Isoeugenol's biotransformation to vanillin using microorganisms

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ABSTRACT

The biotechnological route of producing natural biological food products is preferred over synthetically created ones. One such pathway is biotransformation, which entails the chemical transformation of one substance into another using microorganisms acting as biocatalysts. One crucial process in green chemistry is biotransformation, which results in the biological production of numerous valuable chemicals. Due to its distinct aroma, vanillin is one of the most widely used flavors in the world. It is used in ice cream, cakes, biscuits, chocolates, and cosmetics. Compared to chemically synthesized vanillin, biologically produced vanillin has very few or no radicals, which is why it has very little or no negative effects on humans. Biological precursors such as eugenol and isoeugenol, as well as ferulic acids, can be utilized in the production of vanillin. Pure bacterial cultures were isolated from soil (isolates coded as DSH1001 to DSH1004) and identified by various biochemical reactions as Gram-negative rods. The microorganism identified by 16S ribosomal sequencing with accession number OR140859 can convert isoeugenol to vanillin. Their capacity to biotransform isoeugenol was also investigated. Using HPLC, a final screening of the selected bacterial isolate was carried out at a temperature of 37°C, pH 7.2, agitation rate of 150 rpm, and an initial isoeugenol concentration of 0.01%. The food sector can profit from the commercial production of vanillin by biological means.

Keywords: *Aeromonas veronii, Biotransformation, HPLC, Isoeugenol, Accession number OR140859, Vanillin, NCBI.*

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Highlights of this paper

- Biotransformation is a key green chemistry process that converts isoeugenol to vanillin using microorganisms.
- Pure bacterial cultures from the soil, identified as Gram-negative rods, can convert isoeugenol to vanillin.
- The study highlights the commercial potential of producing vanillin biologically as a safer alternative to synthetic vanillin.

1. INTRODUCTION

Turning less useful or useless goods into beneficial ones through a biological mechanism known as "microbial action" is referred to as "biotransformation." Because it lessens the negative consequences of chemical transformation—such as reducing radical generation or mitigating the impact of radicals—biotransformation is preferred for the manufacturing of a wide range of products. Toxins can also be detoxified through biotransformation, in which a microorganism utilizes the toxin and converts it into a less toxic or non-toxic form.

The precise alteration of a certain molecule into a unique product with a comparable structure by using biological catalysts, such as microorganisms like fungi, is known as biotransformation (Lilly, Pollard, Ison, & Shamlou, 1994).

So, essentially, biotransformation or bioconversion refers to the chemical conversion of a compound into another compound through the action of biocatalysts, such as microorganisms. This process involves converting organic material into an energy source via fermentation. For instance, biogas production from the waste product (dung) of ruminant animals occurs through the action of methanogens. Biotransformation is also recognized as aligning with today's green chemistry strategy. Green chemistry pertains to sustainable chemical manufacturing processes aimed at minimizing waste production and energy consumption (Tang & Zhao, 2011).

Plant-derived phenylpropanoids (e.g., isoeugenol, eugenol, and ferulic acid) are known as natural renewable sources for the synthesis of fine chemicals. Biotransformation of isoeugenol results in the production of vanillin.

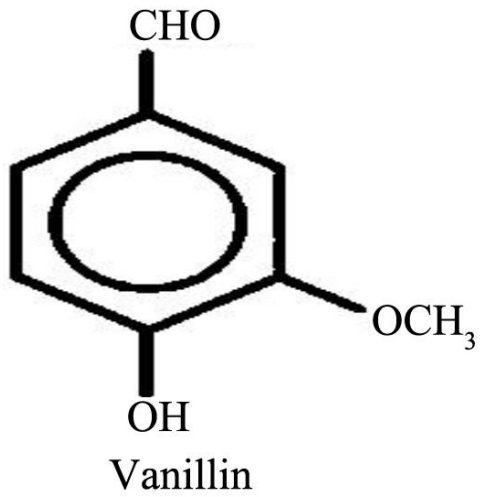


Figure 1. Vanillin (4-hydroxy-3-methoxy benzaldehyde).

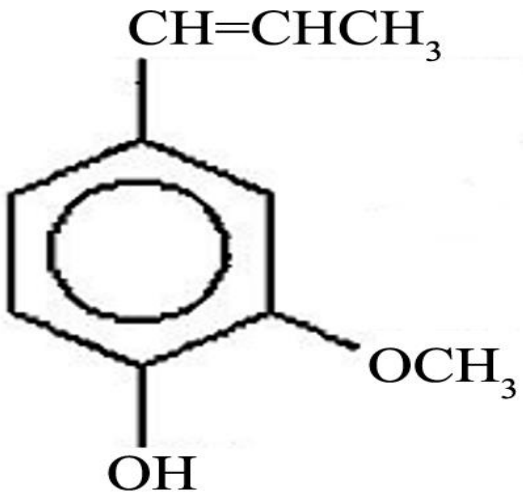
1.1. Vanillin

Vanillin is most widely used as a flavoring in the food industry all over the world. Currently, vanillin is produced from petrochemicals and lignin through chemical synthesis (Hua et al., 2007). Vanillin has a broad range of industrial uses as a flavor in food, confections, ice cream, beverages, chocolate, cookies, and cakes. Vanillin, with the chemical formula C8H8O3, is the most widely used flavoring agent in food and beverages worldwide. (Figure 1) It serves as an alternative to vanilla extract, offering a similar aroma and flavor. Vanillin is both naturally occurring and

synthetically produced (FoodAdditives.net, 2020). The demand for vanillin worldwide exceeds 15,000 tons, of which approximately only 2,000 tons is produced from vanilla beans. The remainder of the demand is satisfied by synthetic vanillin, which is chemically produced from guaiacol and lignin (Liu, Sun, & Huo, 2023). But the use of biological vanillin is preferable, as many countries ban the use of most chemically synthesized products in food (Unno et al., 2007).

Vanillin is also utilized as a fragrance component in perfumes due to its distinctive aroma. Research conducted by Furukawa, Morita, Yoshida, and Nagasawa (2003) reveals that Pseudomonas putida 158 cells are highly effective in converting isoeugenol into vanillic acid. The study emphasizes that the resting cells of P. putida 158 achieved a high molar yield of vanillic acid without the accumulation of vanillin.

This indicates that the biotransformation process is highly efficient and holds promising potential for industrial applications (Furukawa et al., 2003). Vanillin is utilized as an intermediate in agrochemicals and pharmaceuticals (Rana, Mathur, Jain, Sharma, & Mathur, 2013). The requirement for vanillin per year worldwide is about ten to fifteen thousand tons, out of which a few thousand tons are fulfilled by vanilla beans (Vanilla planifolia). There is a significant difference in the production costs of biologically and chemically synthesized vanillin. The current market price of chemically synthesized vanillin is around 10 USD/kg, while biologically synthesized vanillin is approximately 1000 USD/kg (Future Market Insights, 2023). To produce vanillin biologically using cheaper sources, some chemicals such as eugenol, isoeugenol, and ferulic acid are utilized. Through the biotransformation of these compounds with the assistance of microbial strains, vanillin is produced.



Isoeugenol

Figure 2. Isoeugenol (1-hydroxy-2-methoxy-4-propenylbenzene).

1.2. Isoeugenol

Isoeugenol is a pale yellow to colorless oily liquid that occurs in oil of ylang-ylang, nutmeg, and champagne. Isoeugenol can exist in two forms called isomers: cis (Z) isoeugenol and trans (E) isoeugenol. The trans (E) form is crystalline, forming a solid structure. In contrast, the cis (Z) form remains a liquid due to its unique arrangement of atoms (Ashengroph & Amini, 2017). Isoeugenol is slightly soluble in water and soluble in organic solvents like ethyl acetate. The density of isoeugenol is slightly heavier than water (1.080), with a melting point of 10 °C and a boiling point of 266 °C. Isoeugenol is used in medicine, cosmetics, and perfumes because of its aroma. It also exhibits antimicrobial properties. Isoeugenol is thought to be devoid of lignin precursor. Recently, the production of

isoeugenol by sweet basil and petunia flowers was reported (Koeduka et al., 2006). In petunia flowers, the enzyme known as isoeugenol synthase 1 directly facilitates the production of isoeugenol (Koeduka et al., 2006).

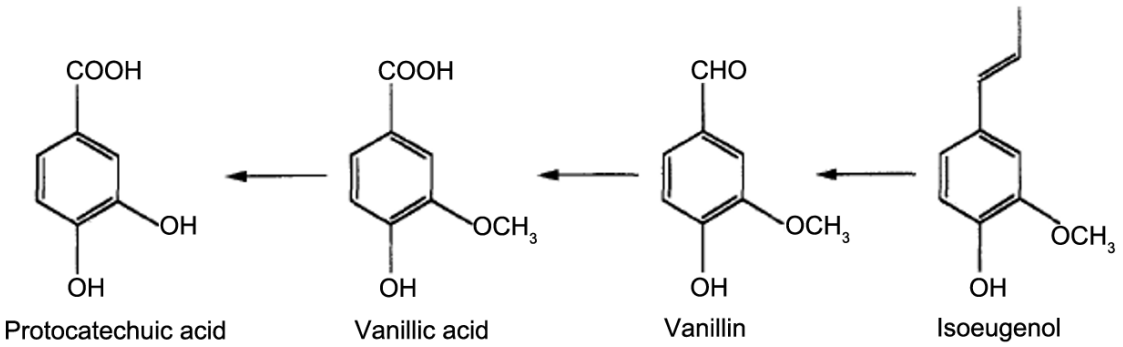


Figure 3. Catabolism of isoeugenol.

Isoeugenol, upon mild oxidation, produces vanillin. Isoeugenol, when treated with various microorganisms or biocatalysts, forms vanillin through bioconversion (Kurtzman, Fell, Boekhout, & Robert, 2011) Figure 3.

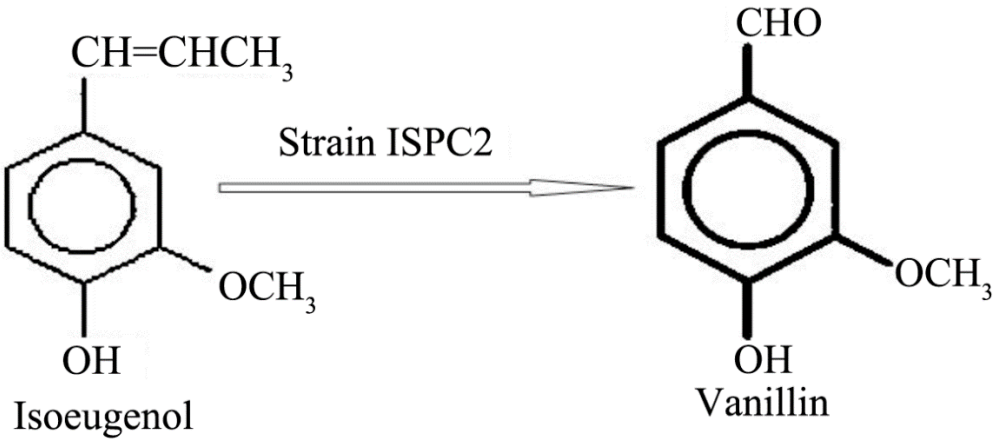


Figure 4. Representation of the bioconversion of isoeugenol into vanillin, catalyzed by strain ISPC2.
Source: Kurtzman et al. (2011).

1.3. Microbes

Biotransformation is a process regulated by biocatalysts, such as microorganisms like bacteria or fungi. The first evidence of the bioconversion of isoeugenol into vanillin was reported in Aspergillus niger ATCC9142. The low yield of vanillin (10%) was attributed to the further metabolization of vanillin into vanillic acid and vanillyl alcohol. The conversion of isoeugenol to vanillin by various bacteria has been reported by several researchers (Zara & Fan, 2023) Figure 4.

- *Serratia marcescens DSM 30126.*
- *Bacillus subtilis.*
- *Pseudomonas putida IE27* (Yamada, Okada, Yoshida, & Nagasawa, 2007).
- *Bacillus subtilis HS8.*
- *Psychobacter sp. strain CSW.*
- *Lysinibacillus fusiformis SW-B9.*

Yeast is generally regarded as safe (GRAS) and is also used for bioconversion. The Trichosporon asahii strain MP24 of yeast provided the first evidence of yeast converting isoeugenol into vanillin (Kurtzman et al., 2011). To achieve cheaper production of vanillin biologically, a variety of biocatalysts, including bacteria and fungi, can be utilized. The biotransformation of isoeugenol into vanillin using various microbes has been reported to meet the increased demand for vanillin and the preference for using biological vanillin (Yamada et al., 2007).

Bioactive flavonoids are regarded as the most significant phytochemicals in food, providing a wide range of biological benefits for humans. Microbial biotransformation strategies for producing flavonoids have garnered significant interest because they enable the creation of novel flavonoids that do not naturally occur. This review summarizes the existing knowledge on flavonoid production and biotransformation by various microbes. Recently, microbial transformation has emerged as an important method for producing natural flavors in high quantities (Ashengroph & Amini, 2017). Because the biotransformation processes are environmentally friendly and the products are considered "natural," flavor production using this method is attracting increasing attention (Yamada et al., 2007).

For the biotransformation of isoeugenol into vanillin, many microbes are used, including Aspergillus niger I-1472. In a study, a stepwise bioconversion of ferulic acid to vanillin takes place. In the first step, A. niger I-1472 converts ferulic acid to vanillic acid (Tan, Liew, Maskat, Aida, & Osman, 2015). In the second step of the bioconversion process, Pycnoporus cinnabarinus is used to convert vanillic acid into vanillin. This two-step process, involving Aspergillus niger I-1472 and Pycnoporus cinnabarinus, is an efficient method for producing natural vanillin (Bernard, Bastin, Stentelaire, Lesage-Meessen, & Asther, 1999). Various bacterial strains, particularly Pseudomonas and Bacillus species, have been isolated and utilized to study the bioconversion of isoeugenol into vanillin (Yamada et al., 2007). Amycolatopsis sp. HR167 and Streptomyces setonii ATCC 39116 exhibited high tolerance towards vanillin (Fleige, Hansen, Kroll, & Steinbüchel, 2013). Using these biocatalysts, efficient processes yielded over 10 g/L vanillin. Pseudomonas nitroreductase Jin1, isolated from soil, was found to metabolize eugenol and isoeugenol as sole carbon and energy sources to produce vanillin.

This is the first report of a bacterium with this metabolic capability. Vanillin produced from isoeugenol by P. nitroreductase Jin1 was stably maintained in the medium, unlike during the metabolism of eugenol (Unno et al., 2007).The first biotransformation of isoeugenol to vanillin was achieved using Aspergillus niger ATCC 9142, with a 10% efficiency (Tan et al., 2015).

Strains of the genera Klebsiella, Enterobacter, and Serratia were utilized to convert eugenol and isoeugenol into vanillin (Cao, Chen, Jassbi, & Xiao, 2015). Using Serratia marcescens (DSM 30126), 20.5% of isoeugenol was converted to vanillin, yielding 3.8 g/L of vanillin after 9 days. The conversion of isoeugenol to vanillin was also reported using Bacillus subtilis (12.4%) and Pseudomonas chlororaphis (12.6%) (Rabenhorst & Hopp, 1991). A group of scientists has developed a bioconversion process using B. subtilis HS8, yielding 1.36 g/L of vanillin (molar yield of 14.7%) (Zhang, Xu, & Han, 2006).

2. MATERIALS AND METHODS

2.1. Culture of Bacteria

The soil sample was collected from a Tulsi (Ocimum tenuiflorum) cultivated field. A master dilution of the soil sample was prepared by dissolving 1 g of the soil sample in a 0.9% Sodium Chloride (NaCl) solution. From the master dilution, a series of dilutions were prepared in the range of (10^{-1} to 10^{-4}). Minimal media was prepared using the salts NaCl, $NaH_2PO_4.7H_2O$, Na2HPO4 .7H2O, NH_4Cl, $MgSO_4.7H_2O$, and $CaCl_2.2H_2O$, along with agar as the solidifying agent. For pouring, 150 mL of media was prepared to have a pH – 7.2.0.01% solution of isoeugenol was added to the prepared media (Smitha, Singh, & Singh, 2017).

2.2. Purification of Culture

Prepared media was poured into four different Petri plates and incubated for 24 hours to check for contamination. 100 µL of the prepared dilutions (10^{-1} to 10^{-4}) were spread onto the Petri plates for primary screening (DSH1001 to DSH1004). The Petri plates were incubated at a temperature of 37°C for 48 hours under agitation conditions of 150 rpm. Purification of the colonies was performed using the streaking method, followed by the preservation of the colonies in nutrient agar slants at 4°C. Gram staining of the selected bacterial culture was performed, and gram-negative cocci were obtained. Further biochemical characterization of the isolates was done using MacConkey agar. Positive growth was observed on the MacConkey agar plate, which confirmed that the selected bacteria were gram-negative.

Biochemical characterization was also performed using MacConkey agar based on the bacterial growth on the plates (Jung & Hoilat, 2024).

2.3. Secondary Screening

Growth of the selected isolates was checked in the liquid mineral salt media supplemented with isoeugenol at a concentration of 0.01%, followed by their selection. The starter culture of the selected isolates was prepared in 25 mL of nutrient broth using a loopful of inoculum and incubated at 37°C for 24 to 48 hours. Liquid mineral salt media was prepared, supplemented with an initial isoeugenol concentration of 0.01%, and inoculated with 4% of the above-prepared starter culture. Cultures were allowed to grow for specified periods at 37°C under agitation conditions of 150 rpm to assess the biotransformation ability of the selected isolate (Khan & Jain, 2019).

2.4. Separation and HPLC Analysis

After fixed days of incubation, the reaction was stopped using concentrated hydrochloric acid (HCl), and the solution was centrifuged at 10,000 rpm for 10 minutes. The resultant supernatant was then extracted using ethyl acetate by mixing in an equal ratio. The collected mixture was concentrated using a rotary vacuum evaporator. Dried samples were then dissolved in 1 mL of 50% methanol. Samples were filter-sterilized and analyzed. For the quantitative and qualitative analysis of the metabolites obtained, the analytical technique of High-Performance Liquid Chromatography (HPLC) was used, employing the RP-HPLC gradient method (Joshi, Kumar, & Rathore, 2015). Methanol and trifluoroacetic acid were used as solvent A and solvent B. The flow rate was maintained at 1 mL/min. Ambient room temperature was used for the separation, utilizing two different detection wavelengths of 254 nm and 310 nm.

3. RESULT AND DISCUSSION

3.1. Isolation and Primary Screening

A total of four morphologically different microorganisms were isolated, of which three were bacteria and one was a fungal colony. The growth of all the isolated microorganisms (isolates coded as DSH1001 to DSH1004) was checked at 600 nm using UV-Vis spectroscopy, followed by their selection for further studies (Koch, 1970; Matlock, 2017). DSH 1001 was identified as a potential bacterial isolate based on its growth in isoeugenol-supplemented media Figure 5, 6, Table 1.

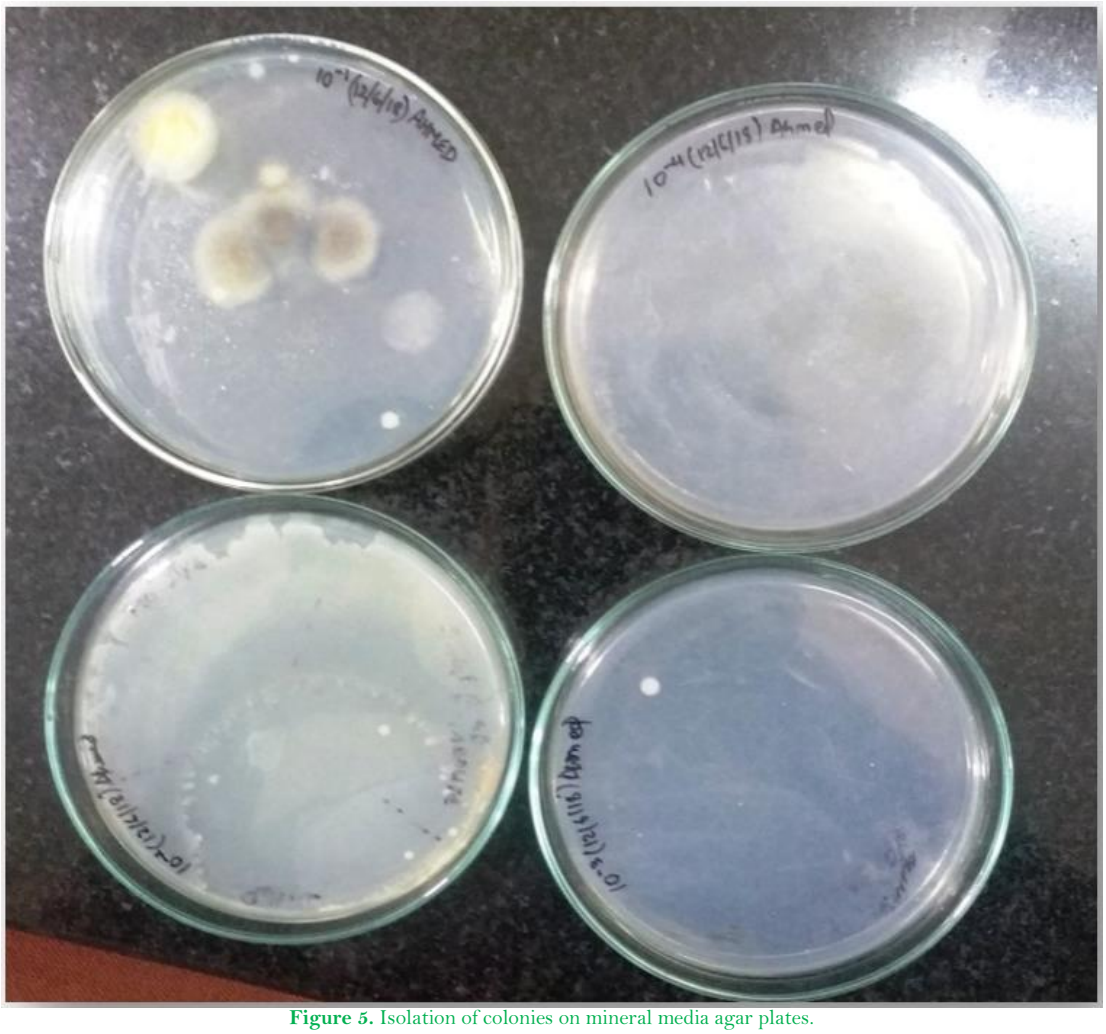


Figure 5. Isolation of colonies on mineral media agar plates.

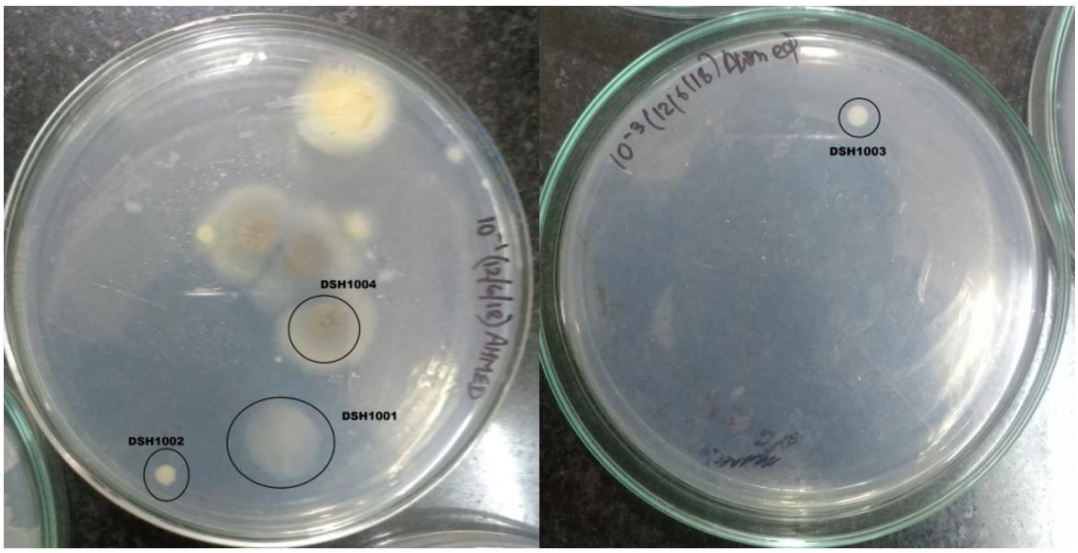


Figure 6. Selected bacterial colonies of the bacterial isolates.

3.2. Identification using 16 s Ribosomal RNA Sequencing

The microorganism identified by 16S ribosomal RNA sequencing (Barcode, Bioscience Pvt. Ltd., Bangalore) with accession number OR140859 was determined using the highly conserved 16S rRNA gene, which allows for

precise identification and classification of bacteria by comparing variable regions unique to each species. https://www.ncbi.nlm.nih.gov/ (National Center for Biotechnology Information (NCBI), 2023).

Table 1. Morphological characteristics of the isolates.

No. of isolates	Morphology	Growth at 600 nm		
		Good	Moderate	No
DSH1001	Large, circular, pale yellow	✓	No growth(x)	No growth(x)
DSH1002	Medium, transparent	No growth(x)	No growth(x)	✓
DSH1003	Small, circular, light yellow	✓	No growth(x)	No growth(x)
DSH1004	Fungal spores	✓	No growth(x)	No growth(x)

3.3. Secondary Screening and HPLC Analysis

The biotransformation ability of the bacterial isolate DSH1001 was assessed using HPLC, based on the formation of metabolites during isoeugenol biotransformation. It was found that isoeugenol was directly converted into vanillin after 24 hours of incubation, reaching its maximum concentration of 6.17 mg/L after 144 hours of incubation. The biotransformation conditions used for this transformation were as follows: substrate concentration: 0.01%, incubation temperature: 37°C, pH 7.2, agitation condition: 150 rpm (Joshi et al., 2015) Figure 7,8. Table 2.

In the HPLC (High-Performance Liquid Chromatography) profile, isoeugenol and vanillin are two different substances that can be identified by their peaks on the graph: Peak 1 (A) represents isoeugenol, which is the starting material, while Peak 2 (B) represents vanillin, the product formed from isoeugenol. Plots C, D, and E show how the isolates (DSH1001) convert isoeugenol (Peak 1) into vanillin (Peak 2) over time. Each plot likely represents a different time point or condition under which the conversion was observed Figure 7.

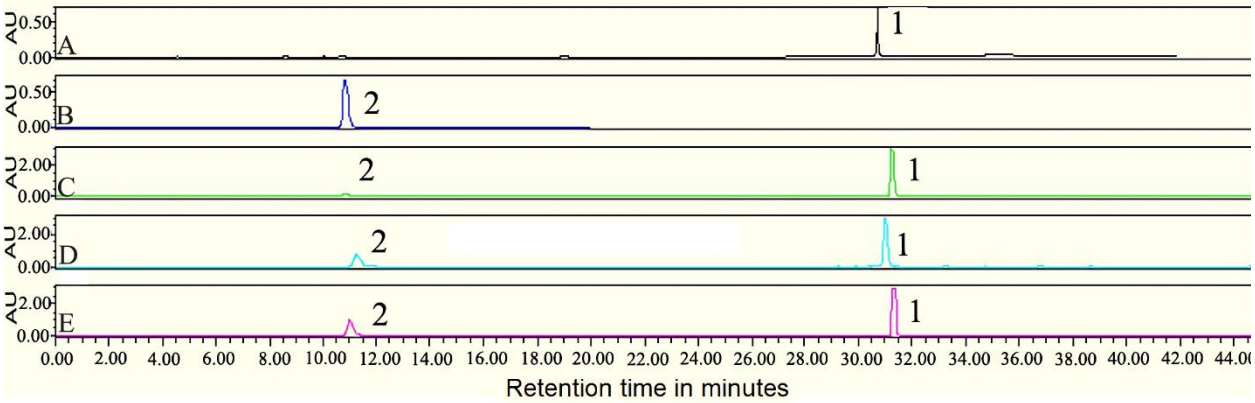


Figure 7. This is the X-axis retention time when a particular analyte travels through the column and reaches the detector. On the Y-axis, Absorbance Units (AU) represent the absorbance or the signal intensity detected by the HPLC detector. Isoeugenol is indicated as peak 1 and vanillin is indicated as peak 2.

Table 2. HPLC data for the production of vanillin.

S.no	Days of incubation	Concentration (mg/L)			
		1st trial	2nd trial	3rd trial	Average
1	2	1.23	1.50	1.30	1.34
2	4	2.90	2.66	2.75	2.77
3	6	6.07	6.23	6.22	6.17
4	8	5.01	5.30	4.87	5.06

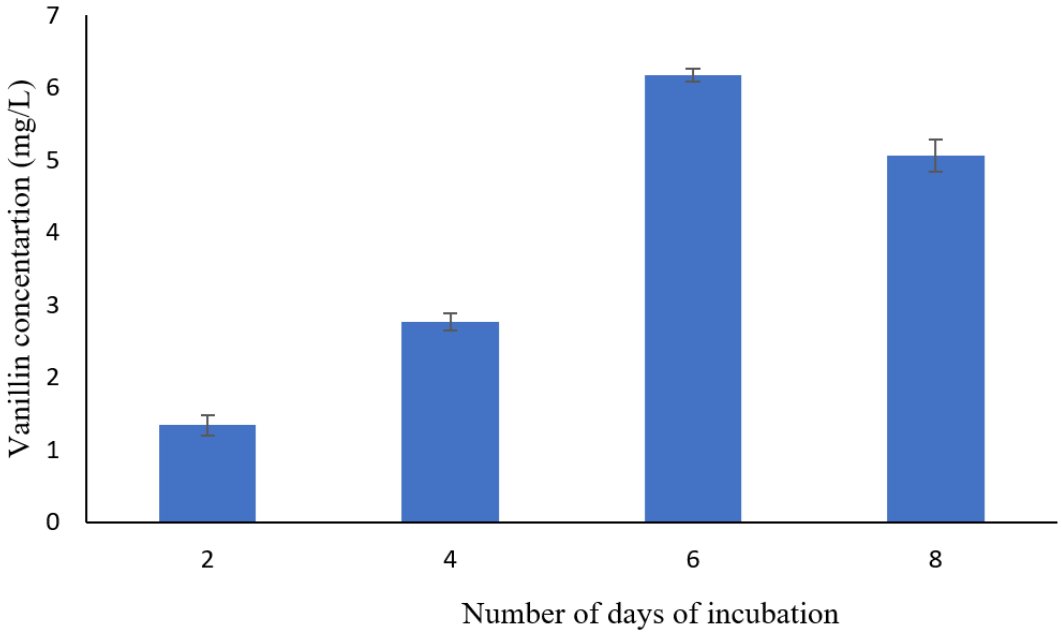


Figure 8. Clustered bar graph showing the effect of different incubation times on the concentration of vanillin using isoeugenol at a concentration of 0.01% at 37 °C under shaking conditions of 150 rpm.

3.4. Statistical Analysis

When isoeugenol at a concentration of 0.01% is incubated at 37°C under shaking conditions of 150 rpm, the concentration of vanillin shows a gradual increase during the first two days. Between days 4 and 6, vanillin production increases significantly as bacterial activity peaks. From the 6th day onward, the concentration of vanillin continues to rise but at a slower rate after days 7 to 8, as the bacteria start to exhaust the available isoeugenol. After 8 days, the vanillin concentration bar graph indicates that the bacteria have consumed most of the isoeugenol, and the production rate stabilizes Figure 8.

4. CONCLUSION

A total of four bacterial cultures were isolated using primary screening. Out of all, one potential bacterial isolate was selected based on its ability to grow in the presence of isoeugenol at a concentration of 0.01% and biotransform it into the value-added metabolite vanillin after 144 hours of incubation under shaking conditions at 150 rpm. The culture was identified as gram-negative bacilli through various biochemical characterizations, and genomic identification of the strain by 16S ribosomal sequencing revealed that the bacteria belong to Aeromonas veronii, which is part of Gammaproteobacteria. The strain has been submitted to the NCBI site, and the GeneBank accession number assigned is OR140859.

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