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IMMOBILIZATION OF MICROBES FOR VANILLIN PRODUCTION

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ABSTRACT The current study aimed to successfully immobilize microbes especially lactic acid bacteria to produce vanillin, The vanillin polyphenol, which is well-known for its use in a variety of industries including food and beverage, flavorings, perfumery, and cosmetics, is becoming increasingly popular. Despite rising need, there is a persistent gap between supply and demand for vanillin in the economy. Natural vanilla, made from Vanilla planifolia orchids, is an essential spice. However, environmental factors such as soil erosion, biodiversity loss, and deforestation place severe limits on vanilla production and harvesting. Recognizing the critical need for environmentally friendly practices, scientific advances have resulted in a ground-breaking approach for continuous vanilla manufacturing. This novel technique makes use of immobilized cells and their fermentation activity on ferulic acid, a well-known precursor of vanilla. Ferulic acid is derived from various agricultural wastes and leftovers, which reduces environmental effect. Immobilization, a long-established approach for increasing bioprocess productivity and stability, is critical to this sustainable vanilla production method. This scientific endeavor addresses not only the economic imbalance in vanillin supply and demand, but also the major environmental challenges linked with traditional vanilla growing. The approaches used in this work provide useful insights into vanillin synthesis via bioprocessing, enabling a better understanding of sustainable production processes.

KEYWORDS : Lactic acid Bacteria, Immobilization, Sodium alginate beads, Loofah, Vanillin, Standard

I. INTRODUCTION

Vanillin, one of the most common flavoring chemicals, is used in a variety of industries, including food, fragrance, and pharmaceuticals. Its distinct smell and taste make it a popular ingredient in consumer items around the world. Traditionally, vanillin has been largely synthesized using fossil fuels, which not only creates environmental problems but also fails to meet the growing demand for natural and sustainable alternatives. As a result, there is an increasing interest in finding environmentally friendly and cost-effective methods of producing vanillin.[1]

Microbial fermentation has emerged as a viable method for the biosynthesis of vanillin, with various advantages over chemical synthesis, including environmental sustainability, stereochemical precision, and the possibility to use renewable feedstock. In recent years, significant study has been focused on optimizing microbial systems to increase vanillin yield and productivity. Among the different solutions investigated, the immobilization of microbial cells has received substantial interest due to its potential to improve bioprocess performance and operational stability.[2][3]

Immobilization is the confinement of microbial cells within a solid matrix or support material, allowing for their retention and continued utilization in bioreactors. This approach has various advantages over traditional free-cell systems, including increased cell viability, protection against shear stresses, improved mass transfer, and easy product recovery. Furthermore, immobilized cells can be reused for several fermentation cycles, lowering operational costs while increasing overall process efficiency. Several immobilization strategies and matrices have been tested for the generation of vanillin by microbial fermentation. Alginate, agarose, polyurethane foam, and silica gel are among the most often used matrices due to their biocompatibility, porosity, and mechanical strength. These matrices create an optimal milieu for microbial growth and metabolism, making vanillin synthesis more efficient.[4]

Sodium alginate immobilization encapsulates microbial cells within alginate gel beads using a gelation process produced by calcium ions. This approach creates a favorable milieu for cell development and metabolism while providing benefits such as ease of use, biocompatibility, and scalability. The porous nature of alginate beads allows for effective nutrition and metabolite exchange, while their mechanical durability shields cells from shear stresses. Optimizing bead size, alginate concentration, and crosslinking conditions is critical for achieving peak performance and long-term stability. Regardless of its effectiveness, eliminating diffusion restrictions and improving mass transfer inside the beads are critical concerns for increasing vanillin production and productivity. Overall, sodium alginate immobilization is a versatile and efficient approach for using microbial cells in a variety of bioprocessing applications, including environmentally friendly vanillin synthesis.[5]

Luffa sponge, produced from the fibrous skeleton of Luffa cylindrica, has gained popularity as a natural and sustainable immobilization matrix for microbial cells due to its porous structure, biocompatibility, and low cost. The linked network of fibers in luffa sponge provides a large surface area for cell adhesion and growth while also facilitating effective nutrition and substrate transfer. Recent research has established the viability of employing luffa sponge as a support matrix for immobilizing microbial cells in a variety of bioprocesses, including enzyme production, wastewater treatment, and biofuel synthesis. In the context of vanillin production, luffa sponge immobilization has the potential to improve the stability and activity of microorganisms that produce vanillin, hence improving overall bioreactor performance and productivity.[6]

Despite tremendous advances in immobilized microbial systems for vanillin synthesis, various problems remain to be addressed. These include optimizing immobilization techniques for various microbial species, increasing mass transfer rates inside the immobilization matrix, and assuring immobilized cells' long-term durability and performance at industrial scales. Furthermore, the scalability and cost-effectiveness of immobilized bioreactors must be improved to promote commercialization.[7]

II. MATERIALS AND METHODOLOGY

Isolation and Preparation of Inoculum: Milk was utilized to collect and culture bacteria, specifically Lactic Acid Bacteria, for ferulic acid production. The technique involved mixing 10 mL of milk with 50 mL of distilled water. This dilution is critical for creating an optimal growing environment for the bacteria. To achieve consistency, the mixture was transferred to a conical flask and placed on a rotating shaker. The shakers were designed to rotate the flasks for 48 hours. This shaking action promotes microbe development and increases yields by uniformly dispersing them throughout the liquid. After 48 hours of incubation, the culture needed to be transferred to solid media so that the essential bacteria could continue to multiply and be isolated. MRS Agar plates were used for this. The culture from the milk and water mixture was streaked over the agar plates with a sterile loop. The culture is spread across the agar surface to produce individual colonies. Following that, the agar plates were incubated at 30 degrees Celsius for 24 hours. The target bacteria thrived, proliferated, and formed visible colonies on the plates during the incubation period. Following incubation, a loopful of the culture was removed from the MRS Agar and transferred to sterile MRS Broth. This approach was used in liquid culture to cultivate the targeted bacterium. MRS Broth, a nutrient-rich medium, can promote the growth of lactic acid bacteria, which are commonly utilized in fermentation processes. The MRS Broth cultures were then placed in an orbital shaker incubator that revolved at 130 revolutions per minute and maintained a temperature of 30°C. The

shaker's rotation facilitates equal culture mixing and a sufficient supply of oxygen, both of which are required for bacterial growth and metabolism. The broth cultures were incubated for twenty-four hours.[8][9]

Immobilization of Cells

Using Sodium Alginate Beads: The immobilization matrix for the cells was first created in a concentrated sodium alginate solution. This was accomplished by blending 150 mL distilled water and 150 g sodium alginate. The solution-containing beakers were then placed on a magnetic stirrer and swirled at 500 rpm for 20 minutes. This stirring method guaranteed that the sodium alginate was fully dissolved and evenly distributed throughout the water. The inoculum, which contained the cells to be encapsulated, was prepared and introduced to the sodium alginate solution in 2 ml quantities. The cells were included into the immobilization matrix by adding the inoculum to a sodium alginate solution. The immobilized cells were then created by adding the sodium alginate-inoculum mixture dropwise to a cold calcium chloride solution. This approach resulted in a double replacement reaction between calcium chloride and sodium alginate. The sodium ions in sodium alginate were transformed into calcium ions because calcium chloride has a divalent form of calcium. Calcium alginate, a gelatinous substance that closely binds and surrounds the cells in bead-like forms, is thus formed. These beads, also known as immobilized cells, provide physical support for the cells, preventing them from dispersing during subsequent processes.[10][11]

Using Loofah: The loofah will be cut into small, uniform pieces, ensuring consistency in size. The loofah pieces will be sterilized through autoclaving or another appropriate method to eliminate contaminants. The desired bacteria will be cultivated in a nutrient medium The desired bacteria will be cultivated in a nutrient medium under optimal conditions, including temperature and pH. The sterilized loofah pieces will be immersed into the bacterial culture, allowing the bacteria to adhere or colonize the loofah surface. The loofah will be incubated with bacteria for an appropriate period to facilitate attachment. The loofah pieces will be rinsed to eliminate excess bacteria that have not attached, ensuring a controlled and defined immobilization of the bacteria. [12][13]

Fermentation: The manufactured beads and loofah were then placed in two separate flasks filled with MHA medium. This nutrient-rich solution provides immobilized bacteria with the necessary habitat and nourishment to complete fermentation. The constituents of MHA medium provide the minerals, essential nutrients, and carbon and nitrogen sources that cells require for growth and metabolism. Ferulic acid is also added as this is converted to vanillin by microbes. The fermentation process began by allowing the immobilized cells to execute metabolic functions in the media at a temperature of 32°C in an orbital shaker incubator with a speed of 130 rpm. At the end of the process, the product was extracted from the medium. Meanwhile, the solution's absorbance was monitored every 3 to 4 days to determine the production of vanillin.[14]

Thiobarbituric Acid Assay: Thio barbituric acid (TBA) assay is a colorimetric method used to detect chemical compounds such as vanillin by formation of a complex with TBA and the compound being detected. Vanillin, a flavor compound commonly found in foods such as vanilla, can be detected using this method. The TBA assay is quick, easy, and inexpensive, making it a popular choice for preliminary screening or quantitative analysis of vanillin in food or other products. The medium obtained after fermentation was centrifuged at 6000 rpm for ten minutes. Mix 50 µl of supernatant with 950 µl of thiobarbituric acid. However, after heating it over a water bath for 1 hour at 55 degrees Celsius, the reddish orange colour is expected which will indicate the presence of vanillin. [15][16]

Standard Graph for Vanillin: To construct a standard graph for determining the concentration of pure vanillin, a series of solutions with known vanillin concentrations is prepared. The absorbance of each solution is then measured at a given wavelength with a spectrophotometer. Typically, a wavelength of roughly 280 nanometers (nm) is utilized because it corresponds to the absorbance maximum of vanillin. The absorbance values were plotted against the concentrations of the vanillin solutions. The content of vanillin in an unknown solution can be estimated by comparing its absorbance to that of the standard curve and thus yield can be calculated. [17][18]

Yield calculation: After the fermentation process is completed and a standard graph of vanillin is created, the yield of produced vanillin from fermentation is determined using the slope of the standard graph and regular absorbance measurements. The greatest absorbance measured is used to compute the maximal production of vanillin produced from both beads and loofah. After calculating the yield from immobilized microorganisms, it is compared to the vanillin yield from free bacteria. Our sample is cultured in MHA medium containing ferulic acid without immobilization.[19]

III. RESULTS AND DISCUSSION

Isolation and checking the purity of bacteria: Lactic acid bacteria were successfully isolated, and the sample's purity was determined by Gram staining. The Gram staining process revealed the features of the bacterial cell wall, which helped to identify between different species of bacteria. LAB generally have a positive Gram staining result and appear purple under the microscope due to the retention of crystal violet dye in their thick peptidoglycan layer. This positive result reveals the existence of LAB in the cultivated samples and confirms their purity, which is required for optimal vanillin synthesis. Furthermore, the absence of other bacterial contaminants in the culture assures that the vanillin fermentation process is of high quality and consistent.

Immobilization of Cells:

Using Sodium Alginate Beads: The successful development of immobilized beads represented a significant leap in microbial fermentation processes, particularly in the synthesis of vanillin. This novel approach effectively immobilized bacteria within the beads, resulting in a stable environment for fermentation. This immobilization strategy improved not only the duration and effectiveness of the fermentation process, but also the extraction of vanillin, a valuable chemical utilized extensively in the food and fragrance sectors.

Using Loofah: The effective immobilization of microorganisms on loofah matrices has shown promising results for increasing vanillin production via fermentation procedures. By effectively trapping bacteria within the loofah's porous structure, a favorable environment is produced for long-term microbial activity, boosting the conversion of precursor chemicals into vanillin. This novel technique not only preserves the microbial population, but also provides a stable platform for ongoing vanillin production.

Thiobarbituric Acid Assay: The Thiobarbituric Acid Assay showed positive results, as evidenced by a noticeable change in color to reddish-orange, confirming the successful immobilization of microorganisms for vanillin synthesis. This result demonstrates the efficacy of the immobilization method in aiding the microbial population's bioconversion of substrates into vanillin. The brilliant color shift provides a visual proof of the metabolic activity within the immobilized microbial cells, indicating their ability to efficiently transform precursor chemicals into the desired product. This crucial finding underlines the great potential of using immobilized microorganisms as a powerful biotechnological tool for increasing vanillin production, providing a sustainable and ecologically friendly solution to meet rising demand for this valuable molecule.

Standard Graph of Vanillin: The standard graph was created using various concentrations of pure vanillin and their corresponding absorbances. A linear graph is generated, which may then be used to compute the concentration of vanillin in an unknown solution obtained from fermentation, as well as the yield.

Yield Calculation: Yield of vanillin produced from immobilized microbes in beads and loofah is calculated. Highest absorbance value in beads is 1.1588 and control is 0.2795. So, the actual absorbance is 0.8793, Now using this absorbance value and slope equation of standard vanillin we get vanillin yield of 0.8705 g/ml. Similarly for loofah, maximum absorbance value is 1.3585 and control is 0.2850. So, the actual absorbance is 1.0735. Using this, vanillin yield is 1.0628g/ml. We can see the yield of vanillin produced by microbes immobilized by loofah is higher than beads. Now, for comparing vanillin yield from immobilized microbes to free microbes, we need to find the vanillin yield from free microbes which will be done similarly as to immobilized microbes.

IV. CONCLUSION

In a nutshell the immobilization of microorganisms utilizing beads and luffa for vanillin synthesis, as evidenced by the good TBA assay

results, is a promising method with substantial industrial potential. This approach has various advantages, including improved stability and reusability of the immobilized microorganisms, which allows for continuous vanillin synthesis while lowering costs. Furthermore, the utilization of natural materials like luffa provides an environmentally beneficial alternative to standard immobilization matrices. The positive TBA assay results verify the immobilization technique's efficiency in increasing microbe vanillin production. Also after yield calculation it was found that vanillin produced from immobilized microbes from loofah is higher than sodium alginate beads. Further optimization and scaling up of this technology could lead to its broad implementation in biotechnological vanillin production, helping to ensure the long-term availability of this valuable molecule. Also, growth kinetics of the microbes will be calculated to get information about the growth pattern of microbes.

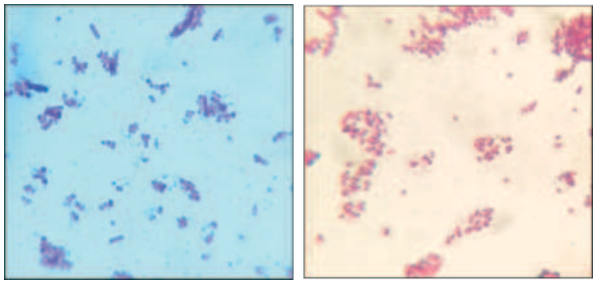


Figure 1: Gram Staining Of Lactic Acid Bacteria-pediococcus

Figure 2: Immobilization By Forming Sodium Alginate Beads

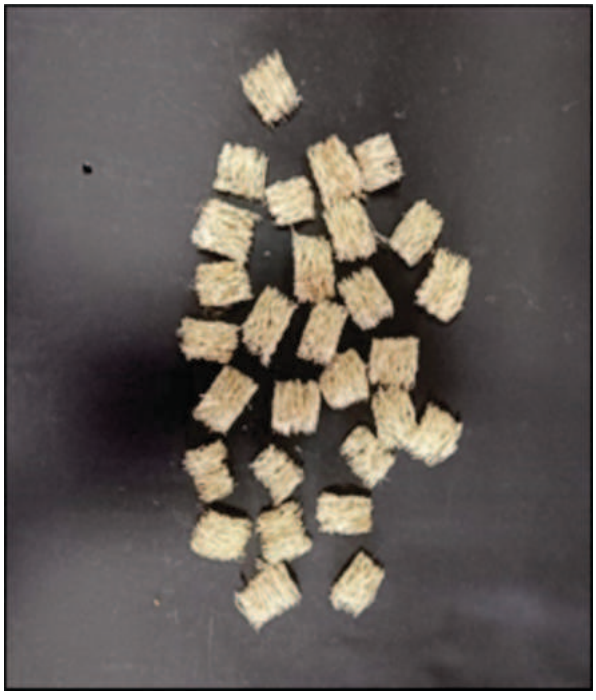


Figure 3: Immobilization By Loofah

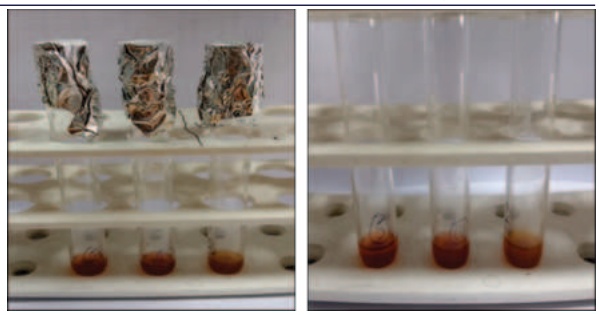
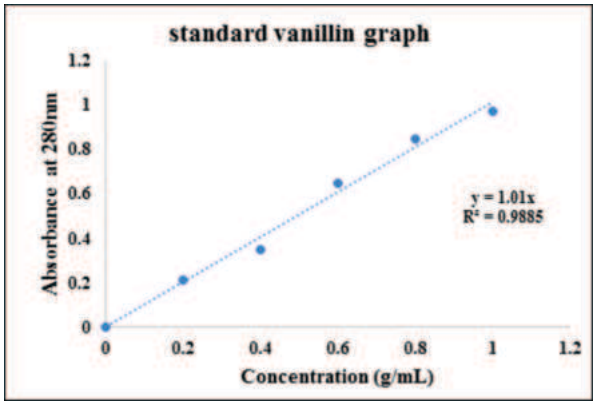


Figure 4: The Thiobarbituric Acid Assay

Table1: Standard Absorbance For Vanillin At 280 Nm

S.No.	Concentration of vanillin (g/mL)	Absorbance
1.	0	0
2.	0.2	0.21
3.	0.4	0.35
4.	0.6	0.65
5.	0.8	0.85
6.	1.0	0.97



Graph 1: Standard Graph For Vanillin

Author Contribution

Prachi Singh and Kritika Jha have contributed equally to this paper and designated as first author and Dr. Ramesh Pathy M is the corresponding author.

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