International Journal of Advanced Biochemistry Research 2025; 9(4): 836-840

International Journal of Advanced Biochemistry Research

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating: 5.29
IJABR 2025; 9(4): 836-840
www.biochemjournal.com
Received: 09-02-2025
Accepted: 12-03-2025

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# Microbial conversion of vanillin for enhanced antimicrobial activity: A comprehensive study

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**DOI:** https://www.doi.org/10.33545/26174693.2025.v9.i4j.4197

**Abstract**
The research investigates the microbial conversion of vanillin to create new products with improved activity for developing an antimicrobial agent using various fungal species. Twenty-two fungal cultures were assessed for their ability to transform vanillin, revealing that *Paecilomyces variotii* and *Debaryomyces* species can convert vanillin into two metabolites. The chemical structures of the metabolites were identified through Nuclear Magnetic Resonance (NMR) spectroscopy, with metabolite 1 identified as vanillic acid and metabolite 2 as vanillyl alcohol. The antimicrobial efficacy of the metabolites was tested against bacterial and fungal pathogens, showing significant antibacterial activity against *Staphylococcus* (inhibition zone of 22.67 mm) and antifungal activity against *Alternaria alternata* (zone of 20.00 mm). A combination of the two metabolites was administered systemically to infected Silkworm larvae, resulting in increased 15% pupal survival compared to untreated. The converted compounds displayed enhanced activity compared to the original compounds, indicating that vanillin derivatives could serve as potential broad-spectrum antimicrobial agents for managing pathogens.

**Keywords:** Vanillin, fungal species, bioconversion, metabolites, antimicrobial agents

## Introduction

Vanillin, a phenolic compound derived from *Vanilla planifolia*, plays a vital role in natural flavors and is extensively employed in the food, cosmetics, pharmaceutical, and medical sectors. It serves as a flavor enhancer and food preservative owing to its antimicrobial and antioxidant characteristics. Although traditionally obtained from beans, synthetic production is prevalent due to cost efficiency. Synthetic vanillin is also used in the manufacturing of herbicides, antifoaming agents, household items, deodorants, air fresheners, and cleaning supplies. Nevertheless, its potent scent at high concentrations restricts its possible uses.

Vanillin and its related aromatic compounds have been shown to have inhibitory effects on the growth of fungi and bacteria (Adeboye *et al.*, 2014) [1]. While a small amount of vanillin is naturally sourced, the majority is produced through chemical synthesis (Priefert *et al.*, 2001) [9]. Current research is focused on developing new methods for producing natural vanillin through biosynthesis using microorganisms. Efforts are being made to find efficient ways to convert various precursors into vanillin through microbial biotransformation. Microbial transformation involves the specific modification or chemical alteration of a compound using biological catalysts such as fungi, bacteria, dead microorganisms, cellular extracts, and isolated enzymes (Bains, 1993; Lilly, 1994) [3, 5].

The resulting compounds may have enhanced properties compared to the original compound or may be identical to its metabolites. Therefore, microbial transformation can be used as a substitute for chemical synthesis to produce drug metabolites, resulting in the discovery of new products with distinct activities. In recent years, there has been a significant increase in consumer demand for natural ingredients. As a result, alternative natural sources of ingredients have been explored, and production methods involving microbial biotransformation of natural precursors have become more appealing. This approach is essential for the sustainable use of natural resources to develop commercially valuable products (Alvarado *et al.*, 2003; Mishra *et al.*, 2016) [2, 6].

The breakdown of vanillin and its derivatives by microorganisms has been extensively researched, as vanillin can be toxic to many microorganisms even at low levels.

Therefore, the ability to break down vanillin is crucial for microorganisms in natural settings or industrial processes where vanillin is present. Vanillin and ethyl vanillin have antibacterial properties due to their phenolic groups. Ongoing efforts are being made to enhance their antimicrobial effects, as bacteria are highly vulnerable. A combination of vanillin and cinnamon has been found to have a synergistic impact on antibacterial activity against *E. coli* and antifungal activity against *L. monocytogenes* (Roda *et al.*, 2012) [11]. The oxidation of vanillin to produce its carboxylic acid has been carried out to enhance its antibacterial properties (Mourtzinos *et al.*, 2009) [8]. A vanillin-derived piperidin-4-one oxime ester has been synthesized and shown to have superior antibacterial activity compared to the positive control (Harini *et al.*, 2012) [4]. Acetyl vanillin derivatives have also been synthesized and displayed antibacterial properties (Yadav *et al.*, 2018) [14]. This research investigates the transformation of vanillin using fungal species to improve its effectiveness and evaluate its antimicrobial properties against silkworm pathogens.

## Materials and Methods

### Chemicals and fungal strains

Culture media, solvents, and 4-hydroxy-3-methoxybenzaldehyde (vanillin) were obtained from HiMedia Laboratories (Mumbai, India). Thin-layer chromatography precoated plates (Silica gel, Merck, PF254) were utilized. Fungal cultures *Debaromyces* species (MCC 1072) and *Paecilomyces* variotii (NCIM 1217) were acquired from the culture collection centers NCCS and NCL in Pune, India, respectively. These cultures were sub cultured on Potato Dextrose Agar and allowed to incubation for 5 days, with the marginal area culture being chosen for the conversion process.

### Conversion and product extraction

The conversion experiments were carried out in 100 ml of Potato Dextrose Broth with fungal cultures. Vanillin was added to the established culture medium at a concentration of 20 mg/ml. The culture control consisted of only fungal culture, while the substrate control contained only the parent compound. The reaction was conducted at 120 rpm at 26±1ºC in a shaking incubator for seven days. Product formation was monitored starting from the second day. Products were extracted by adding solvents to the culture medium. The fungal mycelium and culture medium were separated by filtration using Whatman No. 3 filter paper. The filtered reaction mixture was extracted three times with an equal volume of ethyl acetate. The combined organic layer extract was dried with anhydrous $Na_2SO_4$ and evaporated under reduced vacuum pressure in a rotary evaporator to obtain the crude extract.

### Analytical methods

The converted products were detected through Thin Layer Chromatography (TLC) (Merck, Germany) and compared with the original compound. The extracts were applied to TLC silica plates and eluted with a mixture of ethyl acetate and hexane in an 8:2 ratio. The products were visualized using iodine vapor. The crude extract of the products was then further purified using column chromatography with silica gel (60-100 mesh size). The metabolites were confirmed through $^1$H NMR analysis using Bruker Biospin (IISc, Bangalore, India). The NMR spectra were analyzed using MestreNova 12.0.0 software.

### Antimicrobial activity assays

A bacterial pathogen ($1 \times 10^9$ cells/ml) was applied to a Muller Hinton agar plate, while a fungal pathogen ($1 \times 10^7$ conidia/ml) was applied to a potato dextrose agar plate. Discs with a diameter of 6 mm, soaked in varying concentrations of the converted product solution, were placed on the inoculated plates and then incubated at 32±1 °C for 24 hours. The fungal plates were incubated at 25±1 °C for 5 days. The presence of a zone around the disc was measured to assess antibacterial properties.

### Systemic activity of the converted product

Various ratios of a mixture of derived product and mulberry leaf juice were combined and tested *in vivo* against pathogens. Third instar first-day silkworm larvae were orally administered one milliliter of silkworm pathogens at a concentration of $1 \times 10^9$ cells/ml (*Bacillus, Streptococcus, Serratia, and Staphylococcus)*, and rearing continued until infection onset. In the control group, live pathogens were administered separately for comparison. Three replicates were maintained, with 200 larvae in each treatment group. Infected larvae were fed mulberry leaves sprayed with 0.5% and 1% concentration of the blend for three days, and observations were made on disease progression and mortality.

### Statistical analysis

Statistical analysis of the data was carried out using standard procedures and reported as the mean±standard deviation. Post-hoc analysis was performed using Duncan's test, with a significance level of $p<0.05$.

## Results

### Screening of fungal strains and conversion of vanillin

A total of twenty-two fungal cultures were tested with the vanillin compound to investigate microbial conversion for novel products. *Paecilomyces variotii* and *Debaromyces* species were found to be capable of converting vanillin (Fig.1) and producing two distinct new metabolites. *Paecilomyces variotii* transformed vanillin into metabolites 1 ($R_f$ 0.78) and metabolite 2 ($R_f$ 0.92), while *Debaromyces* sp. specifically converted metabolite 1 ($R_f$ 0.78) from the parent compound (Rf 0.82) (Fig.2). Metabolite 1 was produced on the 3rd day, and metabolite 2 on the 5th day of the reaction. The parent compound was completely consumed on 7th day, leaving only the newly formed products.

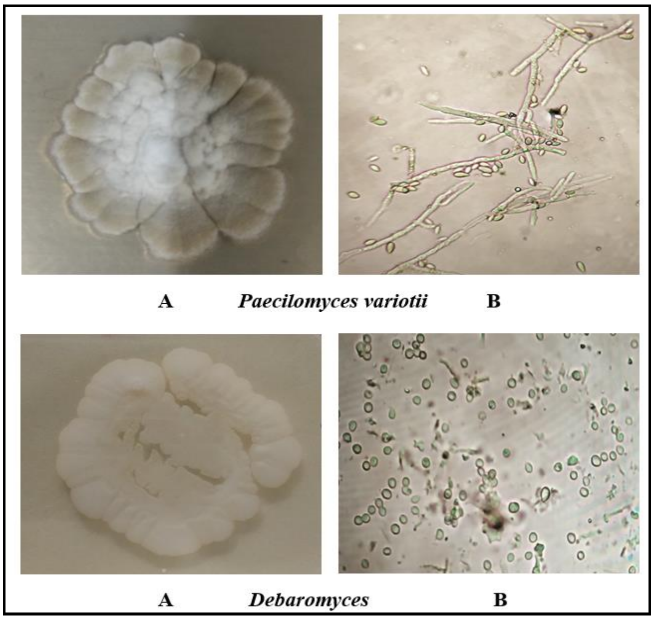



**Fig 1:** Culture (A) and microscopic view (B) of fungal growth used for conversion process.

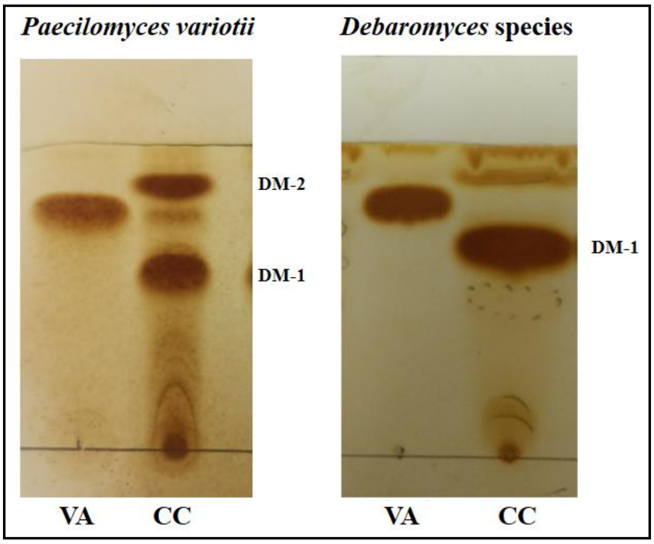
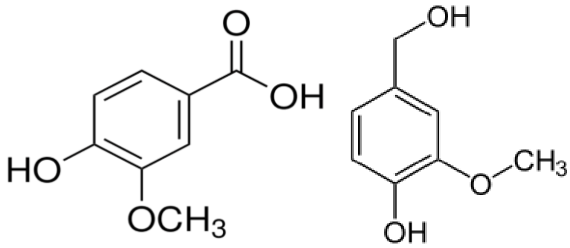



**Fig 2:** Metabolites derived from vanillin through different fungal species identified through Thin Layer Chromatography

VA-Vanillin CC-Converted Compounds DM-Derived Metabolite. The $R_f$ value of derived metabolite is calculated as the distance traveled by the compound divided by the distance traveled by the solvent front.

### Structural elucidation of products

The parent compound and its metabolites were purified and analyzed using NMR spectroscopy. The $^1$H-NMR spectra were recorded on a 400 MHz Bruker biospin spectrometer. The chemical shifts (δ values) are reported in ppm, and coupling constants are in Hertz. The metabolite signals observed in the $^1$H-NMR spectra of isolated metabolites displayed characteristic peaks. Differences in certain chemical shift regions were attributed to the microorganisms involved in the conversion process. Metabolite 1 identified as vanillic acid $^1$H NMR (400 MHz) showed peaks at δ 12.51 (broad singlet, 1H), 9.86 (broad peak, 1H), 7.54-7.34 (multiplet, 2H), 6.84 (doublet, J = 8.4 Hz, 1H), 3.84 (singlet, 3H). Metabolite 2 identified as vanillyl alcohol using $^1$H NMR displayed peaks at δ 3.90 (singlet, 3H, OCH3), 4.62 (singlet, 2H, CH2OH), 6.83-6.94 (multiplet, 3H, H-2, H-5, H-6) (Fig.3). The resulting compound exhibited more signals with higher intensities.




1. Vanillic Acid 2. Vanillyl Alcohol

**Fig 3:** Structure of derived compounds converted from vanillin

### Antimicrobial activity of converted metabolites

The antibacterial activity of the compounds was tested using *in vitro* well diffusion method against both Gram-positive and Gram-negative bacteria. The diameter of the inhibition zone around the disc in Metabolite No.1 demonstrated the highest antibacterial activity against *Staphylococcus* sp. with a 22.67 mm, while the control vanillin had an activity of 12.5 mm. The minimum inhibitory concentration of the metabolites effectively inhibited the growth of all pathogens at 1%, indicating the presence of bioactive compounds in the extract. Furthermore, metabolite No.1 exhibited highest antifungal activity against *Alternaria alternata* with a 20.0 mm inhibition zone compared to the control 15.0 mm (Table 1&2).

### *In vivo* efficacy studies using Silkworm larvae

The efficacy of the metabolites was assessed through *in vivo* experiments on PMxCSR2 Silkworm breeds. A blend of modified compounds mixed in mulberry leaf juice was administered to fresh Mulberry leaves at varying concentrations (0.5 and 1%), which were readily consumed by silkworms in infected groups. Pupal survival rates increased in treated groups exposed to *Bacillus* by 15% and followed by *Staphylococcus* 13% in PMxCSR2 breeds at a 1% concentration compared to the pathogen inoculated control group. There was significant enhancement in survival rates observed against pathogens except *Serratia* species (Table 3).

**Table 1:** Antibacterial activity of converted metabolites tested against bacterial pathogens

| Compounds | *Bacillus* sp. | *Serratia* sp. | *Staphylococcus* sp. | *Streptococcus* sp. |
|---|---|---|---|---|
| | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| DM-1 | 21.66±0.57$^{a}$ | 7.24±0.0$^{b}$ | 22.67±0.58$^{a}$ | 15.33±1.15$^{a}$ |
| DM-2 | 17.00±.00$^{b}$ | 0.00$^{c}$ | 22.33±0.50$^{a}$ | 13.66±1.52$^{a}$ |
| Vanillin | 10.00±0.0$^{c}$ | 0.00$^{c}$ | 12.5±1.52$^{b}$ | 8.66±0.57$^{a}$ |

**Table 2:** Antifungal activity of converted metabolites treated against fungal pathogens

| Compounds | *Beauveria bassiana* | *Aspergillus niger* | *Alternaria alternata* | *Fusarium oxysporum* |
|---|---|---|---|---|
| | **Mean±SD** | **Mean±SD** | **Mean±SD** | **Mean±SD** |
| DM-1 | 14.66±1.15[a] | 14±0.58[a] | 20.00±1.00[a] | 15.22±0.57[a] |
| DM-2 | 0.00[c] | 10±1.5[a] | 0.00[c] | 0.00[c] |
| Vanillin | 7.66±0.58[b] | 12±0.0[a] | 15.0±0.83[b] | 8.4±1.15[b] |

DM-Metabolites derived from Vanillin. Values are represented as Mean±SD of Zone of inhibition (mm), n = 3 per treatment. Superscripts *abc* indicate multiple comparisons and common letter not significantly different ($p$<0.05). 'a' represents the maximum value and 'c' represents the minimum value.

**Table 3:** Effectiveness of derived products tested in (PMxCSR2) silkworm and pupal survival

| Treatment | *Bacillus sp.* | *Serratia sp.* | *Staphylococcus sp.* | *Streptococcus sp.* |
|---|---|---|---|---|
| | **Mean±SD** | **Mean±SD** | **Mean±SD** | **Mean±SD** |
| 0.5% blend | 61.50±2.60[b] | 71.40±3.5[a] | 62.00±3.61[ab] | 56.12±1.53[b] |
| 1% blend | 68.10±1.61[a] | 75.50±2.45[a] | 65.00±4.00[a] | 61.67±3.06[ab] |
| Control (Inoculated) | 53.00±1.15[c] | 73.20±4.90[a] | 52.00±3.61[c] | 56.50±1.15[b] |

Values are represented as a percentage of the Mean±SD of 3 replications. 0.5% and 1% are blends of different concentrations. Superscripts *abc* indicate multiple comparisons, where common letter indicates no significant difference ($p$<0.05). 'a' represents the maximum value and 'c' represents the minimum value.

## Discussion

The study demonstrates the biotransformation of vanillin by *Paecilomyces variotii* and *Debaromyces* fungal strains, resulting in the formation of two major metabolites with enhanced antimicrobial activity. The identified metabolites, vanillic acid and vanillyl alcohol, represent the oxidation and reduction products of vanillin, respectively. These biotransformation reactions are consistent with the known metabolic pathways of aromatic aldehydes in microorganisms, where aldehydes can be either oxidized to carboxylic acids by aldehyde dehydrogenases or reduced to alcohols by aldehyde reductases (Ziadlou *et al*., 2020; Moon and Liu, 2015) [15, 7]. The ability of *P. variotii* to efficiently transform vanillin may be attributed to their robust enzymatic systems for metabolizing aromatic compounds (Shashwati *et al*., 2006) [12]. These fungi are known to inhabit diverse ecological niches, including plant materials rich in phenolic compounds, and have evolved efficient detoxification mechanisms for such compounds (Mishra *et al*., 2016) [6]. The differential production of vanillic acid and vanillyl alcohol by various fungal strains likely reflects the relative activities of aldehyde dehydrogenase and aldehyde reductase enzymes in these organisms.

The enhanced antimicrobial activity of the converted products compared to vanillin is a significant finding of this study. Vanillic acid exhibited stronger antibacterial activity, particularly against Gram-positive bacteria (Vale *et al.*, 2019) [13] while vanillyl alcohol showed lesser antifungal activity. These differences in antimicrobial spectrum may be related to the structural features and physicochemical properties of these compounds, which influence their interactions with microbial cell components. Vanillic acid, with its carboxylic acid group, may disrupt bacterial cell membranes more effectively, possibly by interfering with membrane proteins or by chelating essential metal ions (Mourtzinos *et al*., 2009) [8]. The stronger activity against Gram-positive bacteria compared to Gram-negative bacteria is consistent with previous reports (Qian *et al*., 2020) [10] on phenolic acids and may be attributed to differences in cell wall structure and composition between these bacterial groups (Roda *et al.*, 2012) [11]. Vanillyl alcohol, with its hydroxyl group, may have better penetration into fungal cell walls, possibly due to its higher lipophilicity compared to vanillic acid. The antifungal mechanism may involve disruption of membrane integrity, inhibition of ergosterol biosynthesis, or interference with cell wall synthesis (Harini *et al*., 2012) [4].

The synergistic antimicrobial effect observed with the combination of vanillic acid and vanillyl alcohol is particularly interesting. This synergism may result from the complementary modes of action of these compounds, targeting different cellular components or metabolic pathways in the microbial cells. Such synergistic effects have been reported for other phenolic compounds and are of considerable interest for developing more effective antimicrobial formulations (Yadav *et al*., 2018) [14]. The *in vivo* efficacy studies using silkworm larvae provide valuable insights into the potential therapeutic applications of these compounds. The enhanced survival rates observed in infected larvae treated with vanillic acid, vanillyl alcohol, or their combination, along with the reduced microbial loads confirm the *in vivo* antimicrobial efficacy of these compounds.

This study demonstrates that fungal strains have the ability to convert vanillin into vanillic acid and vanillyl alcohol, both of which exhibit enhanced antimicrobial properties. These derivatives have the potential to serve as potent broad-spectrum antimicrobial agents effective against a range of bacteria and fungi.

## Acknowledgments

The authors express their gratitude to the Central Silk Board for their financial support (Project ARP 3634) and appreciate the assistance of Smt. K. R. Rathnamma, Senior Technical Assistant and Harish H.N, Field Assistant of Silkworm Seed Technology Laboratory, in conducting experiments and rearing of silkworms.

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