different white-rot basidiomycetes [32]. According to research by [33], there are two biotechnological routes to obtain vanillin: the one-step bioconversion technique (Figure. 2.5), which involves the direct conversion of FA into vanillin by a single microbe (either bacteria or fungi), and the two-step bioconversion technique, which involves the employment of fungi strains like *Pycnoporus cinnabarinus* MUCL39533 and *Aspergillus niger* I-1472 [34].

### III. MATERIAL AND METHOD

#### A. Material

Figure 2 shows 500 g pineapple peels bought from a local market in Nilai, Negeri Sembilan.



Figure 2. Pineapple peels are bought at a local market.

Chemicals and reagents used in this study were Ferulic acid reference standard (Sigma-Aldrich, Switzerland), vanillin reference standard (Sigma-Aldrich, Switzerland), vanillic acid standard reference (Sigma-Aldrich, Switzerland), Methanol and Acetic acid were obtained from (HiPerSolv CHROMANORM, New Zealand) used were of the HPLC grade. Maltose, Calcium chloride ( $\text{CaCl}_2$ ), Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), and Magnesium sulphate ( $\text{MgSO}_4$ ) were purchased from R & M Chemicals, Malaysia.

The culture strain used in this research project was acquired from the Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM). The fungal strain employed in this project was

*Aspergillus niger*. The strain was grown and maintained on potato dextrose agar at 25 °C.

#### B. Method

1) *Pre-treatment sample of pineapple peel*: Pineapple peels (PP) were obtained from the local store in Nilai, Negeri Sembilan. The fresh pineapple was put on a weighing scale and weighed approximately 500g. It was then rinsed with distilled water. The pineapple peels were subsequently dried in the oven (Memmert, Germany) for 12-14 hours at 105 degrees Celsius until the moisture content reached 5-8 percent [35]. The dried pineapple peels were ground in a blender till they turned into a powdery texture.

2) *Alkaline Hydrolysis Treatment*: The pineapple peel powder was treated in an alkaline treatment. The experiment was carried out in an Erlenmeyer flask, and approximately 5 grams of pineapple peel powder were treated in (40 gL<sup>-1</sup>) and (60 gL<sup>-1</sup>) of aqueous sodium hydroxide solution in a fume hood (Viralink Corporation Sdn Bhd, Malaysia) and autoclaved at 120 °C for an hour to obtain the black liquor of PP.

Next, the residues were filtered off from the black liquor under vacuum suction along with a muslin cloth to avoid leakage during the suction process. Initially, the pH of the black liquor was reduced with strong hydrochloric acid HCl into pH 2, accompanied by 5 minutes of centrifugation with a centrifuge machine (Beckman Coulter, United States) at 1468g to recuperate the dissolved lignin from the black liquor. The pH of the black liquor was once again altered to 5-6 by calcium carbonate,  $\text{CaCO}_3$  [36].

3) *Liquid-liquid extraction (Ferulic acid extraction)*: To completely extract FA in this step, ethyl acetate was used as an extraction solvent. The neutralized liquor was combined with an equivalent volume of ethyl acetate and vigorously shaken at ambient temperature [36]. The mixture of black liquors (supernatant) and ethyl acetate formed two layers of solution; hence, centrifugation at the speed of 1468xg for 5 minutes was carried out to separate these two layers. The ethyl acetate layer was removed under vacuum using a rotary evaporator until only an insoluble yellowish layer remained at the flask's bottom. The insoluble yellowish layer was observed as a phenolic compound.

To solubilize the phenolic compound, a 50% v/v methanol solution was added and thoroughly shaken to ensure the mixture was homogeneous, then HPLC analysis was performed. Before analysis, the supernatant samples were filtered into vials via 0.22 µm nylon membrane, and the FA standard was used as a reference. The extraction was repeated in triplicates by using an equivalent amount of ethyl acetate with black liquors [36].

4) *Microbial conversion of ferulic acid & Fermentation of vanillic acid to vanillin*: Obtaining spore suspension: The spores were produced in five days on Potato Dextrose Agar (HiMedia, India) medium at 30°C. A suspension for spores was obtained by scraping the spores' culture in a petri dish and rinsing with 14 mL of sterilized 0.9 percent sodium chloride NaCl containing 1 percent Tween80. The suspension

was filtered to separate conidia from hyphal fragments and then centrifuged using a microcentrifuge (Beckman Coulter, Germany) at 3000 rpm for 5 minutes to separate the cell pellets. 1 mL of the suspension was collected and mixed with 200 mL of sterile distilled water to be used in the fermentation method later [37].

Preparation of basal medium: Maltose (20 gL<sup>-1</sup>), as the carbon source, and ammonium sulphate (1.8 gL<sup>-1</sup>), as the nitrogen source, were utilized as the major ingredients to prepare basal medium. Besides, other necessary nutrients required for the medium, including magnesium sulphate  $MgSO_4$  (0.5 gL<sup>-1</sup>), Potassium dihydrogen phosphate  $KH_2PO_4$  (0.2 gL<sup>-1</sup>), Calcium chloride  $CaCl_2$  (0.132 gL<sup>-1</sup>), and 5 gL<sup>-1</sup> yeast extract were also introduced. The basal medium's pH was adjusted to 5.5, and it was then autoclaved.

*Aspergillus niger* was grown on potato dextrose agar (containing 39 gL<sup>-1</sup>) potato dextrose powder in distilled water at 25 °C. After five days of growth, the spores were harvested, and one mL of conidial suspension (at a concentration of 10<sup>6</sup> to 10<sup>7</sup>cfu per mL) was added to 150 mL of potato dextrose broth. After three days, one mL of the sub-cultured *A. niger* in potato dextrose broth was inoculated into 100 mL of basal medium. The pH of the medium was then adjusted to 5 [36].

The *A. niger* was incubated in the shaking incubator at 28 °C with 150 rpm for four days. *A. niger* was then fed approximately 10 mL of a neutralized pineapple peel liquor concentration constantly from day 3 to day 4. On day five, fermentation ended. To separate the hyphal fragments, the fermentation medium was filtered with Whatman no. 1 filter paper. Vanillin, vanillic acid, and FA residues in the medium upon fermentation were measured using HPLC. Each experiment was performed three times.

5) *Vanillin detection by High-performance liquid chromatography (HPLC)*: Vanillic acid, vanillin, and FA concentrations were determined using an HPLC (Agilent Technologies, Malaysia) fitted with a UV detector at 280 nm wavelength. A 250 mm x 4.6 mm HPLC column with 5 µm particles was utilized. The samples were eluted using solvent A (1% acetic acid) and methanol (solvent B). From 0 to 24 minutes, the mobile phase gradient was 80:20 (A: B), 60:40 (A: B) from 24 to 27 minutes, 20:80 (A: B) from 27 to 36 minutes, and 80:20 (A: B) from 36 to 40 minutes. The mobile phase was degassed for 30 minutes following filtration over a 47 mm nylon membrane with 0.45 µm pores. Before analysis, all standards and samples were filtered through a 0.22 µm nylon membrane into a vial, with vanillic acid, vanillin, and FA serving as the reference standards. 10 µL was used for the injection volume during analysis [36]. The analytical flow rate was set to 1 mL min<sup>-1</sup>. For the analysis of vanillin in culture supernatant, 500 µl of the culture supernatant was centrifuged at 5000 rpm for 20 minutes. Before being injected into the HPLC system for analysis, the supernatant was autoclaved, dissolved in 500 µl of methanol, and filtered through a 0.2-micron filter.

6) *Ferulic acid detection by Ultraviolet-visible Spectroscopy (UV-Vis)*: Ultraviolet-visible spectroscopy analysis was utilized to identify the presence of ferulic acid in the hydrolysate solution of black liquor. At 320 nm, the

absorbance of standard ferulic acid solutions was measured. The ferulic acid concentration in the sample was evaluated against a standard graph of ferulic acid that was plotted using known values ranging from 1 to 10 µg/ml.

7) *Quantitative estimation of vanillin produced by the thiobarbituric acid method*: Thiobarbituric acid was used to estimate the yield of vanillin. The values of vanillin were calculated with a high absorbance at 434 nm [8]. As for the preparation of the sample, approximately 2 mL of distilled water was added with 1 mL of the culture supernatant, 2 ml of 2% thiobarbituric acid, 5 ml of 24% hydrochloric acid, and mixed. At 55 degrees Celsius in a water bath, the test tubes were incubated for an hour and kept at ambient temperature for 20 minutes afterward. To determine the concentration of vanillin present in the supernatant, absorbance was measured at 434 nm and compared with a vanillin standard using an obtained standard graph. The quantity of vanillin in the culture supernatant was calculated [23].

8) *Statistics Data Analysis*: Analysis of variance method (ANOVA) was used to analyse the data using Minitab Version 9. Significant differences among variables based on One-way ANOVA and variables were compared using least significance difference (LSD) at P<0.05. The results and discussion were based on the mean data of the experimental trial.

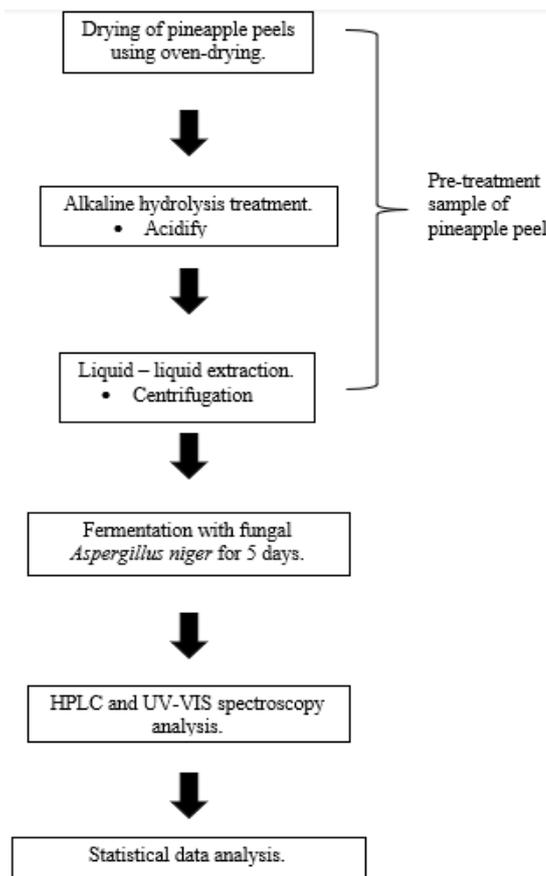


Figure 3. Overall process of vanillin production [2]

### III. RESULTS AND DISCUSSIONS

#### A. Pineapple peel pre-treatment with NaOH solution.

There are two fundamental chemical procedures for hydrolysing biomass, which is acids and bases treatment. The choice normally depends on the material structure and desired product attributes. In general, alkaline-based treatments are more efficient than acid-based treatments because they can dissolve a greater proportion of lignin [38]. This study investigated the solubilization of FA, one of the main components produced from pineapple peels by alkaline treatments with different concentrations of NaOH. According to [39], among many different alkalines available, sodium hydroxide was chosen since it was considered to be more selective in terms of phenolic substances. This led to the selection of sodium hydroxide. In addition, phenolic components such as vanillin, syringic, and ferulic are frequently linked with lignin via ether bonds in agro-industrial wastes [9]. For instance, FA is attached to lignin via ether as well as ester linkages [41].

Even though ferulic acid is covalently associated with the structure of lignocellulosic materials by ether and ester bonds, conventional methods (such as solid-liquid extraction) are ineffective in separating it from the matrix. In order to break these bonds and free the ferulic acid, further methods, such as alkaline hydrolysis treatments, are required. According to [42], alkaline hydrolysis is capable of cleaving the structure of lignin/phenolic-carbohydrate complexes, which results in the formation of a phenolic element, soluble sugars, insoluble lignin, and carbohydrates. In addition, alkaline extraction is the simplest, most cost-effective, and common method of releasing ferulic acid from agro-industrial by-products [33].

The high levels of phenolic compounds that were maintained after alkaline treatment were due to the release of phenolic compounds from polysaccharides by breaking the ester bond linking phenolic acids to the cell wall. Extraction of phenolic compounds by hydrolysing the covalent ester linkages was demonstrated to be the most effective when performed by alkaline hydrolysis. After the extraction procedure was complete, the pH was lowered to 2.0 to induce hemicellulose precipitation (Table 1). The black liquor's pH was then adjusted with calcium carbonate between 5.0 to 6.0 so that the extracted ferulic acid could be used in a microbial fermentation process in which the conditions were not too acidic. During the fermentation procedure, pH plays a vital function. As stated by [42], the pH of the broth culture media has the ability to affect the substrate chemistry and fermentation enzymes during the process. The results reveal that the highest vanillin yield was achieved at a pH of 6.0 and that when the pH was lowered (from 6.0 to 3.5), the yield was decreased [43].

Table 1. pH value of the black liquor samples.

No.	Sample Concentration	Initial PH	pH after added HCl	pH after added CaCO <sub>3</sub>
1	1.0 M NaOH + PP powder	11.87	2.22	5.19
2	2.0 M NaOH + PP powder	11.38	2.22	5.28

Black liquor produced from the treatment was very high in concentration, which reduced lignin availability. Therefore, ethyl acetate was utilized as the extraction solvent for ferulic acid. Liquid-liquid extraction (LLE) could also be employed to enhance high-molecular-weight phenolics and purify extracts using suitable solvents [44]. LLE is one of the most prevalent techniques for extracting phenolics from various cereals [45]. Failure to remove lignin completely from the generated liquor prevented it from being removed from the HPLC analysis. To recover ferulic acid from neutralised liquor, an equivalent volume of ethyl acetate was added, and the mixture was vigorously shaken at room temperature. The supernatant was evaporated under vacuum to eliminate excess solvent. The extract, which contained phenolic compounds, was then tested by HPLC for total phenolic compounds [46].

#### B. Biotransformation process of ferulic acid to vanillin

Microbial conversion of FA to vanillin is one of the most effective approaches for the industrial output of vanillin. Using ANOVA, the validity of the obtained FA in 1.0 M NaOH and 2.0 M NaOH before and after fermentation was examined.

Table 2. Yields of ferulic acid [ $\mu\text{g/ml}$ ] before fermentation with *Aspergillus niger* (n=1)

Concentration of NaOH (M)	Yields of ferulic acid [ $\mu\text{g/ml}$ ]
1.0	57.09 $\pm$ 1.84
2.0	83.84 $\pm$ 4.01

In addition, the observations of ethyl acetate extraction of FA showed that an equivalent amount of ethyl acetate must be repeated several times on PP black liquor to achieve the maximum FA extraction. As stated by [36], PP was discovered as an excellent source of FA, with 5 mg of FA yielded from one gram of PP powder. Due to its structural similarities, FA is the appropriate precursor for vanillin synthesis. According to [41], FA is linked to hemicellulose and lignin in herbaceous plants with ester and ether linkages that act as bridges. The alkali breaks the ester linkage of the ferulate bridge, thereby releasing the lignin compound and FA separate from the hemicellulose. Also, alkaline treatment at high temperatures (85–100°C) can liberate FA from plant cell walls [47]. In this study, these essential compounds were isolated from the phenolics-carbohydrate complexes of pineapple peels using an alkaline solvent. Cell wall components like cellulose and hemicellulose are connected to FA via ester linkages, which can be hydrolysed by enzymes called feruloyl esterases. These enzymes could be derived from a variety of fungi, yeast, and bacteria. For instance, feruloyl esterases derived from *Aspergillus niger* facilitate the extraction of FA from agricultural wastes such as coffee pulp and wheat straw [48].

After fermentation, the remaining yield of ferulic acid is not detected. This is because, during fermentation, *Aspergillus niger* used all of its ferulic acid (pineapple peel black liquor) as a nutrient source to produce vanillin acid, and under the stress of inadequate nutrients after all the ferulic acid has been used, *A.niger* tend to convert the vanillic acid into vanillin. The amount of vanillin detected after fermentation is shown

in Table 3. Despite this, [49] reported that the vanillin manufacturing mechanism is partly related to the side chain cleavage of FA via feruloyl-CoA hydratase and Coenzyme A (CoA) to produce vanillin. This was determined based on the recent discovery of phenolic compounds and associated genes in *A. luchuensis*. Additionally, feruloyl-CoA synthetase catalyses the transfer of coenzyme A (CoA) to the carboxyl group of FA, resulting in the synthesis of feruloyl-CoA. Feruloyl-CoA is degraded by feruloyl-CoA hydratases (sometimes referred to as enoyl-CoA hydratases), which results in the synthesis of 4-hydroxy-3-methoxyphenyl-hydroxypropionyl-CoA. This molecule is cleaved to produce vanillin during this process. Since this study utilized only one microorganism, which is *Aspergillus niger*, it can be assumed that the bioconversion pathway might be similar to the conversion pathway of *Aspergillus luchuensis*, as it is from different species.

Table 3. Yields of vanillin [ $\mu\text{g/ml}$ ] detected after fermentation with *Aspergillus niger* (n=1)

Concentration of NaOH (M)	Yields of vanillin [ $\mu\text{g/ml}$ ]
1.0	1.47 $\pm$ 0.24
2.0	2.83 $\pm$ 0.44

In addition, [36] have demonstrated the transformation of ferulic acid into vanillic acid using one-step bioconversion by *Aspergillus niger*. It shows that *A. niger* can convert ferulic acid released from pineapple peel to vanillic acid. Unfortunately, the metabolic route responsible for this transformation remains unclear. In contrast, this study aims to extract ferulic acid into vanillin directly with *A. niger*. Hence, the metabolic pathway of *A. niger* in the production of vanillin from the study by [30], which used *Pseudomonas fluorescens* as substrate, has been referred. It illustrated the conversion of *P. fluorescens*, which is expected to produce vanillin from eugenol through a metabolic pathway with ferulic acid as an intermediate metabolite.

Other than that, there is a two-step conversion route in which *A. niger* converted FA to vanillic acid, and afterward, *P. cinnabarinus* converted vanillic acid to vanillin [36]. According to [50], it can be postulated that the CoA-dependent B-oxidative pathway is the primary hydroxycinnamate metabolic system, and the *A. niger* genome has at least one other pathway, most likely the CoA-independent oxidative pathway.

### C. Ferulic acid, vanillic acid, and vanillin analysis

#### 1) Ultraviolet-visible Spectroscopy (UV-Vis)

The ferulic acid concentration in the hydrolysate solution of black liquor was calculated using the linear regression equation,  $y = 0.0903x - 0.2278$ . The R value obtained was 0.9933. The concentration of sodium hydroxide was the parameter in this experiment. Examined concentrations of NaOH were 1.0 M and 2.0 M. Yields of ferulic acid are shown in Table 4. The highest average yields obtained were 45.39 $\pm$ 1.47  $\mu\text{g/ml}$  from 2.0 M NaOH pre-treatment. Further increase of NaOH concentration increases the yield of ferulic acid.

Table 4. Yields of ferulic acid and vanillin [ $\mu\text{g/ml}$ ] after fermentation with *Aspergillus niger* (n=3)

Yields of:	Concentration of NaOH (M)	
	1.0 M	2.0 M
Ferulic acid [ $\mu\text{g/ml}$ ]	37.77 $\pm$ 0.28 (Presence)	45.39 $\pm$ 1.47 (Presence)
Yields of vanillin [ $\mu\text{g/ml}$ ]	12.92 $\pm$ 0.54 (Presence)	15.38 $\pm$ 0.77 (Presence)

#### 2) Thiobarbituric acid method to detect vanillin content

Initially, spectrophotometric analysis and TBA were utilized to identify the presence of vanillin following the method [8]. TBA formed yellow-colored solutions in response to normal vanillin solutions (Figure. 4), and light yellow solutions were identified, indicating the presence of vanillin. At 434 nm, the absorbance of standard vanillin solutions was measured.

Standard curve was calibrated from 4.0 – 10.0  $\mu\text{g/ml}$ . Vanillin concentration in the supernatant culture was calculated using a linear regression equation,  $y = 0.0238x - 0.0373$ . The R value obtained was 0.9828. The examined concentration of NaOH was examined at 1.0 M and 2.0 M in the experiment. The vanillin content in the collected culture supernatant peaks at 434 nm. From the graph, vanillin present in the culture supernatant of 2.0 M NaOH concentration is 15.38 $\pm$ 0.77  $\mu\text{g/ml}$  and 12.92 $\pm$ 0.54  $\mu\text{g/ml}$  in 1.0 M NaOH concentration. Based on the statistics obtained by Minitab 19, there is a significant difference ( $p=0.011$ ) between the yield of bio vanillin produced using ferulic acid of 1.0 M and 2.0 M. This shows that higher concentration of sodium hydroxide from the alkaline hydrolysis treatment resulted in higher vanillin content from pineapple peels. As reported by [8], vanillin yield using the thiobarbituric acid method reached 1.04 mg/ml in the presence of FA using *B. subtilis* as substrate, and 0.87 g/L vanillin was produced by *B. aryabhattai* NCIM 5503 [51].



Figure 4. Light yellow-coloured TBA solution with culture supernatant.

However, the amount of ferulic acid and vanillin detected through ultraviolet-visible spectroscopy might not be as accurate as HPLC. This is because the samples may be contaminated with other chemicals that absorb at the same wavelength to give a falsely high concentration of the desired analyte in a UV protocol. Meanwhile, HPLC gives the possibility of separating those interfering compounds away or quantifying a series of related compounds in a sample. As mentioned above, HPLC also tends to be more sensitive. Both require the preparation of a standard curve. To measure UV, it's a matter of absorbance at a wavelength. For HPLC, the peak area is the response. Hence, using UV-Vis, the presence of ferulic acid and vanillin can be identified but not in the exact quantity.

### 3) HPLC analysis

#### A) Ferulic acid content

The quantity of ferulic acid that was synthesized in the hydrolysate solution of black liquor was measured with HPLC, and the results were compared to the standard amount of ferulic acid. The concentration of newly synthesized ferulic acid produced was measured using retention time and peak area. After centrifugation, the first layer, which is the hydrolysate solution, was collected for analysis. Several research utilized acidified water or hydrochloric acid, which was subsequently combined with methanol as the mobile phase [52].

Table 5. Yields of ferulic acid [ $\mu\text{g/ml}$ ]

Concentration of NaOH (M)	Yields of ferulic acid [ $\mu\text{g/ml}$ ]
1.0	57.09 $\pm$ 1.84
2.0	83.84 $\pm$ 4.01

Alkaline treatment of PP with 40 g L<sup>-1</sup> NaOH and 60 g L<sup>-1</sup> NaOH at 120 °C for an hour successfully yields 57.09 $\pm$ 1.84  $\mu\text{g/ml}$  and 83.84 $\pm$ 4.01  $\mu\text{g/ml}$  FA, respectively. According to HPLC analysis, the peak in the hydrolysate sample of 1.0 M NaOH and 2.0 M NaOH concentration with pineapple peel powder with retention times of 40.42 and 41.25 minutes, respectively, matches the peak in the standard FA with retention times of 40.32 minutes (Table 8). Based on the statistics obtained by Minitab 19, there was a significant difference ( $p=0.001$ ) in the yields of ferulic acid from different concentrations of NaOH. In comparison to [36], they effectively extracted 202  $\pm$  18 mg/L of FA from pineapple peel, which was then raised to 1055  $\pm$  160 mg/L by using response surface methodology (RSM) to optimize the FA content. In addition to the reduced content of FA in black liquors, it was hypothesized that the unknown non-ferulate phenolic compound (NFPC) and other harmful substances inhibited the extraction of FA from PP black liquors.

#### B) Vanillic acid content

Meanwhile, for vanillic acid, the fermented sample peak of 1.0 M NaOH and 2.0 M NaOH concentration with retention times of 15.38 minutes and 16.57 minutes, respectively corresponds to the peak in the standard vanillic acid at 15.57 minutes. The amount of vanillic acid produced

was 3.93 $\pm$ 0.79  $\mu\text{g/ml}$  in 1.0 M NaOH concentration and 6.27 $\pm$ 1.57  $\mu\text{g/ml}$  in 2.0 M NaOH concentration.

Table 6. Yields of vanillic acid [ $\mu\text{g/ml}$ ]

Concentration of NaOH (M)	Yields of vanillic acid [ $\mu\text{g/ml}$ ]
1.0	3.93 $\pm$ 0.79
2.0	6.27 $\pm$ 1.57

Based on the statistics obtained by Minitab 19 (Table 7), there was no significant difference ( $p=0.082$ ) in the yields of vanillic acid from different concentrations of NaOH. According to [53], with fungal *A.niger* K8, the highest concentration of vanillic acid (116.9 mg/l) was produced after 36 hours of bioconversion of FA. This occurred after the bioconversion process had been completed. Aside from this, the conversion of vanillic acid to methoxy hydroquinone, which is harmful to the environment, is the most plausible explanation for the low concentration of vanillic acid produced. Hence, [54] reported that the vanillyl alcohol conversion could be suppressed by adsorbing the vanillin onto HZ816 resin, and vanillin synthesis was increased during the process. It has been demonstrated that a single microbe can successfully synthesis vanillic acid and vanillin, even though the amount produced is on the lower end of the spectrum.

#### C) Vanillin content

The quantity of vanillin that was synthesized in the fermentation broth was measured with HPLC. After centrifugation, the first layer, which is the culture supernatant, was collected for analysis. A standard vanillin measurement was also carried out. Vanillin elution time ranged from almost 7 min to 36 min [55].

Table 7. Yields of vanillin [ $\mu\text{g/ml}$ ]

Concentration of NaOH (M)	Yields of vanillin [ $\mu\text{g/ml}$ ]
1.0	1.47 $\pm$ 0.24
2.0	2.83 $\pm$ 0.44

The fermented sample peak of 1.0 M NaOH and 2.0 M NaOH concentration with retention times of 21.74 minutes and 20.74 minutes, respectively, corresponds to the peak in standard at 20.23 minutes. The amount of vanillin produced at a concentration of 1.0 M NaOH was 1.47 $\pm$ 0.24  $\mu\text{g/ml}$ , while at a concentration of 2.0 M NaOH was 2.83 $\pm$ 0.44  $\mu\text{g/ml}$ . Based on the statistics obtained by Minitab 19, there was a significant difference ( $p=0.009$ ) in the yields of bio vanillin from different concentrations of NaOH. This reveals that vanillin was successfully produced by the bioconversion activity of *Aspergillus niger*, which utilizes FA generated from pineapple peels as an inducer in the fermentation broth. Compared to other journals, the amount of vanillin converted by one fungal was 3.8  $\pm$  0.14 g/L of vanillin [35] and 44.4  $\mu\text{g/g}$  of vanillin [37], which are higher than this study. Some merged peaks were observed close to the retention time. This was most likely caused by structural homologues of vanillin or vanillic acid, the oxidized form of vanillin. This is because vanillin has an inherent propensity to rapidly oxidize into vanillic acid. Therefore, the synthesis of vanillin might be due to the interference with the accurate detection of vanillic acid by high-performance liquid chromatography (HPLC). As a

result, the value of the vanillin concentration that was measured in this investigation is lower than the values detected in other studies.

Table 8. The retention time of a standard mixture of phenolic compound

Standard No.	Type	Retention time (min)
1	Ferulic Acid	40.32
2	Vanillic Acid	15.57
3	Vanillin	20.23

#### 4) *Aspergillus niger* is the agent for microbial fermentation

*Aspergillus niger* is a form of brown-rot fungus that is capable of degrading hemicellulose and cellulose but has no effects on the lignin component in some natural sources [56]. As a result, it can be used as a substrate for the transformation of FA to vanillin. According to [53], *A. niger* K8 was selected as a more suitable fungus for converting FA to vanillic acid since it has the maximum potential to generate a high concentration of vanillic acid (116 mg/l) when compared to other fungi. Moreover, according to [7], vanillin can be generated by single or mixed microbes using several biotechnological methods. Other than that, the two-step conversion route involves the employment of fungi strains like *Pycnoporus cinnabarinus* MUCL39533 and *Aspergillus niger* I-1472 [34].

This metabolic route, in which FA is transformed to vanillic acid by *A. niger*, and subsequently *Pycnoporus cinnabarinus* allows for greater yields of vanillin. Previous research has demonstrated that fungal cells are effective microbiological sources for vanillin synthesis. For instance, *P. chrysosporium* ATCC 24725 produced the highest quantity of vanillin (44.8 mg/l) throughout the bioconversion process after 60 hours [53]. As reported by [37], the highest vanillin output is 52.5 g/g in the solid-state fermentation of *Phanerochaete chrysosporium*, employing green coconut husk as a substrate. *Aspergillus niger* I-1472 uses isoeugenol to generate 0.137 g/L vanillin [27]. As observed by [36], *Aspergillus niger* transforms 1055 mg/L of ferulic acid from pineapple peel extract to 5 mg/L vanillin. [54] discovered a two-step ferulic acid bioconversion method resulting in 767 g/L vanillin from maize bran. A wild strain of the fungus *P. cinnabarinus* utilises ferulic acid to create 126 mg/L vanillin [33]. Nonetheless, the synthesis of vanillin by a fungus has several drawbacks, including mycelium disintegration, increased viscosity of broth, infinite fragmentation, and unfavourable pellet disposition, which inhibits the entire process. However, [57] revealed that the two-step conversion route is inefficient since these two fungi can also convert vanillic acid to methoxy hydroquinone.

Meanwhile, in this study, *A. niger* was able to yield 2.83±0.44 µg/ml vanillin from 2.0 M NaOH concentration and 1.47±0.24 µg/ml from 1.0 M NaOH concentration at the end of the fermentation process. [36] demonstrated that vanillin yield from pineapple peel extracted by *Aspergillus niger* fermentation was 5 ± 1 mg, L<sup>-1</sup> which is considerably higher compared to this study. [35] reported the production of vanillin using banana peel as a substrate and *Enterobacter*

*hormaechei* as the microbe, managed to produce 3.8±0.14 g/L vanillin via submerged fermentation (SmF). This has indicated that a one-step conversion approach can still produce vanillin, despite its lower quantity. It was demonstrated that *A. niger* is capable of successfully converting lignin complex into vanillin on its own. Even though a clear FA metabolic pathway of *A. niger* is yet unknown, researchers had a strong belief that FA content from natural sources affected *A. niger*'s metabolic pathway during FA conversion to vanillin.

#### IV. CONCLUSION AND RECOMMENDATION

In this study, the biotransformation of ferulic acid from pineapple peel as a substrate to vanillin production through microbial fermentation method with *Aspergillus niger* was investigated. Since pineapple peel is easily accessible as a natural raw material with high phenolic compounds such as ferulic acid, it is chosen as a substrate in vanillin manufacturing. Instruments such as Ultraviolet-visible spectroscopy and High-Performance liquid chromatography, ferulic acid, vanillic acid, and vanillin compound were analysed. The vanillin contents determined from dried pineapple were 1.47±0.24 µg/ml from HPLC and 12.92 ± 0.54 µg/ml from UV-VIS of 1.0 M NaOH. Meanwhile, in 2.0 M NaOH concentration, about 2.83±0.44 µg/ml of vanillin content was analysed through HPLC and 15.38±0.77 µg/ml from UV-VIS. A statistical comparison of the quantitative determination of ferulic acid and vanillin showed that the HPLC method could identify, determine, and separate each compound in the supernatant and hydrolysate solution. Meanwhile, UV-Vis is only applicable for detection only. The HPLC technique allows a more accurate means of ferulic acid and vanillin content than the UV-Vis technique.

Besides, the fungal-based approach of vanillin production is gaining more consideration due to its low production cost, easy accessibility, simpler process, and metabolic versatility. Products obtained from the microbial and enzymatic bioconversion processes are rendered the GRAS status; thus, if ferulic acid, obtained from pineapple peel, is used as a substrate in the bioconversion process, catalyzed by *A. niger*, the products formed, namely vanillin and vanillic acid would also be natural. Hence, the biotechnological approach for vanillin production creates an opportunity to replace synthetic vanillin production and promotes the use of nature-identical vanillin produced by utilizing natural substrate at a low price. In the future, to produce a greater quantity of vanillin, the two-step bioconversion route can be utilized in with Amberlite XAD-4 resin during the purification process. Another drawback during vanillin synthesis process is cellular toxicity at higher vanillin yield.

#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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