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FERMENTATIVE PRODUCTION OF BIOVANILLIN FROM FRUIT WASTES USING *Aspergillus niger*

Mrudula N. Iyer[1], Isha H. Patel[2], Sanjay N. Parekh[3]
[1,2,3]*Microbiology department, Shree Ramkrishna Institute of Computer Education & Applied Sciences, Sarvajanik University, Surat (India)*

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ABSTRACT

The growing demand for natural and sustainable flavoring agents has intensified interest in biotechnological routes for vanillin production. This study explores the bioconversion of fruit waste-derived ferulic acid into vanillin using Aspergillus niger, a GRAS (Generally Recognized As Safe) fungal strain. Various fruit wastes – including banana, beetroot, pineapple, and orange peels were used to extract ferulic acid. The extracted ferulic acid was subjected to microbial fermentation using A. niger under optimized conditions of pH, temperature, inoculum size, and incubation time to maximize vanillin yield. Vanillin production was qualitatively confirmed by FTIR and quantitatively assessed through UV-Vis spectrophotometry and HPLC analysis Among the substrates tested, banana peel yielded the highest vanillin concentration.The study highlights a scalable and sustainable approach to value-added bioproduct generation from food waste, contributing to circular bioeconomy efforts.

INTRODUCTION

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a highly valued aromatic compound extensively used as a flavouring agent in foods, beverages, and confectionery products (approximately 60%), as well as in perfumes, cosmetics (33%), and pharmaceuticals (7%) (Lomascolo et al., 1999; Muheim and Lerch, 1999). Traditionally, natural vanillin is extracted from the cured beans of the tropical orchid *Vanilla planifolia*, and to a lesser extent from *Vanilla tahitiensis* and *Vanilla pompona*. However, the extraction process is labor-intensive, time-consuming, and yields low quantities of vanillin, contributing to its high cost—ranging between $1,200 to $4,000 per kilogram—compared to synthetic vanillin, which costs less than $15 per kilogram (Rosazza et al., 1995). As a result, natural vanillin accounts for less than 1% of global production.

Biotechnological production of vanillin, particularly through microbial biotransformation, offers a viable alternative to traditional methods. This approach utilizes microorganisms such as bacteria, fungi, and yeast to convert phenylpropanoid precursors—including ferulic acid, eugenol, and isoeugenol—into vanillin through enzymatic pathways (Rana et al., 2013). Among these microorganisms, *Aspergillus niger* has emerged as a promising candidate due to its metabolic versatility, extracellular enzyme production, and ability to convert ferulic acid into vanillin (Banerjee and Chattopadhyay, 2019). Notably, Tang and Hassan (2020) demonstrated that *A. niger* could convert 1055 mg/L of ferulic acid derived from pineapple peel extract into 5 mg/L of vanillin, highlighting its potential in valorizing fruit waste into high-value compounds.

MATERIALS AND METHODOLOGY

1. FRUIT WASTE COLLECTION & CHEMICAL PRETREATMENT

Fruit waste was collected from both domestic and commercial sources, primarily focusing on banana peels, beetroot peels and pulp, orange peels, and pineapple peels. The collected fruit wastes were then placed onto a metal tray covered with aluminium foil before undergoing the process of oven drying at an temperature of 105 ˚C for 12 – 14 hours (Saeed S. *et al.,* 2021; Tang and Hassan, 2020). This drying process was performed until the remaining moisture content ranged between 3 – 5%. (Rifaiea *et al.,* 2023) Before the pretreatment, the dried fruit wastes were ground into powdered state using an electrical grinder that acts as a source of ferulic acid. In the pretreatment stage, with a ratio of 1:30, the hydrolysis process was carried out by introducing 5 gm. of the ground form of dried fruit wastes to 150 ml aqueous solutions of sodium hydroxide at two different concentrations; 1.0 M and 2.0 M. The fruit waste 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℃ for one hour in an autoclave (Tang and Hassan, 2020; Saeed S. *et al.,* 2021, Mehmood T *et al.,* 2022). 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 (Tang and Hassan, 2020). 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

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a neutral state of pH 5 – 6 using calcium carbonate. (Rifaiea *et al.,* 2023)

2. EXTRACTION OF FERULIC ACID

In 15 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). 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. (Rejani, C. T. and Radhakrishnan, S. 2022., Saeed S. *et al.,* 2021; Tang and Hassan, 2020)

3. FERMENTATIVE PRODUCTION OF VANILLIN USING VARIOUS SUBSTRATE

3.1 FUNGAL CULTURE

The fungal culture of *Aspergillus niger* were obtained from the Microbiology Laboratory, Shree Ramkrishhna Institute of Computer Education and Applied Sciences (Surat). The culture was grown on Czapek Dox agar at 30℃. The spore suspension (10^6– 10^7 cfu/mL) of *Aspergillus niger* were prepared in sterilised water containing Tween 80. 1 mL of this mixture of spore suspension was inoculated into the basal medium.

3.2 FERMENTATIVE PRODUCTION OF VANILLIN

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 of different fruit waste containing ferulic acid 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 (Small Feeding Volume) samples and 20 mL of medium was removed for LFV (Large Feeding Volume) samples.

After the fermentation period ends, the fermented samples were filtered to separate the cell pellets from the fermentation medium. Vanillin content was qualitatively assessed by FTIR analysis and quantified using HPLC analysis and UV-VIS spectrophotometer. (Rifaiea *et al.,* 2023)

4. VANILLIN ASSAY

4.1 PREPARATION OF SAMPLES

A standard stock solution of 1mg mL^{-1} vanillin was diluted to 500µg mL^{-1}, 400µg mL^{-1}, 300µg mL^{-1}, 200µg mL^{-1},100µg mL^{-1}, 75µg mL^{-1}, 50µg mL^{-1}, 25µg mL^{-1} 1µg mL^{-1}. The samples were prepared as follows. One millilitre of sample was added into a 15 mL centrifuge tube and dissolved with 3.0 mL of Tris buffer. Acetonitrile (7 mL) then was added into this sample solution and mixed by vigorously vortexed for 1 min. The mixture was centrifuged at 10,000 rpm at room temperature for 10 min (Ma J *et al*., 2014). The supernatant was transferred to another 15 mL centrifuge tube, and 2 mL of n-hexane was added to the supernatant. The mixture was vortexed for 1 min and then centrifuged at 10,000 rpm at room temperature for 5 min. The supernatant was taken and filtered through a 0.22µm nylon membrane and stored at 4°C in the dark. (Jin Zhao *et al.,* 2018)

4.2 ASSAY

1 mL of vanillin solution and 200µL of O-toluidine were added to a 1.5 mL Eppendorf tube, and the mixture was shaken for 5 min at room temperature. Acetic acid solution (200µL) was then added to the mixture, and the tube was placed in a boiling water bath for 15 min at 100°C. After the tube cooled to room temperature, the absorbance was measured at 363nm by a UV spectrophotometer. (Jin Zhao *et al.,* 2018)

5. OPTIMIZATION OF FERMENTATION PARAMETERS FOR BIOVANILLIN PRODUCTION

5.1 EFECT OF INCUBATION TIME

For the optimization of fermentation period in the fermentative production of vanillin, the fermentation process was carried out for varying durations: 18, 24, 42, 48, 66, and 72 hours. A fixed volume of inoculum was added to the fermentation medium under standardized conditions, and samples were withdrawn at each specified time point. Vanillin concentration was then measured to evaluate the effect of fermentation duration on product yield. (Mehmood T *et al.,* 2022)

5.2 EFFECT OF INOCULUM SIZE

For the optimization of inoculum size in the fermentative production of vanillin, varying volumes of inoculum ranging from 1 mL to 6 mL were introduced into identical fermentation media and vanillin production was measured at the end of the incubation period. (Mehmood T *et al.,* 2022)

5.3 EFEECT OF pH

For the optimization of pH in the fermentative production of vanillin, experiments were conducted using a range of pH values: 5.5, 6.5, 7.0, 9.5, 10.5, and 11.0. The fermentation medium was adjusted to the desired pH using either 1N HCl or 1N NaOH prior to sterilization. (Mehmood T *et al.,* 2022).

5.4 EFFECT OF TEMPERATURE

For the optimization of incubation temperature in the fermentative production of vanillin, experiments were conducted at 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. The cultures were monitored for vanillin production at the end of the incubation period (Mehmood T *et al.,* 2022).

RESULT

1.1 EXTRACTION OF FERULIC ACID FROM FRUIT WASTE

Fruit wastes (banana peels, beetroot, pineapple peels, and orange peels) were used as potential substrates due to their richness in phenolic compounds, particularly ferulic acid for the production of vanillin (FIGURE 1). These fruit wastes served

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as raw materials for the production of hydrolysates used in both small and large feeding mode fermentations.


Banana Peel


Beet Peel


Orange Peel


Pineapple Peel

FIGURE 1. FRUIT WASTE SAMPLE

The drying process was carried out in a hot-air oven at a consistent temperature of 105°C to ensure the retention of bioactive compounds. The dried peels were then ground into a fine powder using an electric grinder.

The chemical pretreatment using sodium hydroxide was conducted on 5 grams of dried fruit peel powder mixed in 150 mL of NaOH solution at two different concentrations—1.0 M and 2.0 M—yielding a solid-to-liquid ratio of 1:30. The hydrolysis was carried out at 120 °C for 1 hour in an autoclave. This step promoted the degradation of lignin and other phenolic-bound structures, leading to the release of ferulic acid and other soluble phenolics.

The reaction mixture formed a dark-coloured liquor, commonly referred to as black liquor, indicating the presence of dissolved lignin, phenolic compounds, and other degradation products. The visible swelling of fruit residues during hydrolysis confirmed the effective disruption of the lignocellulosic matrix due to NaOH treatment. Following hydrolysis, all mixtures were filtered using a colander lined with 400-mesh nylon cloth to separate the solid residues from the black liquor. The filtrates were then acidified to pH 2 using 10% HCl. Upon acidification, precipitation of soluble lignin fractions was observed. Centrifugation at 3000 rpm for 10 minutes allowed successful recovery of these lignin-rich precipitates. The supernatant was then adjusted to pH 5–6 using calcium carbonate to stabilize the phenolic compounds in preparation for further analysis.

Equal volumes of ethyl acetate and hydrolysate (7 mL each) were combined in 15 mL falcon tubes, vigorously shaken, and centrifuged at 3000 rpm for 10 minutes. The ethyl acetate layer (containing extracted ferulic acid) was carefully recovered in flasks for quantification and potential downstream conversion to vanillin.

1.6 FERMENTATIVE PRODUCTION OF VANILLIN

Fermentation was carried out using *Aspergillus niger* cultures in 250 mL Erlenmeyer flasks under controlled conditions in a shaking incubator at 30 °C and 110 rpm agitation. In the SFV mode, 1 mL of the neutralized hydrolysate solution (containing ferulic acid extracted from fruit waste) was introduced daily from day 3 to day 6, followed by termination of the fermentation on day 7. To maintain constant volume and reduce medium saturation, 4 mL of culture medium was removed before feeding. In contrast, the LFV mode involved the addition of 10 mL hydrolysate on day 3 and day 4, with 20 mL of medium removed prior to the addition. Fermentation in this case was terminated earlier, on day 5.

At the end of the fermentation periods, all cultures were filtered to separate the fungal biomass (cell pellets) from the fermentation broth. The fermentation supernatants were analysed to detect and quantify the production of vanillin, the targeted biotransformation product. (FIGURE 2 & 3).

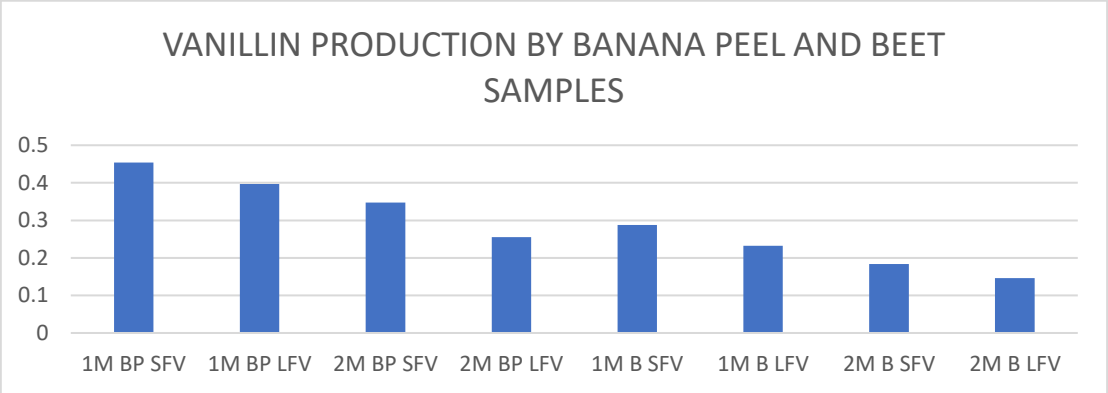


FIGURE 2. Vanillin production by banana peel and beet samples (BP = BANANA PEELS, B= BEET PEELS/PULP, OR= ORANGE PEELS, PP= PINEAPPLE PEELS, SFV= SMALL FEEDING VOLUME, LFV= LARGE FEEDING VOLUME)

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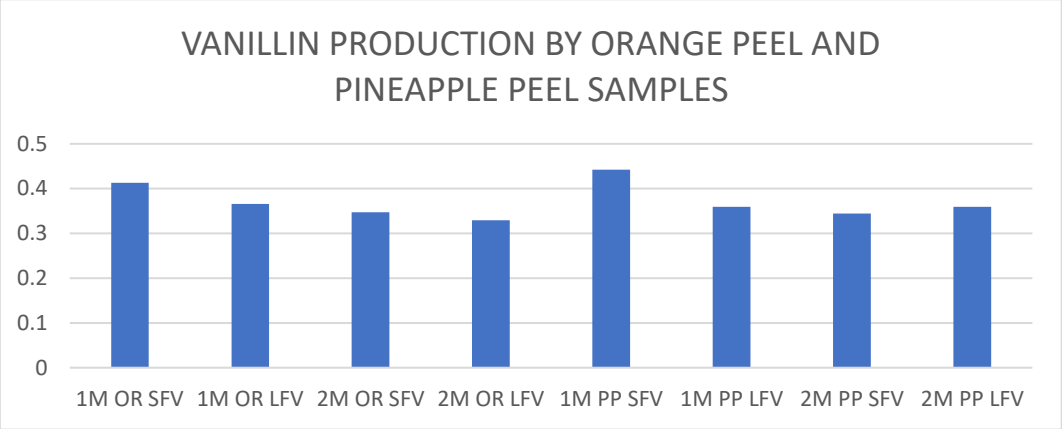


FIGURE 3. Vanillin production by orange peel and pineapple peel samples

These findings demonstrate that both feeding strategies successfully supported the microbial bioconversion of ferulic acid into vanillin by *Aspergillus niger*, although variations in feeding volume and duration had notable effects on product yield, which are discussed in subsequent sections.

1.7 OPTIMIZATION OF FERMENTATION PARAMETERS

1.7.1 EFFECT OF INCUBATION TIME

The vanillin yield was found to be highly dependent on the duration of fermentation. Among the various time intervals tested (18, 24, 42, 48, 66, and 72 hours), the highest vanillin concentration was observed at 48 hours, indicating the most efficient period for microbial conversion of ferulic acid into vanillin. (FIGURE 4).

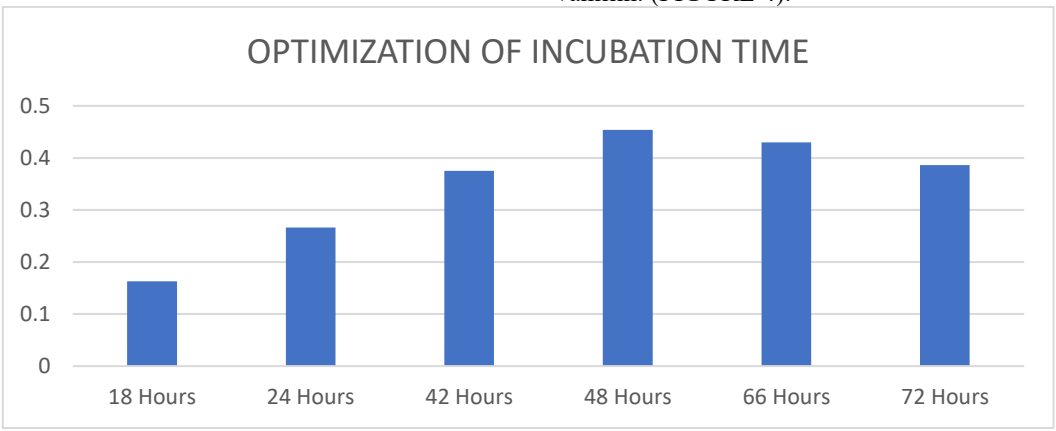


FIGURE 4. Effect of incubation time

1.7.2 EFFECT OF INOCULUM SIZE

Different inoculum volumes (1 mL to 6 mL) were evaluated to determine the optimal microbial load for maximum bioconversion efficiency. The results showed that 4 mL of inoculum produced the highest vanillin yield. Lower inoculum sizes may not provide sufficient enzymatic activity, while higher volumes may result in nutrient competition and accumulation of metabolic by-products, reducing vanillin synthesis. Thus, a moderate inoculum size ensures balanced growth and efficient ferulic acid metabolism (FIGURE 5).

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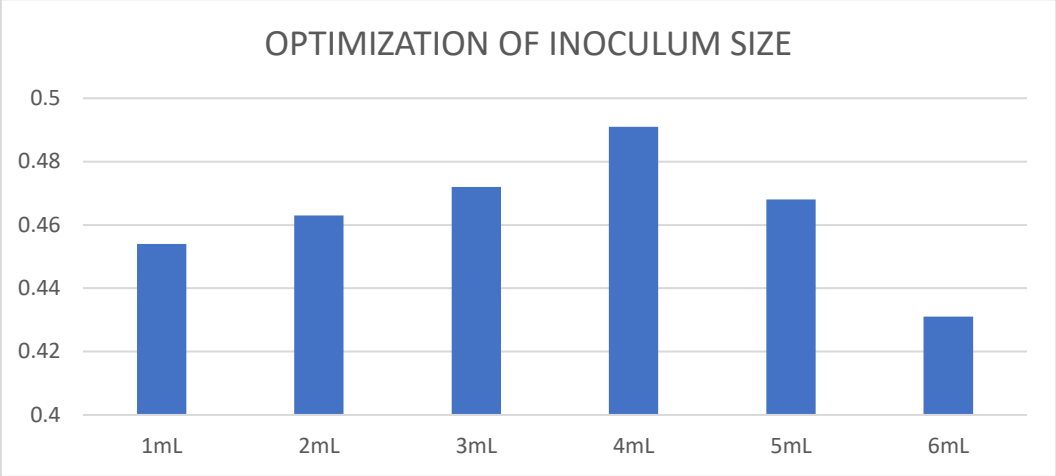


FIGURE 5. Optimization of inoculum size

1.7.3 EFFECT OF pH

The influence of initial pH on vanillin production was examined using a range from pH 5.5 to 11.0. The optimal pH for vanillin production was found to be 6.5, which supports maximum microbial activity and enzyme stability. At extreme pH values, a noticeable reduction in vanillin content was observed, likely due to enzyme denaturation or inhibition of fungal growth. (FIGURE 6).

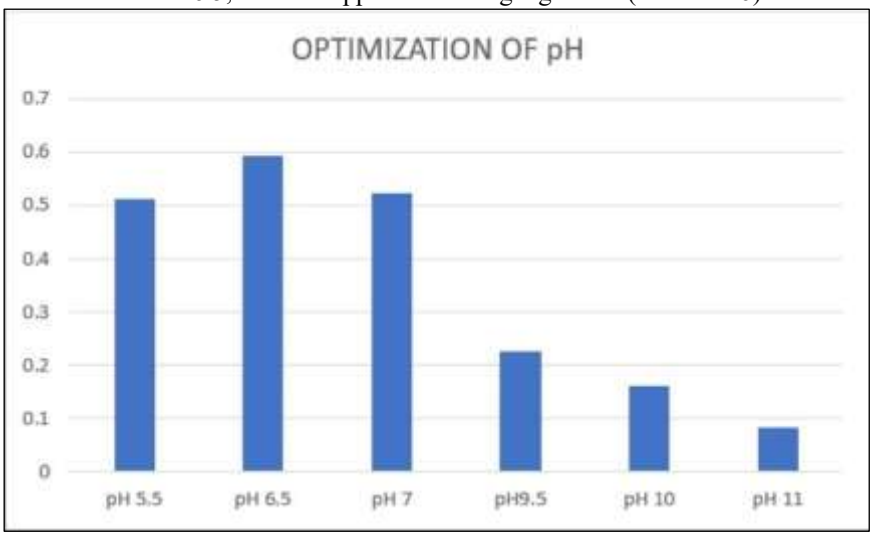


FIGURE 6. Effect of pH

1.7.4 EFFECT OF TEMPERATURE

Among the tested temperatures, 30°C yielded the highest concentration of vanillin. This is consistent with the optimal growth conditions of *Aspergillus niger*, which thrives and exhibits peak enzymatic activity around this temperature. At higher temperatures (above 40°C), vanillin production declined sharply, likely due to thermal stress on the fungal cells and enzyme inactivation (FIGURE 7).

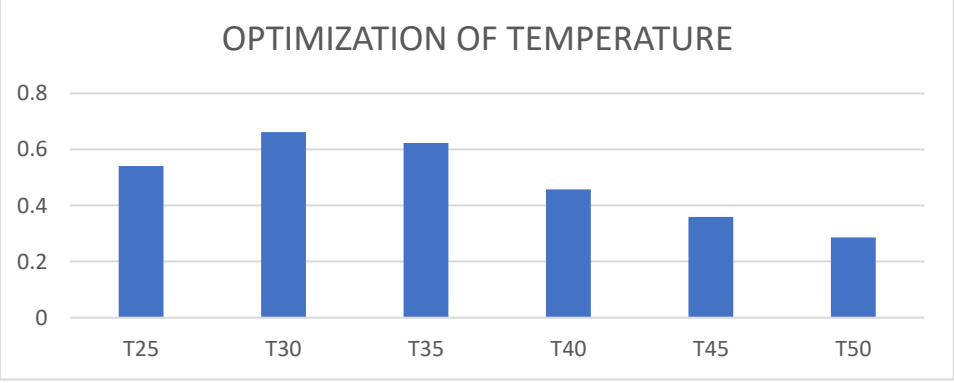


FIGURE 7. Effect of Temperature

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1.8 QUALITATIVE ANALYSIS OF VANILIN

1.8.1 FTIR ANALYSIS

The FTIR spectrum (FIGURE 8) shows several prominent peaks that are in good agreement with the known molecular structure of vanillin (4-hydroxy-3-methoxybenzaldehyde).

The broad absorption band observed at approximately 3400 cm^{-1} corresponds to O–H stretching vibrations typical of phenolic compounds. The peak around 1690 cm^{-1} is indicative of the carbonyl (C=O) group stretching, confirming the aldehyde functionality of vanillin. Additional peaks between 1500–1600 cm^{-1} are associated with aromatic C=C stretching vibrations. The fingerprint region (below 1500 cm^{-1}) further shows distinct bands between 1260–1020 cm^{-1}, which are attributed to the C–O stretching vibrations from both the phenolic –OH and ether $–OCH_3$ groups. The absence of unexpected or additional peaks suggests that the sample was relatively pure, and no major impurities or degradation products were present.

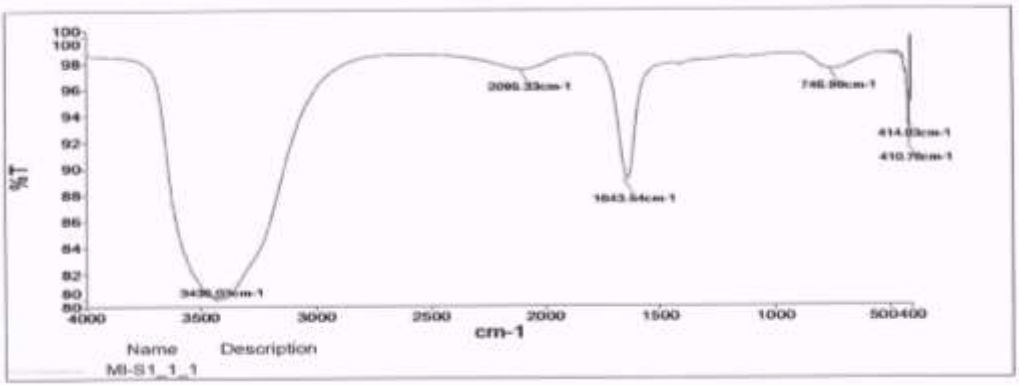


FIGURE 8. FTIR analysis

The FTIR results are consistent with the expected structure of vanillin and confirm the presence of key functional groups such as hydroxyl, aldehyde, methoxy, and aromatic moieties. These findings validate the identity and chemical integrity of the synthesized or extracted vanillin sample.

1.8.2 HPLC ANALYSIS

The vanillin was separated on a reverse – phase C18 Column using methanol-acidified water (10 + 90) and were detected at 280 nm. The flow rate of HPLC is approximately 4 mL/min. Preparation of Standard Solutions. Standard solutions containing 0.5, 1.0, 2.0, 3.0 and 4.0 ppm of each standard were prepared in 99.9 % ethanol for HPLC. In the standard chromatogram, a prominent peak was observed at a retention time (Rt) of 2.194 minutes, with an area of 1078.98 mAU·s, comprising 96.39% of the total chromatographic area. This confirms the purity of the standard and establishes the retention time as a reference for identifying the target compound in the sample.

The sample chromatogram (FIGURE 9) showed a major peak at Rt of 2.247 minutes, closely matching the standard's retention time. This peak accounted for 90.91% of the total chromatographic area, with an area of 4481.09 mAU·s, indicating a high concentration of the target compound in the sample. The slight shift in retention time (0.053 min) is within acceptable experimental variation and suggests that the compound in the sample is indeed the same as that in the standard.

In addition to the major peak, the sample chromatogram exhibited several minor peaks at retention times of 1.950, 2.696, 2.829, 3.282, 3.995, and 4.429 minutes. These peaks likely represent by-products, residual substrate, or intermediate compounds formed during the bioconversion process. Their presence indicates that while the conversion was largely successful, it was not entirely selective or complete. The estimated concentration of the target compound in the sample is approximately 415.32 µg/mL.

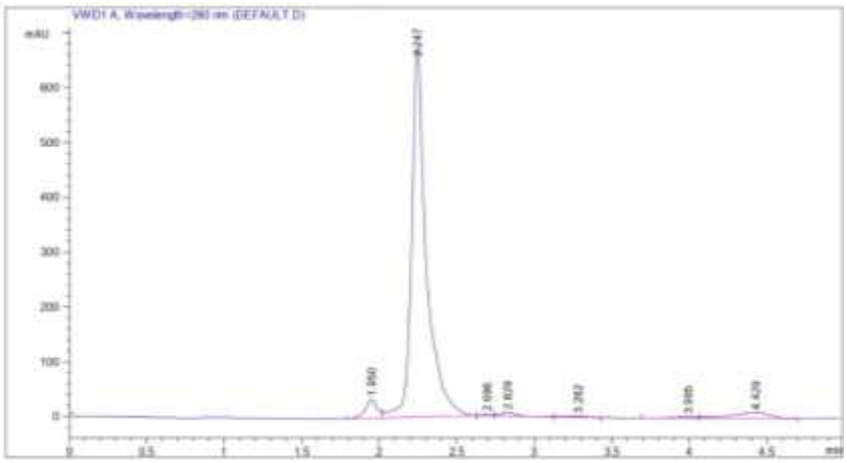


FIGURE 9. HPLC result

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CONCLUSION

This study successfully demonstrated the potential of fruit waste as a substrate for the fermentative bioproduction of vanillin using *Aspergillus niger*. Among the various fruit peels tested (banana, beetroot, pineapple, and orange) banana peels treated with 1M NaOH and subjected to small feeding volume (SFV) fermentation yielded the highest vanillin concentration.

The study also highlighted the critical importance of optimizing fermentation parameters. Maximum vanillin yield was achieved at an incubation time of 48 hours, pH 6.5, a temperature of 30 °C, and an inoculum size of 4 mL. Analytical techniques such as FTIR, UV-Vis spectrophotometry [And HPLC] confirmed the successful synthesis and purity of vanillin, aligning with established structural markers.

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