Bioconversion of Fruit Waste to Vanillin-related Compounds

Project report submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE (M.Sc)

IN

BIOTECHNOLOGY

by

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Under the Supervision

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DECLARATION

I hereby declare that the work reported in the M. Sc thesis entitled **“Bioconversion of Fruit Waste to Vanillin-related Compounds“** submitted at Jaypee University of Information Technology, Waknaghat, India, is an authentic record of my work carried out under the supervision of Dr. Garlapati Vijay Kumar, Dept. of Biotechnology and Bioinformatics, JUIT, Waknaghat, HP-173234, India. I have not submitted this workelsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M. Sc report entitled **"Bioconversion of Fruit Waste to Vanillin-related Compounds",** submitted by **KM Shivani (217805)** at Jaypee University of Information Technology, Waknaghat, India, is a bonafide record of the work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Thank you,

(**KM Shivani, 217805**)

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ABSTRACT

Vanillin is used in nutrition, refreshments, and industrial applications as a fragrance and condiment. *Vanilla. planifolia* is the most crucial resource of vanillin., with. a current value ranging from $1200 to $4000 per kg. To meet customer requirements, low-cost vanillin is produced synthetically (Fifteen $/kg.); however, US and European legislation encourages synthesis via fermentation. Ferulic acid is vanillin producers' primary and most expensive pre-cursor. It occurs naturally in lignocellulose agricultural residues. The current study was thus aimed at extracting ferulic acid from Kinnow peels. And. Afterward, converting it into bio vanillin with the help of *Rhizopus oryzae*. During this optimization to know the hyperproduction of vanillin, the parameters were pH, temperature, incubation period, and different concentrations of ferulic acid obtained from the waste.

Keywords: Ferulic acid; Vanillin; Bioconversion; *Rhizopus oryzae*; waste; flavour

CHAPTER 1

Introduction

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Vegetables and fruits are considered good sources of vitamins and minerals as well as fibers. Furthermore, they are also rich in bioactive compounds more likely polyphenols and they have many health-promoting effects. The scavenging capacity of these compounds and synergistic effects contribute to the antiinflammatory, antimicrobial, antitumor, neuroprotective properties, and antimutagenic properties. Recently, there's been a surge in fascination and utilization of bio-active compounds either from raw or extracted forms from fruits and vegetables in different industries for instance food and pharmaceuticals. Now during the processing of fruits, they are commonly processed into pulp which leaves a large number of waste in the form of peels. Although these leftover are organic waste they are the leading cause of environmental pollution as they are usually disposed of in open areas or municipal dustbins. Using these wastes as they carry many beneficial minerals and fibers can add value to many industries but will also decrease the threat to the environment. There have been suggestive studies that the antioxidant properties and other effects are supposed to be helpgul in the cosmetic and pharmaceutical industries in response they can replace synthetic antioxidants. These phenolic compounds can also be used as flavors. The global market supply of flavors and fragrances, which have been widely used within the food, livestock, pharmaceutical, as well as the cosmetic industries, is constantly increasing. The majority of accessible flavoring compounds are already manufactured through synthetic chemistry, with only a minor contribution from the manufacturing of "natural" flavors extracted from native plant source materials or cell cultures. Throughout the past generations, there has emerged a growing consumer pattern to "eco-friendly" and "environmentally" procedures, in addition to "healthy" activities connected to "bio" or "organic" product lines.Despite its substantial yields, flavor production by chemical reactions has several drawbacks, including high ecological consequences as well as low-end product quality.

Furthermore, synthetic chemical substances have been labeled as "natural resemblance" or "artificial," reducing their commercial value. As a result, notwithstanding their vastly greater costs, organic flavors are preferred in the global market. Natural flavor generation by removing contaminants from botanic sources, on the other hand, can no longer meet the large market demand due to the low concentration levels of the desired product in plants, which increases removal and purification procedures, in addition to harvest dependence on seasonal, meteorological, and political circumstances. Effective renewable supplies of flavor are required, as well as biotechnology is unquestionably the most appealing field of investigation. Overall, bioengineering approaches have had the economic benefits of moderate reaction conditions, and high regio- and enantio- specificity, resulting only in each product isomer, neither any form of toxic wastes, and therefore very few environmental issues. Furthermore, as per the latest US Food and European legislation, the definition of "natural" seems to be no longer confined to flavors derived from botanical components.

The European flavor regulation (EEC No 1334/2008) characterizes contained in article 3 (2) c)"Natural flavoring active ingredient" means a flavoring component acquired by acceptable physical, metabolic enzymes, or microbiological methods from the material of vegetable, wildlife, or microbiological origin , in either native format or after handling for human utilization by one or more conventional preparing food procedures". The latest flavors have been added to the GRAS list (generally recognized as safe).As a result, considerable research has been done, primarily in the area of flavor creation from various organic precursors using multiple microorganisms or solitary enzymes. Vanilla flavoring is without a doubt the most essential flavor for biotechnological applications.

CHAPTER 2
Literature review

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2.1 Ferulic acid

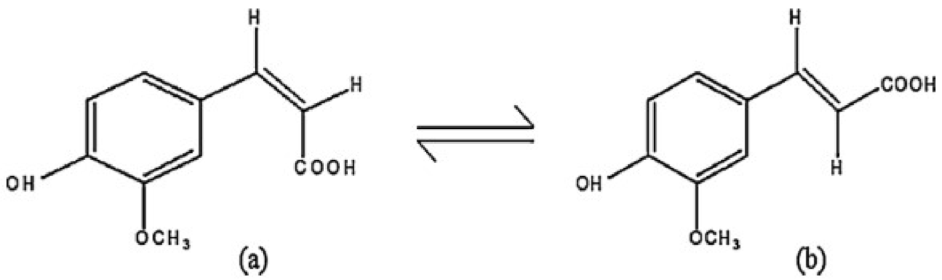

Fig 1. (a) cis conformation (b) trans conformation of ferulic acid

Polyphenols, which are vital food components that make up our nutrition, are synthesized by vegetation. They are divided into various categories based on their chemical makeup. There are four major categories of polyphenols that are flavonoids, lignans, stilbenes, and phenolic acid. Polyphenols play an important role in protecting the body from stimuli from the outside and in eliminating reactive oxygen species (ROS), which have been identified as the cause of many diseases. Polyphenols, which are found in tea, chocolate, fruits, and veggies, can possibly improve people's health. Cocoa flavan-3-ols, for example, have been linked to a lower risk of myocardial infarction, and diabetes. Adding them to one's diet can help in improving blood pressure, the resistance of insulin, inflammation, and lipid profiles also. They have a significant association with gut microbes because they affect the composition of those microbes which leads to maintaining the good health of humans. Natural polyphenols are studied to know their potential to treat or prevent metabolic syndrome. For instance, the following are quercetin, cyanidin, hydroxycinnamic acid, and many more. Ferulic acid is one of the polyphenols and will get more information in this article about it. It is chemically synthesized around 1925 and its structure was confirmed using spectroscopic techniques. Its chemical name is 4-hydroxy-3-methoxy-cinnamic acid , being a phenolic compound, it is very useful. Generally found in commelinid plants such as *Oryza sativa*, *Triticum*, and *Avena sativa*. It was first isolated from *Ferulafoetida*. FA has an unsaturated side chain, and both cis- and trans-isomers exist due to the double bond present in it and this resonance stabilized the radicals which states for its effective antioxidant properties. The higher degree of resonance stability of the phenolate anion across the conjugated molecule hence increases its acidity as compared to similar phenolic acids. It is also referred to as string dibasic acid. Ferulic acid has great importance in industries because of its property of antioxidants. it can prevent discoloration in food industries, for instance, it prevents of oxidation of bananas which on oxidation turns black color. UV-induced peroxidative reaction chains in the membranes are disrupted. As some of the UV is absorbed by the Ferulic acid that's why it's a weak sunscreen by itself. However, there are studies related to the aging of the skin which include prevention and treatment for the same.FA has been discovered to be related to a wide range of carbohydrates such as Glycosidic conjugates, Different Esters , and amides, along with a widerange of organic products Because it is included in the components of various drugs, functional-foods , and nutritional supplements, FA extraction, a lot of it comes with business opportunities as well as additional environmental and economic encouragement for industry sectors. FA which is extracted by use of chemical synthesis doesn't count as natural, that's why various attempts at enzymatic release have been done from natural sources and there are other methods also used to extract it for instance alkaline and acidic processes. For higher yields it needs to be optimized, the parameters for the same can be pH, and temperature. Now if we look more into it the solubility of FA is less in water, however is miscible in hot water, ethyl ethanoate , C_2H_6O, etc. The literature which has been published has shown that FA is present in the cell wall of wood, grasses however, it is difficult to make it available from these sources effortlessly as they are covalently linked with different carbohydrates furthermore, it is only possible if alkaline hydrolysis is done to extract it from these natural resources. Extracting it for commercial production by using the enzyme extraction method has been a challenge as it is connected with lignin and other bio-polymers. Although, currently Uraji et. al upgrade this method successfully from defatted rice bran and mentioned that Streptomyces can also be used for extraction of FA from other sources also The separation of the extracts and the visualization using UltraViolet light gives a rapid method for substantial efficiency identification of ferulic acid. In 12 hours, the incubation period is approx. >45% of FA content was released from a sweet potato from the enzymatic treatment. Using *Saccharomyces cerevisiae* around 90% of yields were got from eugenol and coniferyl alcohol, a study done by Lambert et al..

Now the metabolic pathway of ferulic acid firstly, the amino acids phenylalanine and tyrosine undergoes a transformation into p-coumaric acid and cinnamic acid which include the help of the enzyme’s tyrosine ammonialyase and phenylalanine ammonialyase. Methylation and hydroxylation reaction then covert the p-coumaric acid in ferulic acid. Studies which are done in vivo show that ferulic acid converted into various substance such as ferulic acid sulfate , vanillic acid , and dihydro-ferulic acid. In human beings, a tiny quantity of FA is metabolized in the liver. This occurs via -oxidation.

Overhage et al. performed away research in which they used *Pseudomonas sp.* Strain HR199 at the tail end of the 20th century disclosed that the genes were associated with the cata-bolic mechanism of ferulic acid was there in the DNA . There are many applications related to it for example anti-aging. anti-cancerous, an anti-inflammatory absorber, and many more. As it can be obtained from different plants, here we are going to extract FA from the waste of fruits.

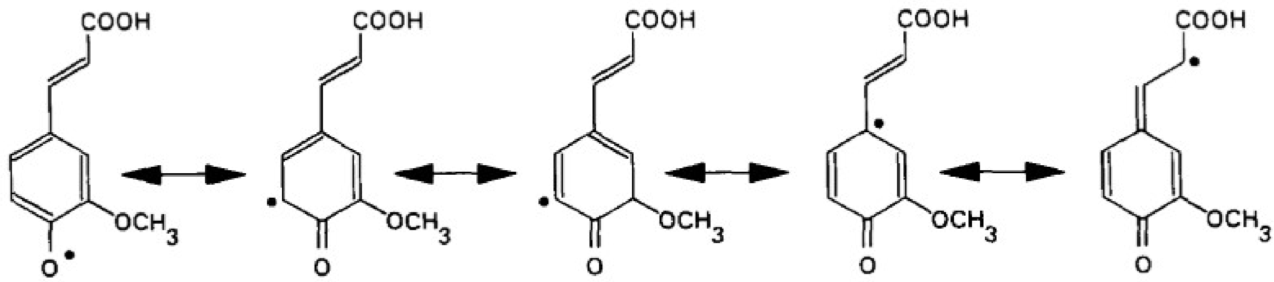

Fig 2. Stabilization of ferulic acid radical (Resonating structure).

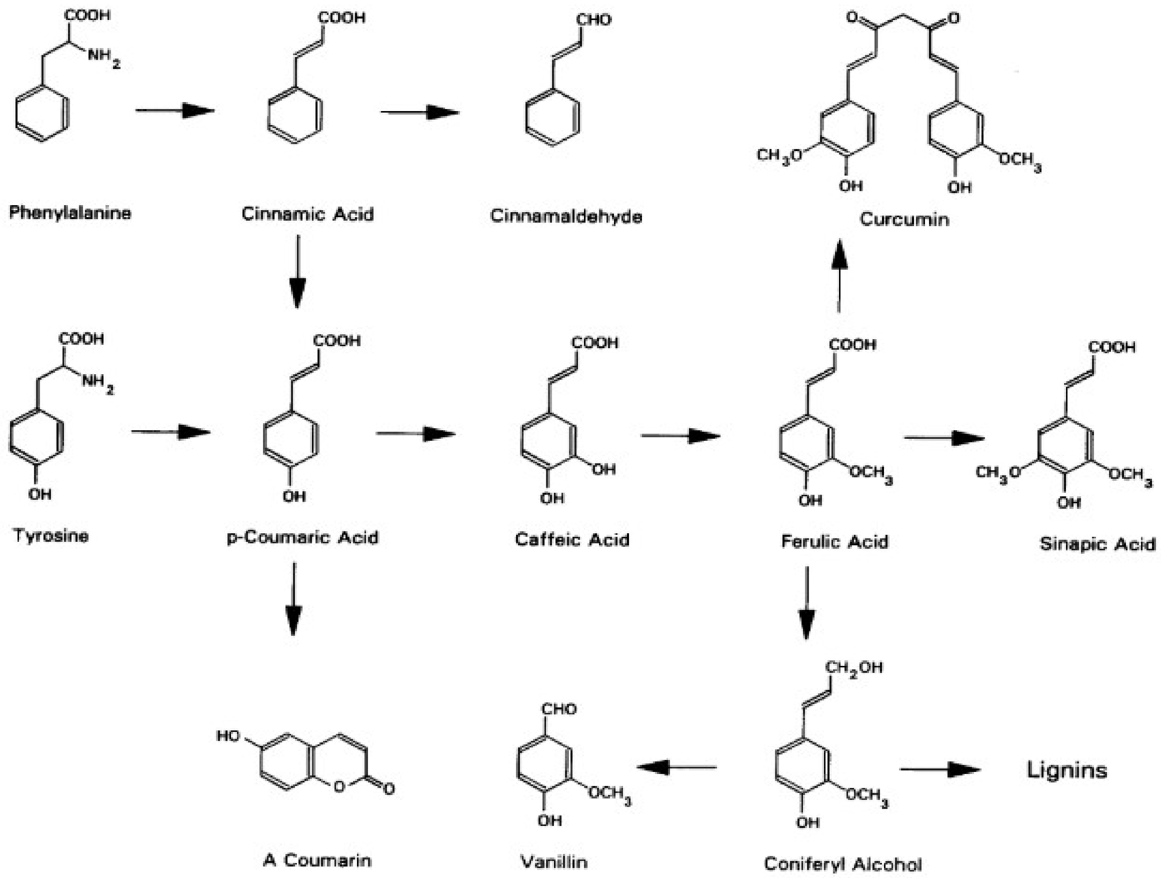

Fig 3. Pathways for the formation of ferulic acid in plants

2.3 Application of ferulic acid

As we already know about the chemical properties and where ferulic acid is presented or found, let's briefly shed some light on its application.

Ferulic acid has a wide range of applications in various fields, including the food industry, skincare that is cosmetics, pharmaceuticals, and agriculture.

1) Food additive: - FA is a natural food additive that is commonly used to stabilize food products and prevent rancidity. It's additionally utilized as an ingredient for flavouring in drinks and also as a color stabilizer in meat products. It stabilizes the coloration of the food and prevents them from discoloration. For example, it prevents green tea from discoloration.
2) Antimicrobial: - In today's world of increasing resistance of microorganisms which are highly pathogenic in a response attributed to the frequent use of antibiotics inadequately and transmission between individuals. To cope with the situation of the emergence of resistant microorganisms there have been studies done on plant

secondary metabolites which are involved in defense mechanisms and some of them have beneficial effects on health, which include antimicrobial properties. Ferulic acid is one of the secondary metabolites which help in this process. It is a potential natural alternative to synthetic antimicrobial agents as it has antimicrobial properties against a wide range of fungi and bacteria.

3) Cancer prevention: - Ferulic acid has been associated with anti-cancer properties, which include breast, liver, and colorectal cancer prevention. There are different studies showing that ferulic acid can significantly stop the proliferation of cells and the invasion of hela and caski cells By hindering the autophagy process and causing cell-cycle detention in the cervical cancerous cells in humans it may be acted as an anticancer drug.
4) Anti-inflammatory: - Conditions such as asthma, arthritis, and inflammatory bowel disease ferulic acid makes useful contributions in treating these types of inflammatory conditions. In in-vitro conditions, FA has been experimentally demonstrated to significantly inhibit the development of pro-inflammatory factors. The treatment of benzo pyrene-induced microglia with ferulic acid, for instance, it can help in reducing the neuro-inflammation and also the cellular factors responsible for inflammation such as IL-6, NO,ROS, IL-1β, which shows that ferulic acid can in fact play a role of neuroprotective by inhibiting the microglium -mediated proinflammatory responses.
5) Antioxidant: - Antioxidants belong to the most common bioactive components in anti-aging as well as anti-pollution and skincare items. While antioxidants are used in skin care products, they frequently cause stability issues. Due to this instability of active antioxidants, it ultimately reduces the efficacy of the product and, over time, the product changes its color during storage. Hence it automatically reduces the product acceptance in the market .Over the past few years, there seems to be a lot of curiosity about naturally generated active substances for skin care items.. Ferulic acid is a great source of antioxidants that really can offer protection from free radical oxidative damage. It has strong free radical scavenging activity. It has many potential benefits for the skin for instance anti photoaging, antiwrinkle, reduce pigmentation, skin lightening, anti-collagenase, and anti-tyrosinase efficacies, and in addition, it also absorbs harmful ultraviolet light which provides protection against the sun. However, it has limited in these products because of its poor solubility and stability.

2.2 Ferulic acid content

Recently the phenolic content of various fruits has been evaluated such as banana kinnow, mango, apple, pomegranate, and carrot. Banana pulp has the highest amount of polyphenols among them. Other authors noted that Kinnow peels had a larger amount of phenolic activity meanwhile Rai et al. show that under modified conditions jambolana has increasing phenolic content.Amount of FA in fruit waste in different portions.

Table 1. Content of ferulic acid in Indian fruits and vegetables

Fruits and vegetable	portion	Ferulic acid
Black carrot	whole	21.8±0.1
Musa	Peel Pulp	61.7±0.4 16.6±0.2
Kinnow	Peel Pulp	33.9±0.2 12.3±0.2
Mangifera	Peel Pulp	16.6±0.3 10.2±0.1
Orange carrot	whole	12.1
Apple	Peel	1.2 ±0.1

2.3 What are the stats on waste in India?

India is the world's second-largest producer of vegetables and fruits. It is also published in FAOSTAT that India is the world's leading producer of fruits. and the second-largest farmer of vegetables in the world. A variety of fruits and vegetables are grown in India for instance, *Mangifera indica*, *Musa sp.*, *Kinnow mandarin*, and *Vitis vinifera* are common fruits. *Solanum melongena* , *Daucus carota* , and *Momordica charantia* are some kinds of vegetables grown in India. Data of the Central Institute of Post-Harvest Engineering and Technology (CIPHET), Ludhiana. Punjab total shows that of 13,300 crores of waste is generated annually.

Table 2. Production of food waste and its by-product in vegetables and fruits.

Process of production	**By-product and waste (%)**
▪ White wine	20-30
▪ Red wine	20-30
▪ Fruit and vegetable juice	30-50
▪ Vegetable and fruit processing and preservation	5-30
▪ Vegetable oil production	40-70
▪ Production of sugar from sugar beets	85

Table 3. Different fruit wastes stats

Fruit	Waste's nature	Production(content)(tons)	Approx. Waste (%)	Potential amount of throw away (tones
Mango	Peels, seeds	6987.7	45	3144.4
Musa	Peels	2378.0	35	832.3
Citrus	Peels, rag,& seed	1211.9	50	6-06.0
Ananas	Skin , core	75.7	33	24.7
Vitis	Stem , skin &Seed	565	20	N/A
Guavas	Peels, core& Seed	565	10	N/A
Apple	Peel, pomace, and seeds	1376.0	N/A	412.0

2.4.1Kinnowwaste

In this experiment, we are going to use kinnow peel waste for the extraction of FA.It belongs to the Rutaceae family and member of the Magnoliopsida class. Kinnow is a hybrid of *Citrus nobilis* × *Citrusdeliciosa*. The citrus genus plants are usually cultivated in tropical and subtropical regions of the earth. According to reports in 2020, the total production of citrus across the world was 158.49 million MT, and India alone produced 139.97 million MT. Fruits of the citrus genus have many uses. If we look globally about 1/5th of all these fruits go into industrial processes for the production of juices which produce huge amounts of waste. kinnow waste especially peels have many bioactive compounds such as flavonoids, polyphenols, pectin, and essential oils. In the context of bioeconomy waste can be used to generate biofuels also. On a commercial level, the juice from citrus fruits is extracted from food processing plants, and for future consumption, it is packed. Furthermore, at a small scale, the juice is extracted from small to medium-sized juicers by fruit juice vendors and freshly served. In plants of food processing after the juice is extracted, they are left with the kinnow waste and this waste includes peels, seeds, and residual pulp. However, in later cases, the leftover material is called citrus pulp without peels. Studies have done that after sun drying this waste can be used as a component of a total mixed ration for livestock and poultry feed. In another study, it is shown that this waste could play an essential role in re-establishing the quality of the soil, water, along with air, as well as preventing groundwater depletion. It is mostly grown in the region of Haryana, Punjab, Jammu, Himachal Pradesh, Rajasthan, and Uttar Pradesh. Rich in vitamin C and antioxidants which are essential for a good and healthy life. Not only edible parts but nonedible parts like peels are also of great importance in pharmacology and rich in bioactive constituents. Currently, during juice processing, the peels were thrown out however recently food scientists and researchers looking for their use in different industries such as food industries, cosmetics industries, and pharmaceuticals industries. Moreover, there have been techniques to dry the kinnow peels, sun-drying, and oven drying and a new study has been done to dry it with the help of infrared and to check its influence on the quality characteristics.

The characteristics which were evaluated were total phenolic content, moisture, water activity, total flavonoid content, and DPPH radical scavenging activity.

Fig 4. *Citrus deliciosa*

Fig 5. *Citrus nobilis*

Fig 6. Kinnow

2.4.2Constitutes of Kinnows

Table 4. Nutritional profile

Part of fruit	Specific-nutrients/minerals	Quantity
Pulp	Bio-active compounds TPC TFC	 3.54 mg GAE g^{-1} 2.61 mg QE g^{-1}
Peel	Total phenolic content TFC Ferulic acid Coumaric acid Gallic acid	7.62 mg GAE g^{-1} 4.43 mg QE g^{-1} 5 mg 100 g^{-1} 1.7 mg 100 g^{-1} 3.78 mg 100 g^{-1}
Pulp	Minerals K Mg Na Ca	 1.35 mg g^{-1} 0.78 mg g^{-1} 0.43 mg g^{-1} 3.20 mg g^{-1}
Peel	Mg Na Ca K	0.73 mg g^{-1} 1.38 mg g^{-1} 0.55 mg g^{-1} 3 mg g^{-1}

2.5 Vanillin

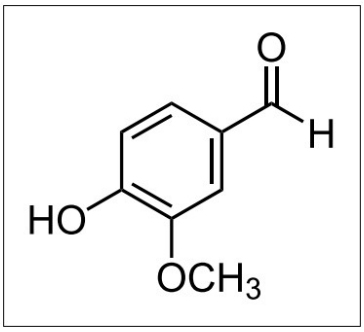

Fig 7. Vanillin

Vanillin was first coined by the scientist Gobley who back in 1858in France first isolated white crystals from *Vanilla planifolia*. The name 'vanillin' comes from the Spanish word vanilla, which is a shortened form of vaina, or layer.The chemical name of vanillin is4-Hydroxy-3-methoxybenzaldehyde and the molecular weight is $C_8H_8O_3$ .Vanillin is a common aromatic compound used in foods, beverages, perfumes, and pharmaceutical drugs. It is chemically synthesized by lignin and petrochemicals. It was used by the ancient Aztec Indians in Mexico and Totonaco it was written as “vanilla beans and their extracts”.A drink called chocolatl, has ingredients such as beans of cocoa , and ground corn and is flavored with tlilxochittl which was made from ground black vanilla pods and honey.

In 1874 vanillin was extracted using the oxidation of coniferyl alcohol beta-D-glucoside which is present in softwood pines species by Tiemann and Haarmann. In 1875 this led to the commercialisation of production of vanillin .and ame scitentist in 1876 develpoed other ways to syntheze vanillin from guaiacol and eugenol.Due to the concern to enviorment most of the facilities which were preparing synthetic vanillin had to shut down .In china guaiacol glyoxalic acid route is in use extensively. Supplying natural vanillin has been costly for industries. Vanilla extract alone cost a lot then it affected the cost of vanilla beans Which made it costlier for a small amount of vanillin. That is why the vanilla used in flavor industries is vanilla essence which is not vanilla.

So if we go back, in 1977, a microorganism that was present in soil identified as *Corynebacterium sp*, and *pseudomonas* eventually converted eugenol into ferulic aid and vanillin. The first patent for the natural production f vanillin was applied by Haarmann and Rrrrrr in 1991. They used strains of *Serrati sp*, *Enterobacter sp*. After that many discoveries have benn done related to this.

It is an aromatic compound that is well-known to found naturally as a glycosidebound to carbohydrates. The pods of vanilla have van-illic acid, p-hydroxy-benzaldehyde, p-hydroxy-benzoic acid, sugars, lipids, minerals, and water, apart from vani-llin. Vanilla generally extracted from *Vanilla planifolia* however, there are other varieties also present from which it can be produced. Firstly, it was synthesized from coniferin but as time and technologies get better there were different alternatives were used like sawdust, rice bran, etc. Vanilla extraction solely from plants only makes up to 1-2% of global needs, while other than natural sources it can be produced from various precursors, for instance, lignin waste, ferulic acid, and many more. researchers have found many important applications of the compound, and those studies have shown that it displays anticarcinogenic properties and anticlastogenic properties. In addition, it can stop the cell cycle of CR cancer cells. The term wonder drug is given to it, if it is utilized properly it can stand by that name. Although vanillin is used as a flavouring substance but can also be utilized in preservatives as it has antimicrobial properties also.It has gained the status of being generally recognized as safe. As it has various importance in different industries it is booming for the past several years and demand is also increasing. so if there can be any other methods to produce it, biologically not chemically it can be beneficial for society and its demand can be fulfilled easily. Our work is to bio-convert ferulic acid to vanillin.

CHAPTER 3
Materials and Methods

3.1 Chemicals

Ferulic acid (MP biomedicals, LLC), Standard vanillin (Loba Chemie),2-thiobarbituric acid (Sigma Aldrich), Sodium hydroxide pellets (HIMEDIA), absolute ethanol, Distilled water, ethyl acetate(SRL), Hydrochloric acid(Fisher Scientific), Sulphuric acid (MERCK), Acetonitrile (FINAR), Sodium hydrogen sulfite (MERCK), PDA(HIMEDIA), PDB(HIMEDIA) and Kinnow peels are obtained from the local market Of Waknaghat Himachal Pradesh.

3.2 Instruments

Weighing balance, Hot air oven, Grinder, Orbital shaker, pH meter, Incubator, Rotary vacuum evaporator, Laminar air flow hood, Autoclave, Centrifuge (Eppendorf), Multiskan spectrophotometer, water bath.

3.3 Others

Erlenmeyer flask (250ml, 150ml, or 100ml), dropper, autopipettes, spatula, measuring cylinder, Petri plates, Tarsons, cuvette, Eppendorf tubes, cotton.

3.4 Culture Preparation and Maintenance

Potato dextrose agar was used to culture *Rhizopus oryzae* and kept at 4ºC before use. The culture was firstly kept in 250ml of the conical flask for 3 days in potato dextrose broth on an orbital shaker at 180rpm at 25ºC. After 3 days, Petri plates were prepared of *Rhizopus oryzae* for reviving and for longer use. The culture in the broth will be used as inoculum for further studies in this experiment.

Fig 8. *Rhizopus oryzae* in PDB

Fig 9. *Rhizopus oryzae* in petri plates

3.5 Methodology

3.5.1 Extraction of

3.5.1 Extraction of Ferulic Acid

For Ferulic acid source, kinnow peels are used. Kinnow were peeled manually and weighed 100g after this these peels were kept for drying in an oven at 50ºC for 48 hours until it is completely moisture free. Further,the dried peels were ground in ultrafine particle and are now helpful for the production of the above mentioned compound.

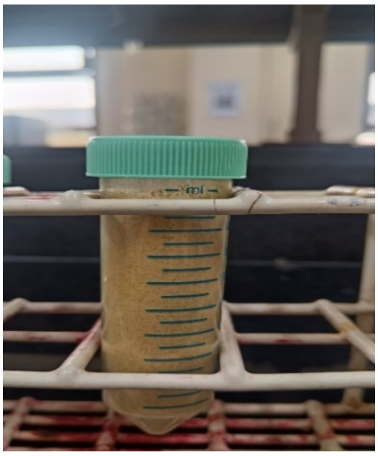

Fig 10. Kinnow peel powder

2g of powder was taken in a 250ml conical flask and mixed with NaOH solution. Ferulic acid can get oxidized so 0.001g $NaHSO_3$ was put in to the mixture. For the next 24 hours, the composition was kept on an orbital shaker at the speed of 108 rpm, 25°C. After one-day centrifugation basic hydrolysate was done on 7000rpm for 15 min.

Fig 11. Alkaline hydrosylate

Then the supernatant was acidified by 2M HCl (less than 2 pH). Ethyl acetate(60ml) was added to isolate the ferulic acid-rich fraction thrice to the solution.

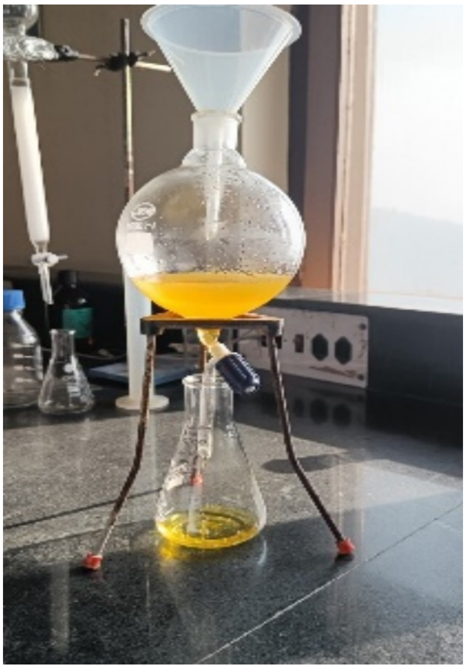

Fig 12. Filtration to isolate FA

Now rotary vacuum evaporator is used and concentrated crystals of ferulic acid were obtained which then dissolve in acetonitrile:water (1:1) for quantification of the compound. Furthermore, a standard curve was also prepared of known concentration to find out the sample concentration known concentration (5-40µg/ml).

Fig 13. Rotary vacuum evaporator

3.5.2 Production of Bio-vanillin by Fermentation

1 ml of inoculum was taken into 25 ml of autoclaved potato dextrose media which was mixed in pure water.

Fig 14. Inoculum in PDB and FA

The sterilized(using a membrane filter) ferulic acid which was taken from Kinnow peel was taken in various concentrations(0.3,0.5,0.7,0.9,1.1%) and was put into the media used for fermentation to enhance the concentration for hyperproduction of vanillin.

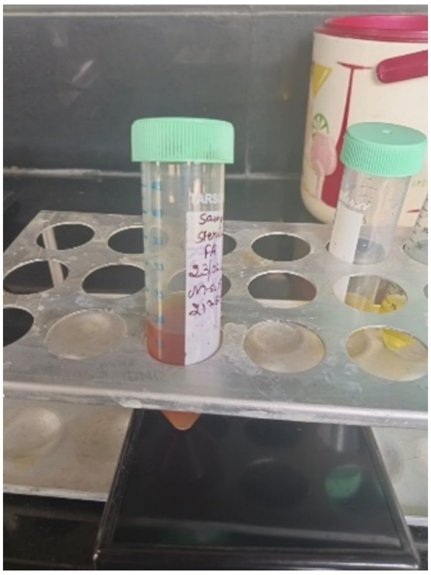

Fig 15. Sterilised Ferulic acid

All this procedure takes place in 150ml of conical flask i.e., Erlenmeyer flask. The fermentation media is then kept for incubation in an orbital shaker at 30ºC, pH5. After ending this process, the compound was taken out from the fermentation media and then assessed quantitively by the spectro-photometric analysis.

3.5.3 Optimisation of conditions for vanillin production

The physical parameters were optimized for hyperproduction like pH(5,6,7,8 and 9), Temperature(20,30,40,50,and60ºC), and incubation period(16,20,24,38 and 44 hours) by using one factor at a time.

3.5.4 Extraction of bio-vanillin from fermentation media

At the end of fermentation, the sample was taken in tarson for centrifuge (7000rpm for 10 minutes) to separate fungus from it, after the pH was set between 2-3 and an equal amount of ethyl acetate was added and again went for centrifugation at 3000 rpm for 1 minute the organic layer formed will be taken for further analysis.

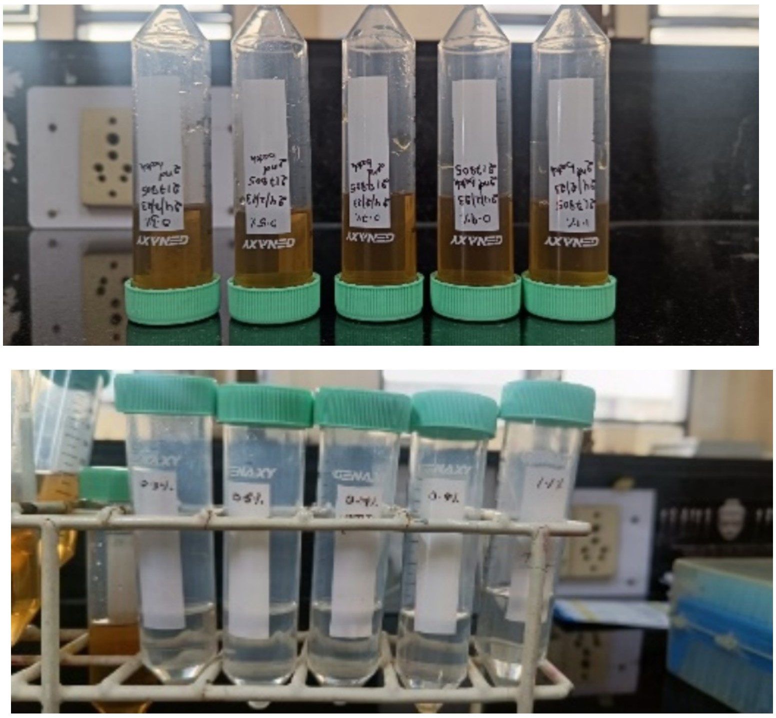

Fig 16. Organic layer separated using ethyl acetate

3.5.5 Analysis of the Samples

3.5.5.1 Quantitative analysis: - Acid colorimetric method

The sample is taken for analysis of whether vanillin is present on not for this 2-thiobarbituric acid was used. When thiobarbituric acid reacts with standard vanillin it is reported that it gives yellow orange color now the presence of vanillin is confirmed using the same method in fermentation media.

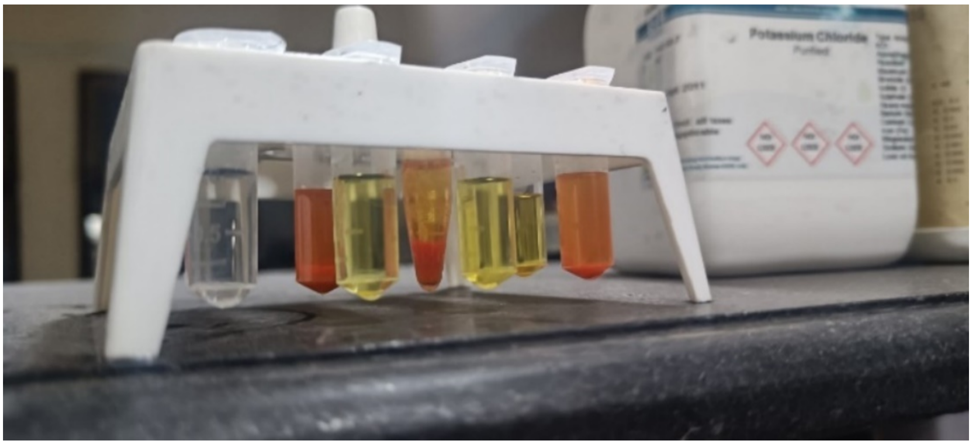

Fig 17. Yellow and orange colour of standard vanillin

3.5.5.2 Spectrophotometric analysis

In Eppendorf tubes,50µl of sample and 950µl of thiobarbituric acid reagent was added. The composition of thiobarbituric acid was 24% HCl,1% thiobarbituric acid, and distilled water. The tubes were kept in a water bath at 55ºC for 1 hour. After one hour let it cool for 20 minutes at room temperature then take the optical density of the samples at 434nm using a UV-vis spectrophotometer. Using the same method standard of vanillin was prepared and using the standard of vanillin, the amount of vanillin which is present in liquid media was calculated.

CHAPTER 4
Results and Discussion

As we mentioned above that people are moving to the idea where they are looking for more natural products whether they are skincare or diet. They are interested in the natural flavor; vanillin is one of the most famous and consumed flavors. Now as the biotechnology sector is growing they have given many alternatives to produce natural flavours. Amycolatopsis sp. Stain HR166 has given satisfying results using 19.9g/l of ferulic acid has given 11.5g/l of vanillin with-in 32 hours.

4.1 Estimation of ferulic acid

The ferulic acid standard graph was prepared with known concentrations and concentrations of the sample were calculated using the standard graph. From 2g of kinnow peel powder 2.35±0.55 mg ferulic acid was obtained.

Table 5. Quantitative estimation by spectrophotometer

Concentration (µg/ml)	Absorbance(310nm)
5	0.39
10	0.842
15	1.062
20	1.382
25	1.731
30	2.128
35	2.176
40	2.535
Unknown sample	
23.55	1.564

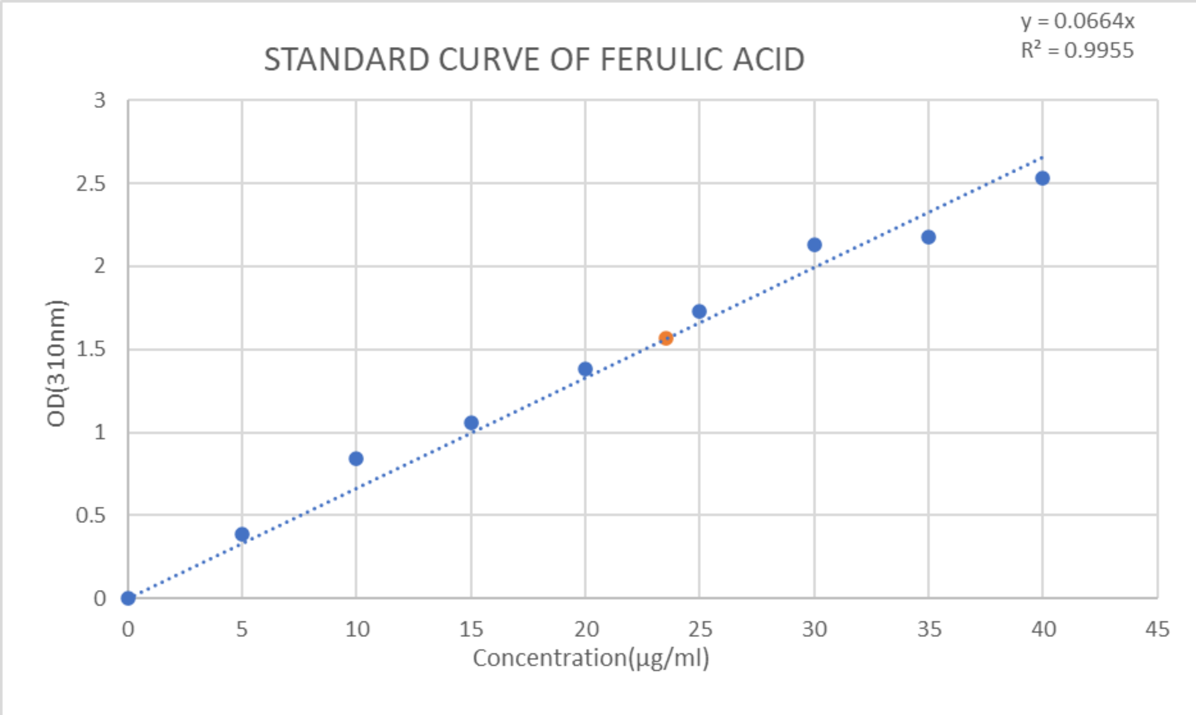

Fig18. Standard curve of ferulic acid

4.2 Estimation of bio-vanillin

4.2.1 Acid colorimetric analysis

The reaction of 2-thiobarbituric acid with vanillin showed a change in color i.e., yellow which confirms the presence of vanillin in the samples.

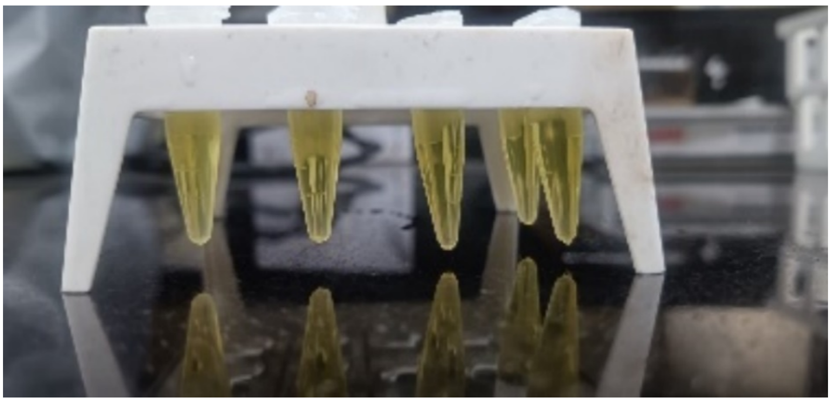

Fig 19. Colorimetric test

4.2.2 Spectrophotometric analysis

The standard graph for vanillin was prepared and using this graph we can find out the concentration of the unknown sample (Fig.20).

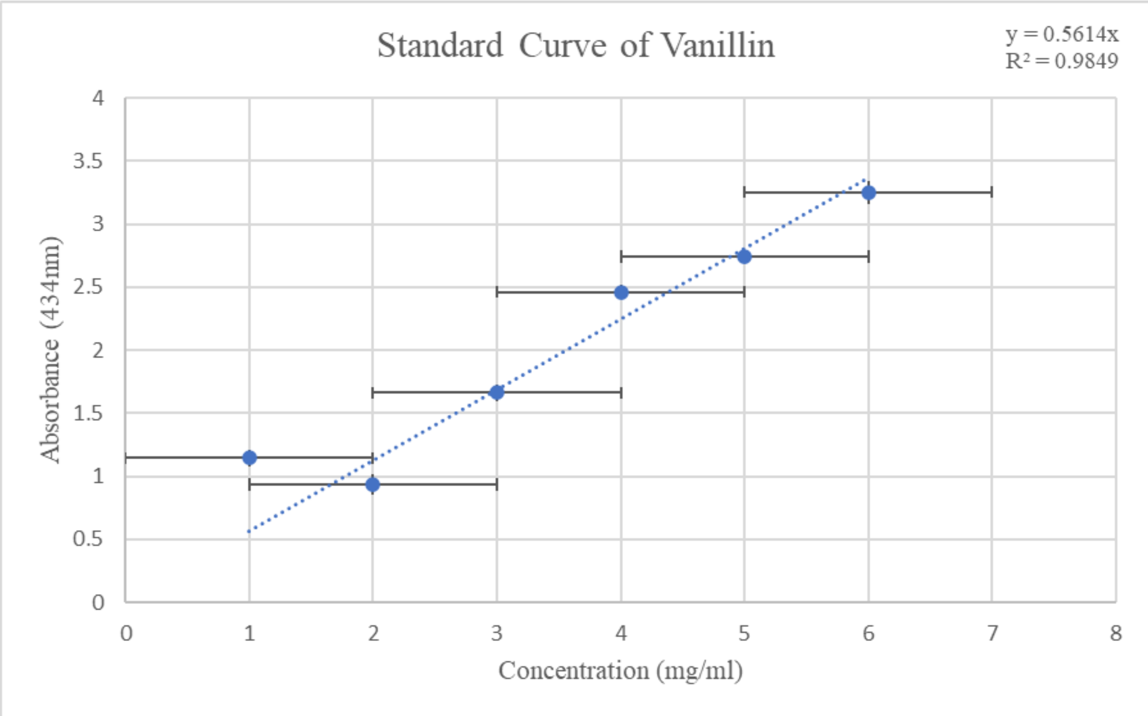

Fig 20. Standard curve of Vanillin

4.3 Optimization of substrate concertation

Rhizopus Oryzae brings out a maximum bio-vanillin of 0.43mg/ml at a0.5% of ferulic acid. Vanillin production first increases however when concentration gets increased it starts declining. As ferulic acid is harmful to micro-organisms at higher quantity this resulted in low production of vanillin.

Other studies show that 2.5g/l of ferulic acid mass was potent because it produced maximal bio-vanillin that a transformation the yield of 10percent from *Pseudomonas reinovorans* strain SPR1 Zaho et al. used *Bacillus fusiformis* CGMCC1347 , it is an a extremely isoeugenol-tolerant strain, the formation of vanillin their result showed that substrate concentration was increased from two percent to sixty percent, and the production of vanillin elevated from 1.67 gram per litre to 32.5g/l. Although there is opposition to the finding two researchers Vaithanomsat and Apiwatanapiwat found 10g/L concentration is more effective for high production of vanillin they used *A. niger* and *P. cinnabarinus*.

The same result is obtained in this study when FA concentration increased more than 0.5% there is a decline in vanillin production.

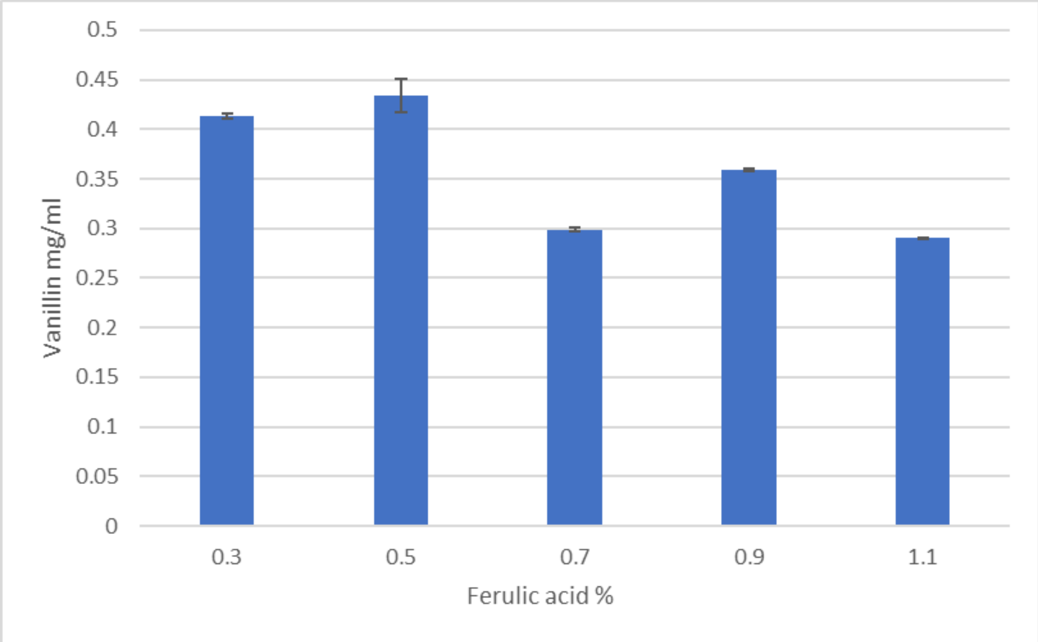

Fig 21. Effect of ferulic acid %

4.4 Optimisation of pH

Notably at pH 6 a higher production of vanillin was observed as shown in Fig. 22 *Rhizopus oryzae* works more efficiently at pH 6 for vanillin production i.e.,0.92mg/ml.

Vanillin which is produced before and after this pH is low. In agreement with this study, experiments were done to check the effect of pH on the production Tilay et al. run fermentation at pH ranging from 4 to 7 and they got a better yield at 6.5 pH, a low quantity was seen at lower pH. However, contrary, to this experiment, Mazhar et al. reported that at Ph 7 they got optimum vanillin it was done using bacteria *E.hormaechei* (KT385666). Chattopadhyay et al. revealed at pH 7.5 using *Streptomyces sannanensis* (MTCC6637) they got the highest production of vanillin.

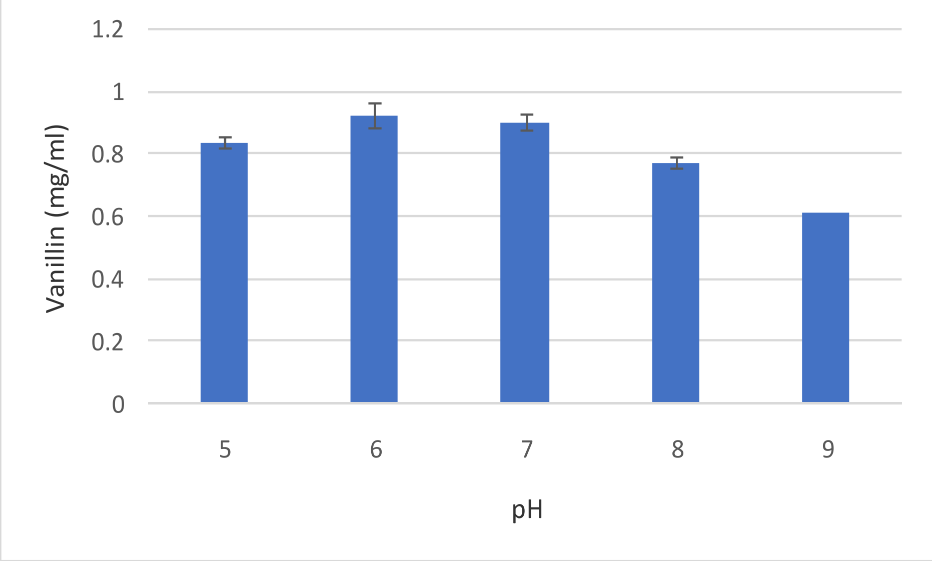

Fig 22. Effect of pH

4.4 Optimization of temperature

While working with microbial systems temperature plays a remarkable role in the growth of micro-organisms or to achieve a specific target, similarly for optimization temperature is important to maintain. An elevation can be seen at 30°C as shown in Fig. 23.

Similar results were obtained by *E.coli* JM109 at a temperature of 30°C. As Mazhar et al. also got the same results at this temperature. In contrary to this, Wangrangsimagul et al. observed that at the temperature of forty-five degrees for *Brevibacillus agri* 13 it gets converted into bio-vanillin from eugenol.

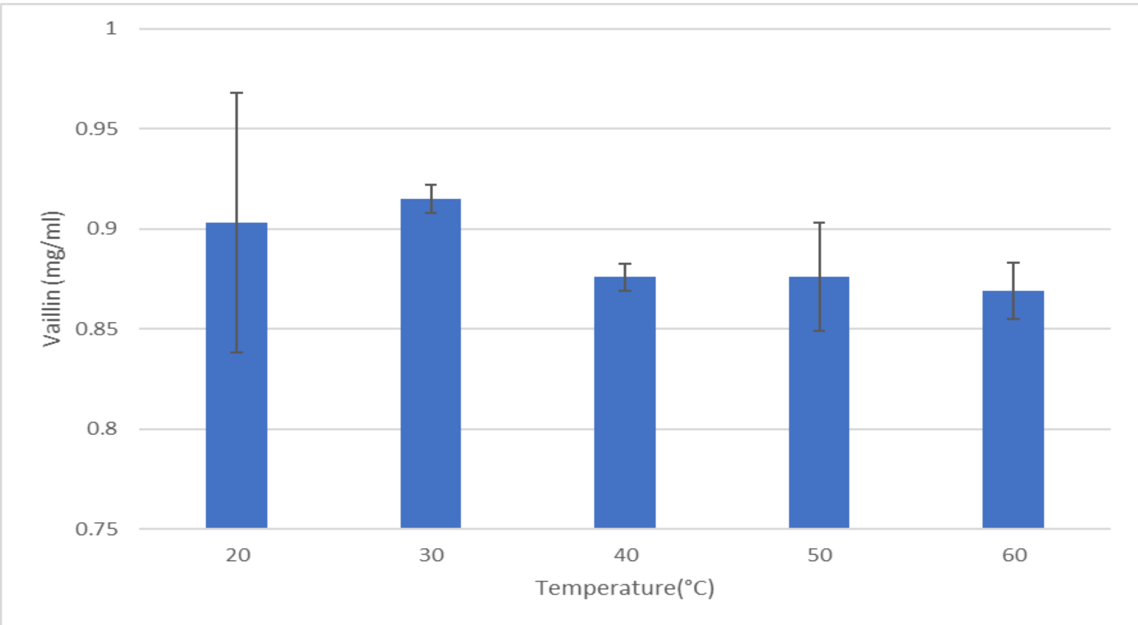

Fig 23. Effect of temperature

4.5 Optimization of incubation periods

Rhizopus oryzae shows maximum production at an incubation period of 16 hours which was 0.69mg/ml as shown in Fig. 24. It can also be seen that the concentration of vanillin starts to decrease with an increase in the number of hours with time it accredit to its malignant identity Chakraborty et al. according to his studies vanillin is an aldehyde also an aromatic one, that exhibits poisonous behavior on cells at higher concentrations because of that micro-organisms transform it into a less malignant chemical which is vanillic acid rather than vanillin , that's why decreases in concentration can be seen after 16 hours of incubation.

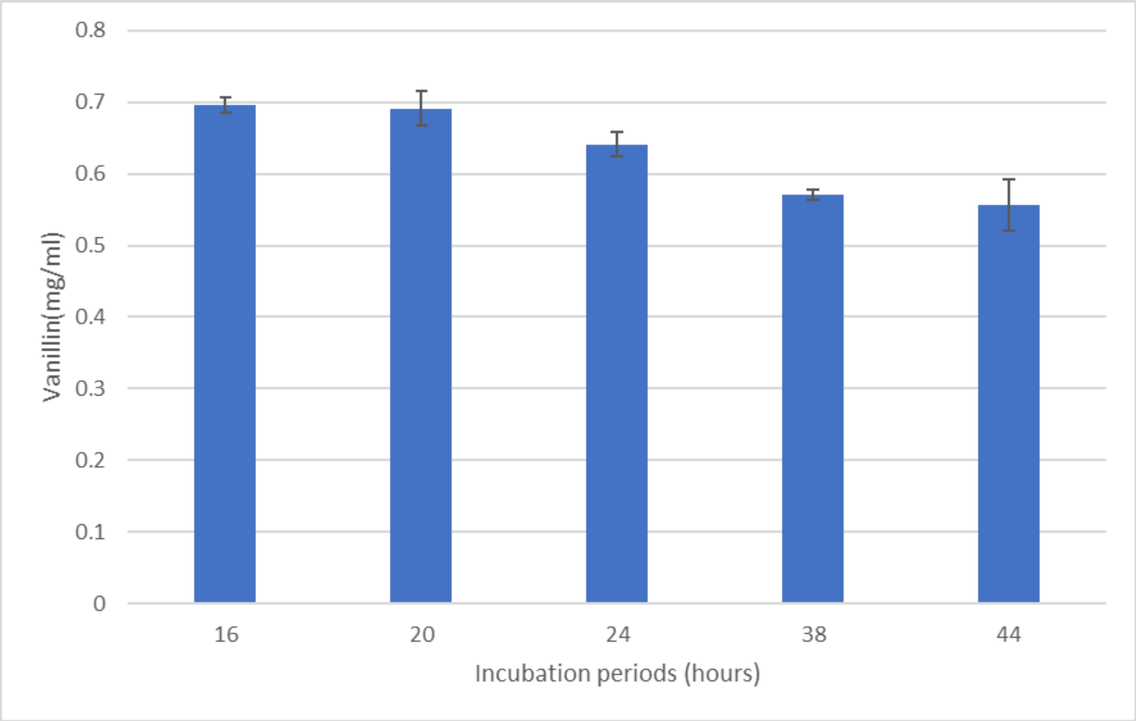

Fig 24. Effect of incubation period

CHAPTER 5

Conclusions

- Ferulic acid is a costly precursor for vanillin it is important to extract it from sources that are cheap and can easily be available for which waste of fruits is a good alternative, as it is easily available and using waste is eco-friendly.
- 2.35mg of ferulic acid was extracted from 2g of the powder of kinnow peel waste.
- The fermentation was improved by optimizing the following conditions i.e. pH, temperature, incubation time, and concentration for *Rhizopus oryzae*.
- The maximum production of vanillin was at 0.5% FA, pH 6, temperature 30°C, 16h incubation period.
- *Rhizopus oryzae* helps convert ferulic acid in the intermediate compound i.e., vanillin.

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