This reproduction of the Aztec calendar stone found in the National Museum of Anthropology and History in Mexico City. In the third ring from the outside are depicted the 20 symbols of the days of the sacred almanac. They are meant to be read in a counterclockwise direction, beginning at the top left.
Chapter 1: Preliminary studies

Location of study area

Preliminary study and the samples for the preliminary were obtained from the following farms,

1. Mangala farms, Karkala, Dakshina kannada, Karnataka.
2. Sediypu Agros, Puttur, Dakshina kannada, Karnataka.
3. Ednad Shankar Bhat, Ednad (Kumbla) Kasaragod Dist, Kerala.

(Source: www.tageo.com)

Figure. 1. Location of farms and study area

(Source: www.tageo.com)

Figure. 1. Location of farms and study area
Table 1. Location of the study area.

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Name of the Farms</th>
<th>Location</th>
<th>Elevation (in Feet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sediyapu Agros, Puttur</td>
<td>12°45’18.20” N</td>
<td>419</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75°11.52.84” E</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mangala farms, Karkala</td>
<td>13°05’30.86” N</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td></td>
<td>74°58’36.70” E</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ednad Shankar Bhat, Kumbla</td>
<td>12°35’20.15” N</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75°01’12.14” E</td>
<td></td>
</tr>
</tbody>
</table>

*Google earth

Brief description of the farms:

1. Mangala farms, Karkala, Dakshina kannada, Karnataka (Farm 1)

   Vanilla cultivation is mainly using glyricidia (*Gliricidia sepium* (Jacq.) as support (22,000 veins) and also in a mixed cropping system with areca as support (16,000 veins). Annual production is 6 quintal of green beans and another 1.25 quintals of cured bean are produced by shade net cultivation using concrete pole as support.

2. Sediyapu Agros, Puttur, Dakshina kannada, Karnataka (Farm 2)

   One of the experienced and a progressive farmer involved in the cultivation and curing of vanilla beans with total land holding of 160 Acres. Vanilla is cultivated mainly in areca plantation (1-2 veins/palm) as support in about approx 150 Acres. Another 5 Acre with coconut palm as support and also glyceridia as support in another 5 Acre. Concrete poles are also used as support for vanilla vein with the total cultivation 50,000 veins. Normally 12 flowers are pollinated per vein.

3. Ednad Shankar Bhat farms, Ednad, Kasaragod Dist, Kerala (Farm 3)

   A small scale grower, having 2000 veins producing 25 kg of cured beans. Practicing mixed cropping system with areca and glyricidia as supports (1 vine/palm).

1. Field visit, observation and collection of samples:

   Farms were visited during the months October to January from 2005 to 2008 for the observation, collection of samples on processing (curing) aspects. All the activities of the farm right form harvesting to the curing of bean were recorded.

   The vanilla samples were collected from the above farms from green beans to conditioned beans. Approximately 250 grams of samples were collected of each day during sunning (6-10 days), rack drying and conditioning samples. The samples were collected in polythene bags, labeled and brought to the lab and refrigerated at 4° C until processing.
2. Isolation and identification of the Microorganisms:

(a) Isolation and subculturing of Bacteria and Yeast

Microbial enumeration of vanilla beans was carried out by standard plate count (SPC). Both surface and endogenous microflora were isolated for the studies. The outer tissue of the beans was peeled using a sterile stainless steel blade and approximately 1 gram of the tissue was taken into a test tube containing physiological saline (0.85%) and diluted serially. To isolate the endogenous flora the internal tissue of the bean was scraped with a sterile blade and the same procedure as mentioned above was followed. Exactly 1 ml of the diluents was plated on Nutrient agar (NA) and Potato dextrose agar (PDA) for bacterial and fungal/yeast isolation. Plates were incubated at 35° C and at 27° C for 48 Hrs for bacteria and fungi/yeast respectively and observed for the colony, counted, tabulated and expressed in terms of colony forming units (cfu). Isolated culture were purified and subcultured and stored at 4°C for further studies.

(i) Screening of bacteria and Yeasts for β-Glucosidase production (Plate assay)

Purified isolates were screened for the production of β-glucosidase by plate technique using 4-Methyl Umbelliferyl β-D-Glucopyranoside (4-MUG) as a substrate following the method reported by Fia et al., (2005). The plates were point inoculated and incubated at 25° C for 48 h and plates were observed under long wave UV light, a positive culture will have a Fluorescent halo around the colony. β-glucosidase if produced will break the substrate into 4-Methyl Umbelliferone and β-D-Glucopyranoside. Under UV light the former will give blue fluorescence indicating a positive culture.

(ii) PCR, Gel Electrophoresis and sequencing

Bacterial cultures were identified by Colony polymerase chain reaction (PCR) assay targeting the 16S rRNA followed by sequence analysis. The oligonucleotide sequence of primers used in the study were, forward (Uni1): 5'-AAGGAGGTGATCCAGCCGCA-3' and reverse (Uni 2):5'-AGAGTTTGATCCTGGCTCAG-3'. A well isolated colony was suspended in the reaction mixture using micropipette tip.

(a) PCR conditions

All reactions were carried out in a thermal cycler (Technee, USA) in 25 µl reaction volumes. Composition of the reaction mixture is given in the table 1. Standard amplification conditions for primers UNI 1 and UNI 2 were initial denaturation of 94° C for 5 minute,
followed by 45 cycles of 95° C of 1 min denaturation, annealing at 55° C for 1 min and extension at 72° C for 2 minute, followed by final extension step of 72°C for 10 minute. Negative control reactions without any template DNA were carried out simultaneously.

Table. 2. Composition of PCR for bacterial isolates

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Taq buffer with 15 mM MgCl₂ (10x)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>2 dNTP Mix (2.5 mM Each) (10 mM)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>3 Primer (10 pmoles/µl) F</td>
<td>0.5 ul (5 pmoles)</td>
</tr>
<tr>
<td>R</td>
<td>0.5 ul (5 pmoles)</td>
</tr>
<tr>
<td>4 Taq DNA polymerase (5U/µl)</td>
<td>0.3 ul (1.5 U)</td>
</tr>
<tr>
<td>5 Nuclease free water</td>
<td>19.7 µl</td>
</tr>
<tr>
<td></td>
<td>25.0 µl</td>
</tr>
</tbody>
</table>

(b) Identification of Yeasts by internal transcribed region (ITS):

Yeast strains were identified by colony PCR amplification and sequencing of rRNA region spanning the 5.8 rRNA gene and the two ITS as a rapid and easy method for yeast species identification.

Cells were directly collected from a fresh yeast colony using a micropipette tip and suspended in 25 PCR reaction mix and used as a template in the reactions to amplify a segment of the eukaryotic rRNA operon encompassing the 5.8S rRNA gene and the flanking internal transcribed spacers ITS1 and ITS2. The primer sequence used includes, forward ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3' and Reverse ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'. The composition of PCR mixture is given in the table 2.
Table 3. Composition of PCR for yeast identification

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Taq buffer with 15 mM MgCl₂ (10x)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>2 dNTP Mix (2.5 mM Each) (10 mM)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>3 Primer (2.63 pmoles/µl) F</td>
<td>1.9 µl (5 pmols)</td>
</tr>
<tr>
<td></td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>1.9 µl (5 pmoles)</td>
</tr>
<tr>
<td>4 Taq DNA polymerase (5U/µl)</td>
<td>0.3 µl (1.5 U)</td>
</tr>
<tr>
<td>5 Nuclease free water</td>
<td>16.9 µl</td>
</tr>
<tr>
<td></td>
<td>25.0 µl</td>
</tr>
</tbody>
</table>

(c) PCR conditions:
Amplification was carried out using Techne (USA) thermocycler. PCR conditions were as follows: Initial denaturation of 94° C for 7 minute, followed by 45 cycles at 94° C for 45 sec, of denaturation, 56° C for 1 min annealing and extension at 72° C for 1 minute, followed by final extension at 72° C for 7 minute. Negative control reactions without any template DNA were included during the run.

(d) Electrophoresis:
Amplified products of both Bacterial and Yeast were separated by agarose gel electrophoresis (Genei electrophoresis unit, Bangalore) in 2 % agarose using TAE Buffer with applied voltage 110. DNA Markers (Genei electrophoresis unit, Bangalore) of size 10 kb and 1 kb for bacteria and yeasts was also loaded along with the samples to determine the size of the products obtained. The gels were stained with 2 µl/100 ml ethidium bromide and photographed under UV light with using gel documentation system. The band of interested was excised from the gel and saved to isolate the amplified product using DNA purification kit (Sigma USA). Purified products were once again checked for purity and also to confirm the molecular size of the product before sequencing.

(e) DNA Sequencing:
Purified 16S rRNA and ITS genes were sequenced using the respective primers. Sequencing was performed with the Abi3100 Genetic analyser DNA sequencing system using the software kbasescholar (ver: 5.1.1) facilities available at Bangalore Genei, Bangalore. The identity of the obtained sequences were determined by comparison to DNA sequences within GenBank® using BlastN (http://www.ncbi.nlm.nih.gov/BLAST).
(iii) Scanning Electron microscopy (SEM):

(1) SEM of vanilla beans:

Small pieces of vanilla beans (Green and cured) were fixed overnight at room temperature in 6% glutaraldehyde in Phosphate buffered saline (PBS). The samples were rinsed 3 times in PBS for 15 min each. They were then dehydrated by giving two half-hour immersions in two washes in 100% acetone (Polak-Vogelzang et al., 1979).

(2) SEM of Bacteria and yeast:

Cultures were grown in Nutrient broth (pH 7.2) for bacteria and a medium (YEPD) containing 1.7 g YNB, 5.0 g (NH₄)₂SO₄, 5.0 g yeast extract, 5.0 g peptone, 5.0 g glucose per liter (pH 6.0) for yeast for 24 hrs at 35°C for bacteria and at 27°C for yeast respectively. Cells were harvested by centrifugation at 8000 rpm and the palette was washed 3 times in Phosphate buffered saline (PBS) and fixed in 2.5% glutaraldehyde in Phosphate buffered saline (PBS). Suspension was incubated at room temperature for 2 hours and later was washed 3 times in PBS and collection of the pellet by centrifugation and later fixation in 1% Osmium tetroxide. Finally dehydrolysis of the sample by different ethanol volumes starting; 30%, 50%, 70%, 90% and 100% and for each ethanol volume incubation was done for 10 minutes and final incubation in 100% ethanol for 1 hour (Atkey and Wood, 1983).

A conductive coating of platinum was applied on both the above samples using Emscope sputter coater and the specimens were examined in a scanning electron microscope JSM-6380 (JEOL/EO, Germany) at 5 kV accelerating voltage and images were captured using the computer software (Version 1.1)

(iv) Biochemical characterization of screened bacteria and yeasts:

(a) Growth at different temperatures:

Growth characteristic of the identified organisms were determined at three temperatures i.e., 37°C, 45°C and 60°C for bacteria and 27°C, 37°C and 45°C for yeasts. 24 hours old culture was inoculated to 100 ml of Nutrient broth (pH 7.2) (HiMedia, Mumbai) and YEPD (Yeast extract 10 g, peptone 20 g, Glucose 20 g, Distilled water 1000 ml, pH 5) incubated in temperature controlled orbital shaker (Newtronic orbital shaking incubator 725, Newtronic equipment company, Bombay) with 150 rpm. Optical density of the culture was measured every 2 hour using spectrophotometer 104 (Systronics, Ahmadabad) at 600 nm.
(b) Utilization of complex substrates:

The production cellulase, proteinase, amylase and xylanase were determined through plate assay to find out the hydrolyzing capacity of cellulose, protein, starch and xylan respectively. For bacteria following media was used, whose composition is given in the table 1, 2 and 3.

(i) Cellulose hydrolysis was tested on CMC Agar. Bacterial strains were spot inoculated and after 48 hours the plates were flooded with Iodine solution, positive culture can be seen with the clear halo around the colony due to hydrolysis of cellulose. Diameter of the halo was recorded.

Table. 4. Composition of CMC Agar (g/Lit)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaN03</td>
<td>2.0</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO4</td>
<td>0.5</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Carboxy methyl cellulose (CMC)</td>
<td>2.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.2</td>
</tr>
<tr>
<td>Agar</td>
<td>17.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

(ii) Protease production was determined on casein-gelatin agar plates. Culture was spot inoculated on the plate. Positive cultures can be identified by the clear halo around the colony.

Table. 5. Composition of Casein-gelatin plates (g/Lit)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>10.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>10.0</td>
</tr>
<tr>
<td>Nutrient broth</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
</tr>
</tbody>
</table>

(iii) Starch degradation was tested on starch agar plates. Cultures were spot inoculated, after 48 hours the plates were flooded with Iodine solution, positive cultures can be seen with the clear halo around the colony due to hydrolysis of starch. Unhydrolysed area is stained dark violet.
Table. 6. Composition of starch agar plates (g/Lit)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Starch</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>16.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

(iv) Xylan degradation was tested on Xylan medium, whose composition is same as that of CMC agar, where CMC was replaced by Xylan 2.0 (gm/lit). After 48 hours the plates were flooded with Iodine solution, positive isolates can be seen with the clear halo around the colony due to hydrolysis of xylan.

Yeast isolates were also characterized similarly using potato dextrose agar as media (pH 5) with the respective substrates as mentioned above.

(c) Vanillin resistance:

Vanillin resistance was determined by supplementing nutrient broth with vanillin at fixed temperature of 37° C. Stock solution of vanillin (2.5 M) was prepared in absolute alcohol and added to the nutrient broth so as to get the required concentration (1 Mm, 10Mm and 20Mm) in the broth. Vanillin was added to the sterilized media after autoclaving at 121° C for 20 min. Sterile broth was inoculated using 24 hrs grown culture and the flasks were incubated in orbital shaker at 37° C. Optical density of the culture was recorded every 2 hours using spectrophotometer. Alcohol (0.8 %) was also used as control along with control without vanillin/alcohol.

Similar experiment was carried out for yeast isolates, using the media YEPD (Glucose 20 g, Peptone 20 g, Yeast extract 10 gm, Water 1000 ml, pH 6.2) at 27°C.

(d) Screening of bacteria and yeast for β-Glucosidase production in liquid medium:

Identified bacterial and yeast isolates were further tested for the β-Glucosidase production in liquid medium to use in the controlled fermentation.

Bacterial isolates were grown in CYPE broth in 250 ml Erlenmeyer flask. Composition of CYPE broth is given in the table. Medium was sterilized by autoclaving at 121° C for 20 min and inoculated with 0.5 ml/100 ml containing 10⁶ cells/ml and incubated 37° C in an orbital shaker at 150 rpm for 3 days.
Table. 7. Composition of CYPE broth (g/Lit)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxy methyl cellulose (CMC)</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Yeasts isolates were cultured on YNB-G Medium for the production and estimation of β-glucosidase (Manzanares et al., 2000). Medium was sterilized by autoclaving at 121° C for 20 min and inoculated with overnight cultures cultivated on the same media and incubated at 27° C in an orbital shaker at 150 rpm for 3 days.

Table. 8. Composition of YNB-G Medium (g/Lit)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast nitrogen base</td>
<td>1.7</td>
</tr>
<tr>
<td>NH₄SO₄</td>
<td>5.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
</tr>
</tbody>
</table>

After 24, 48 and 72 hours, 1 ml culture was withdrawn aseptically from both the cultures in laminar air flow unit and centrifuged at 8000 rpm for 10 min at 4° C to get supernatant to test extra cellular activity and the pellet was used to check the cell bound activity. Supernatant was used as such, pellet was washed 3 times using cold distilled water and re suspended in citrate phosphate buffer to test the cell bound activity.

**Assay for β-glucosidase:**

β-glucosidase activity was measured using 2-nitrophenyl β-D-Glucopyranoside (2-pNPG) as substrate. 200 µl of supernatant/cells suspended in citrate buffer (pH 5.0) and 200 µl of 2-nitrophenyl β-D-Glucopyranoside (5mM) was incubated for 1 hour at 40° C. The Reaction was stopped by the addition of 1.2 ml of carbonate buffer (250 Mm, pH 10.2) and the yellow colour developed due to the release of p-nitrophenol (pNP) was read at 400 nm using spectrophotometer (Ferreira et al., 2001). For quantification, a standard curve was
prepared using pNP ranging between 0 and 200 nmol. Enzymatic activity was expressed as nmoles of pNP produced per ml per hour. All the assays were performed in duplicate.

(b) 1. Isolation and identification of fungi involved in the spoilage of vanilla bean by Standard blotter method (SBM):

Fungal contaminated and suspected vanilla beans were subjected to standard blotter method (SBM) for isolation of fungi. Vanilla beans were placed in 190 mm diameter petri dish containing three layers of moist blotter paper using sterilized distilled water. The plates were incubated at 27±1° C for 7 days under cool white fluorescent light with alternating cycles of 12 hours light and 12 hours darkness. The light source was cool day fluorescent tubes emitting 1900 luxes of light (Neergard, 1977; ISTA 1976; Elwakil and Ghoneem, 2002). After seven days of incubation, samples were examined for the presence of fungi and were identified under stereo binocular microscope using standard manuals (Barnett and Hunter, 1972). Fungal cultures were purified and subcultured and stored at 4° C for further studies.

2. Production, Extraction and Analysis of Secondary metabolites (Mycotoxin) of fungal isolates:

All the isolated strains were grown on 10 g shredded sterilized wheat with 6 ml of water in a 250 ml Erlenmeyer flask for two weeks at room temperature. Twenty-five grams of anhydrous Na₂SO₄ and 70 ml of CHCl₃ were added to the flask. Mixture was boiled on a steam bath for 10 min and the CHCl₃ extract was decanted through a double thickness whatman No. 1 filter paper. Metabolites were re extracted from the culture with additional 50 ml of CHCl₃ twice. Extracts were pooled and evaporated on a steam bath to approximately 5 ml. Hexane was added drop wise until the solution became cloudy. The solution was allowed to stand at 0° C for 4 hr. The precipitate which formed was collected and air dried (Rodricks, 1968) and redissolved in Chloroform. Approximately 0.03 ml of the extract was spotted on analytical TLC plate (TLC Silica gel 60 F₂₅₄, Merck, Germany) using Benzene:Acetonitrile (98:2) and observed under long wave UV light and plates photographed.

3. Water activity (aw):

Percent water activity (aw) of cured vanilla samples were measured at fixed temperature of 27 ± 1° C using Aqualab water activity meter (Model Series 3 TE, Decagon

40
4. Moisture content analysis:

Vanilla beans were cut into small pieces, and exactly 5 grams in duplicate weighed (Metler Toledo, Switzerland) and dried in an oven at 105±° C for 16 hours (Clifford, 1985). Moisture content was determined due to the loss of weight due to drying and calculated using the formula given below,

\[
\frac{W_1-W_2}{W_1} \times 100
\]

Where, \(W_1\) = Initial weight and \(W_2\) = Final weight

5. pH

pH of the vanilla beans undergoing curing was determined by grinding 1 part of vanilla beans with 2 part of distilled water (1:2) and pH of the suspension was determined using pH meter (Systronics, Ahmadabad) (Adebajo and Diyaolu, 2003).

6. Estimation of vanillin (By UV spectrophotometric Method):

A standard procedure of AOAC was followed (AOAC, 1990). The most commonly used quantification method by the flavor industry for routine analysis is the AOAC 966.12 method (AOAC, 1990). Quantification is done by the absorbance measurement of an alkaline solution of the extract at 348 nm.

(a) Preparation of standard curve

0.100 g vanillin was dissolved in 5 ml alcohol and diluted to 100 ml with distilled water. Transfer 15, 10 and 5 ml was transferred to 250 ml standard flask and diluted to volume with distilled water and mixed properly (Solns X). 10 ml of soln X was pipetted into 100 ml standard flask and diluted to volume with distilled water. Another set of 10 mL solns X was pipetted into 100 ml standard flask and added approximately 80 ml H2O and 2 ml 0.1 N NaOH, shaken and diluted to volume with H2O. Absorbance of the alkaline solution at 348 nm using spectrophotometer using neutral solution as reference blanks. Standard curve was plotted.
(b) Preparation and analysis of sample:

One gm of vanilla beans was ground using 45 % ethanol. Slurry was centrifuged at 3000 rpm for 10 min and supernatant was used for the estimation. Five ml of the extract was diluted to 100 ml with distilled water further analysis was done as mentioned above.

7. Total phenolics:

(a) Extraction:

200 mg of Vanilla beans + 10 ml of 70% chilled aqueous acetone was ground using cold pestle and mortar. Slurry was transferred to the centrifuge tube and centrifuged at 10,000 RPM at 4° C for 15 min. Supernatant was collected and used for the estimation.

(b) Estimation:

50 μl of the supernatant was taken in test tube and made up to the volume to 0.5 ml with distilled water then added 0.25 ml of the Folin-ciocalteu reagent and 1.25 ml of Sodium carbonate solution. Tubes were vortexed and incubated at room temp for 40 min. Blue colour developed was read at 725 nm. Amount of total phenol was calculated as Gallic acid equivalent from the calibration curve constructed using 0.02, 0.04, 0.06, 0.08 and 0.10 ml of Gallic acid standard (Makkar et al., 1993).

8. Activity of vanilla bean β-Glucosidase:

Finely chopped vanilla beans (5 gm) were ground in the chilled pestle and mortar with 20 ml of cold Sodium citrate buffer (0.1 M, pH 5.0). Resulting slurry was centrifuged at 10,000 RPM at 4° C for 30 min and the supernatant was used as enzyme source.

The reaction mixture consists of 100 μl of the 0.1 M Sodium citrate buffer (pH 5.0) + 200 μl of enzyme extract + 200 μl of 0.0055 M p-Nitophenol-β-D Glucopyranoside (pNPG) as substrate. The mixture was vortexed and incubated at 40° C for 20 min. After 20 min the reaction was terminated by the addition of 1 ml of 1 M Na₂CO₃. Yellow colour thus developed due to the releases of p-nitrophenol was read in the spectrophotometer (Shimazdu, Japan) at 400 nm and quantified using a standard graph constructed using p-Nitophenol.
9. Analysis of vanilla bean volatiles:

(a) Extraction by Simultaneous distillation-extraction (SDE):

Simultaneous distillation-extraction (SDE) method was carried out as per Likens-Nickerson (1964) for the isolation of volatiles. Approximately 100 gm of vanilla beans were finely chopped and taken in a sample flask with 200 ml of distilled water. Before starting the distillation, U-tube separator section of the apparatus was added with distilled water to cover the tube and also approximately 5 ml of diethyl ether. The apparatus was assembled as shown in the figure 2 with a solvent flask containing 100 ml of diethyl ether on the other side (Figure. 2). Distillation was carried out by introducing a jet of steam into the sample flask; the steam generated in the sample flask will carry the free volatiles along with and condense oven the condenser. The volatiles will be transferred to the ether. Ether containing the volatiles will be collected on the other side. This cycle was allowed repeated for 2 hours. Distillation time was evaluated after the first distilled drops of each distillation solvent entering into its original flask. Trace of water if any in the ether was removed through dry ice and evaporated to dryness under nitrogen gas. Finally the oil fraction (20 mg Approx.) was made into 1 percent solution and subjected to Gas chromatography Mass spectrometry (GC/MS).

Figure. 3. Simultaneous distillation extraction (SDE) setup for the isolation of volatile compounds from vanilla beans (Likens-Nickerson, 1964).
(b) Analysis

Extracted oil was analyzed in Shimadzu GC-17A Gas chromatograph attached Mass spectrograph (QP5050A). (Shimazdu, Kyoto, Japan) with a capillary column, DB-5 ((5%-Phenyl)-methylpolysiloxane, L: 30 m, i. d 0.25 mm, film thickness: 0.25 μm) (J&W Scientific, Folsom, CA, USA).

For operation, the conditions of the instrument was programmed as follows: column temperature from 60°C to 200°C at the rate of 4°C/Min, held at initial temperature and at 200°C for 5 min and further 280°C at the rate of 10°C/Min held at final temperature for 20 min. The injector and interface temperature was 210°C and 280°C respectively. The instrument was operated using helium as a carrier gas with the flow rate of 0.9 ml/min. One μl of samples were injected in split mode. The peaks were identified from the spectral library, Wiley/INST Libraries available in the attached computer.

10. Anatomical studies of the bean:

Green and cured vanilla beans were used for the anatomical studies in order to estimate the cellular damage due to traditional curing. Thin cross sections of specimens were obtained by hand held razor blade and transferred to watch glass containing safranin staining solution for 2-3 min and washed once again in distilled water and transferred to a fresh watch glass containing distilled water. Thin sections of the specimen were taken on a glass slide (7.5 cm x 2.5 cm) covered with cover slip and observed under stereomicroscope (Leica DMLB2) with Photo micrographic attachment using digital camera Cannon Powershot S45. The images were captured using remote shooting software (Zoom browser). Internal tissue of the beans were observed using stereomicroscope (Magnus MS 24).
1. Vanilla beans

Only matured, uniform green beans were selected for the experiment. The beans were sub sampled in to small bundles (Approximately 1.5 Kg each) for each treatment and packed in a low density (LD) polythene bags and sealed using the plastic sealing machine. Beans were irradiated to low dose (2.5 kGy) gamma rays for microbial decontamination to eliminate the surface as well as endogenous microflora for controlled fermentation.

2. Production of inoculum

Based on the above biochemical studies following candidate organisms are used in the controlled fermentation,

Bacteria: *Bacillus cereus* and *Bacillus thuringiensis*

Yeast : *Pichia guilliermondii* and *Pichia anomala*

Selected organisms were mass produced in a 250 ml Erlenmeyer flask using Nutrient broth and Potato dextrose broth for bacteria and yeast respectively. Thus inoculated flasks were incubated in an orbital shaker at 32° C for 24 hrs for bacteria and at 26° C for yeast at 150 rpm. Cultures were harvested by centrifugation (Huttuch, Zentrifugen, Germany) at 8,000 RPM at 4° C for 20 Min. Supernatant was discarded and the pellet was washed twice with phosphate buffered saline (PBS) and finally suspended in PBS. The cell strength was adjusted to $10^8$ cells/ml according to the optical density readings. The following terms were used in this study to designate the type of inoculum for the ease of use,

1. Cocktail : Mixed culture containing more then one species and/or genus
2. Co-cultivation : Inoculum containing more then one species

3. Fermentation method:

For controlled fermentation following protocol was used,

(a) Sun drying method

1. Cocktail (Bacteria and yeasts : *Bacillus cereus & Bacillus thuringiensis* and *Pichia guilliermondii & Pichia anomala*)
2. Co-cultivation (Two bacteria : *Bacillus cereus & Bacillus thuringiensis*)
3. Co-cultivation (Two yeasts : *Pichia guilliermondii & Pichia anomala*)
4. Phased addition (Cocktail: first two yeasts followed by two bacteria after 5 days of sun drying)
5. Control (Irradiated beans)

(b) Oven method
1. Cocktail (Bacteria and yeasts: *Bacillus cereus* & *Bacillus thuringiensis* and *Pichia guilliermondii* & *Pichia anomala*)
2. Co-cultivation (Two bacteria: *Bacillus cereus* & *Bacillus thuringiensis*)
3. Co-cultivation (Two yeasts: *Pichia guilliermondii* & *Pichia anomala*)
4. Control (Irradiated beans)

4. Inoculation of beans

Two type of inoculation was followed. In type one the culture were applied on the surface of the bean using atomizer. In another type the culture suspension was introduced inside the bean by making slit along the half of the bean using sterile stainless steel blade. 50 µl each culture was then inoculated into the bean. All the procedures were carried out inside the laminar air flow unit (Klenzaids, India) to avoid any cross contamination and subjected to sun drying and oven drying.

Polythene bags containing inoculated beans were subjected to traditional sun drying on black cloth and exposed to the midday sunlight for approximately 2 hours daily. Moisture accumulated inside the bags was removed by making a small opening in the corner of the bags and drained out. Each day, after sunning the bags were wrapped using the same black cotton cloth and kept inside the thermocole box inside the carton overnight. This procedure was repeated until sufficient moisture was lost from the beans. In oven method, the Polythene bags were incubated in oven set at 40° C continuously until sufficient moisture is lost. Finally both the samples were subjected to rack drying and conditioning.

Approximately 30 grams of beans were sampled regularly at 0, 5, 10, 20 and final cured bean on 40th day, which includes 8-10 days of sunning, 5-6 days of rack drying and approximately 15 days of Conditioning. Rack drying was performed in a processing laboratory on steel rack (RT 30° C) and conditioning inside a carton in an aluminum foil wrapped around the beans. Samples withdrawn at the regular intervals were subjected to moisture analysis, β-Glucosidase activity, microbial enumeration, estimation of vanillin and other phenolics and volatiles.
5. Moisture content of bean: As mentioned above

6. Microbial enumeration:

The Microbial enumeration was carried out as before. 10 grams of beans was crushed in 90 ml of saline using stomacher (England) in a sterile polythene bag and the resulting diluents was serially diluted and plated on a plate count agar and incubated at 32°C for 48 hours and populations was expressed in terms of colony forming unit.

7. Activity of β-Glucosidase: As mentioned above

8. Estimation of vanillin and other phenolics by HPLC:

(a) Standard chemicals

Vanillin (Fluka, France) and vanillic acid, p-Hydroxy benzaldehyde, p-Hydroxy benzoic acid, guaiacol and coumarin were obtained from Aldrich, (Germany). Where as glucovanillin was previously isolated from the green bean through preparative TLC, purified, identified (Acid hydrolysis/HPLC), quantified and used as standard. Methanol and Acetonitrile was of HPLC grade (Merck, Mumbai).

(b) Extraction

25 gram of cured vanilla beans were powdered in pestle and mortar using liquid nitrogen and suspended with 100 ml of 45% ethanol, the resulting slurry was kept overnight, to prepare vanilla extract. Aroma constituents were extracted using omni mixer (Sorval, England) using muslin cloth, cake was further extracted with 100 ml x 2 of 45% ethanol. Extracts were pooled, centrifuged at 8,500 rpm for about 20 min at room temperature; supernatant was used for the estimation.

Above extract was further diluted (0.5 ml of extract + 9.5 ml of 45% ethanol), filtered using 0.45 μm membrane filtered (Millipore, Germany) and injected (25 μl) in to the HPLC system using microsyringe (Hamilton, Switzerland)

(c) Estimation

High Performance Liquid Chromatographic (HPLC) was performed in Jasco, PU 980 Pump with column length of 250 mm x 4.6 mm (ThermaHypersil-Keystone, UK) with UV Detector (UV 975) set at 275 nm and analyzed using Jasco Borwin-PDA Software (version 1.50). The mobile phase consisting of methanol 50 ml, Acetonitrile 100 ml and 850 ml of water acetified with 10 ml of acetic acid with the flow rate 1 ml/Min (Archer, 1989).
A standard containing vanillin 100 mg, vanillic acid 10 mg, \(p\)-hydroxy benzaldehyde 10 mg, Vanillyl alcohol 10 mg and \(p\)-hydroxy, benzoic acid 5 mg was prepared in 100 ml of HPLC grade methanol and diluted appropriately to get 5 point calibrations.

(i) Preparation of Analytical and preparative TLC plates:

30 gram of silica gel (Merck, Germany) was taken in the stoppered conical flask (250 ml). 60 ml of ammonium sulphate solution (3%) was used to prepare the slurry, shaken thoroughly to get homogenous slurry and immediately poured into the applicator (set to 0.25 mm) and drawn on a clean glass plate. Plates were activated 160°C for 1.5 hours and stored in desiccators.

Preparative plates were prepared similarly using distilled water (40 grams of silica gel with 80 ml of distilled water) with the thickness of 0.5 mm.

(ii) Preparation of column:

A clean, dry column was filled with approximately 30 gram of the Amberllite XAD-16 (Sigma, USA) using Methanol. Column was tightly packed by gently taping the column using rubber tube. Initially column was washed with 100 ml each of Methanol, diethyl ether and distilled water respectively. The column was ready for use and sample can be introduced slowly. Once the sample run was complete, once again column was washed in the reverse order i.e., distilled water, diethyl ether and Methanol. Methanol fraction contains glycosides was collected, concentrated under reduced pressure and made into percentage solution.

(a) Isolation, identification and estimation of glucovanillin;

(i) From green beans

Glucovanillin from the fresh green were estimated as described by Dignum, (2002). 5 grams of ground beans was taken in 100 ml volumetric flask with 65 ml of acetate buffer (pH 5.0), mixed well for 5-10 min. The suspension was heated in microwave oven to deactivate the enzymes. Finally the volume was made up to mark with methanol; a small volume was filtered and analyzed through HPLC. HPLC conditions were same as that used for the estimation of vanillin and other phenolics.
(ii) From cured beans:

The aroma glycoside was analyzed as per Gunata, (1985) with slight modification. Cured vanilla beans were collected from three farms. 10 grams of cured vanilla beans in duplicate were powdered using liquid nitrogen and suspended in 100 ml of aqueous methanol (80:20) overnight. The slurry was stirred using omni mixer (Sarvoll, Newtown CT) and filtered through buchner funnel, the cake was extracted twice with 100 ml each of aqueous methanol to get approximately 300 ml of extract. Extracts were pooled, dried by flash evaporator and re suspended in 100 ml of distilled water and passed through XAD-16 column of 26 (L) x3 cm ID (13 cm of resin). Finally methanol fraction (containing glycosides) was collected, concentrated under reduced pressure using flash evaporator and made into percentage solution.

(iii) Identification of Glucovanillin by acid hydrolysis:

Small portion of the above methanol extract was dried using flash evaporator. 0.4 ml of 2 N HCl was added and hydrolyzed for 1 hour over water bath maintained at 60-70°C under reflux. The acidic solution was then neutralized with 4-5 ml of water was extracted with diethyl ether (3x5 ml) in separatory funnel. Ether layer was again washed with distilled water. Finally ether was concentrated and the extract was analyzed by TLC and also injected into GCMS for confirmation.

Portion of the above extract was used for the estimation of glucovanillin though HPLC and also the samples were loaded on to an analytical TLC plate containing Ammonium sulphate, and developed with ethyl acetate/isopropyl acetate/water (65:30:15) to check the total glycosides of the vanilla beans. The compounds were visualized by heating the plate at 120°C for 1.5 hours.

Remaining portion of the above extract was used for the isolation of glucovanillin through preparative TLC. The plates were developed with the above solvent system. The bands were visualized with iodine vapors; band corresponding to glucovanillin (which was previously identified through acid hydrolysis and HPLC) was marked, scraped and eluted with methanol, dried using flash evaporator and finally made into percentage solution and used for the preparation of standard graph in the estimation of glucovanillin through HPLC and also used in the Pulse radiolysis studies.
(b) Radiation processing of Green vanilla beans:

a. Irradiation and Curing

Vanilla beans, 15 kgs were purchased from the farmer, transported immediately to the lab and stored in a cold room until process. The sub samples were made into small bundles of approx. 1.5 kg x 2 for each dosage and irradiated at 5.0, 10.0 and 15.0 kGy excluding unirradiated bean as control. Irradiation was carried out in Gamma irradiator (GC5000).

Soon after irradiation the beans were subjected to the traditional curing. Each bundle of the beans were packed in a polythene bag and subjected to sun drying for approximately 2 hours daily. Moisture deposited inside the polythene bags were removed manually by a making a small opening in the corner of the bag and drained out as it accumulates. After sunning, each day beans were wrapped using the black cotton cloth and transferred to the carton overnight for preserving the heat. The above procedure was repeated until sufficient moisture was lost and ready for the next steps i.e., rack drying. At the end of sunning, beans were hand picked with sufficient moisture loss and subjected to rack drying. In our experiment the sunning lasted for about 8 days. Loss of moisture during different stages of curing was estimated as mentioned before.

Rack drying was performed in a processing laboratory at room temperature (30±1°C). The rack drying lasted for about 7 days, followed by conditioning where the beans were wrapped in butter sheets and kept inside the carton for approximately one month and subsequently analyzed for following,

b. Moisture content of bean : As mentioned above

c. Estimation of Vanillin and other phenolcis (HPLC) : As mentioned above

d. Analysis of Volatiles (SDE/GCMS) : As mentioned above

(B) Radiation processing of cured vanilla beans

Moisture content (%) and water activity (aw) of fungal infected cured beans were determined as mentioned before.
Samples and Irradiation:

5 kg of Cured vanilla beans were procured from one of the farmers and irradiated. Beans were sub sampled into small bundles of 500 gm each and irradiated in the dose range of 1, 2, 3, 4, 5, 10, 15, 20 and 30 kGy, unirradiated beans were treated as control.

(i) Microbial enumeration by standard plate count : As mentioned above

(ii) Estimation of vanillin and other phenolics (HPLC) : As mentioned above

(iii) Extraction and analysis of free fatty acid of irradiated cured vanilla beans:

In order to check the chemical changes induced if any due irradiation, free fatty acid profiles were studied by thin layer chromatography.

(a) Extraction:

50 gm of vanilla beans was grinded using liquid nitrogen to fine powders using pestle and mortar and the sample was extracted with 100 ml of Hexane. Slurry was filtered using Whatman No. 1 Filter paper and once again extracted with 100 ml of hexane. The total extract was approximately 200 ml. Extract was concentrated and made into percentage solution (1%).

(b) Analysis:

Approximately 0.03 ml of the extract was applied on the analytical TLC plate (TLC Silica gel 60 F_{254}, Merck, Germany) in the band form and developed using the mobile phase Hexane:ethyl acetate:Acetic acid (80:20:1). Developed plates were sprayed with 10 % H_{2}SO_{4} and heated in oven for 10 min and observed for banding pattern and photographed.

Further compounds showing degradation upon irradiation as observed in the present study was isolated and purified through preparative TLC and identified by GCMS.

(iv) Mechanism of radio resistance of Glucovanillin (Pulse radiolysis)

(a) Irradiation of purified Glucovanillin:

Small volume of purified glucovanillin was dissolved in distilled water and irradiated at different dosage (10, 30 and 60 kGy) to check the resistance of glucovanillin. Irradiated solution was directly injected into HPLC System to check the products formed.
(b) Pulse radiolysis

Glucovanillin was dissolved in N$_2$O saturated medium and subjected to pulse radiolysis studies. Pulse radiolysis experiments was carried out using 7 MeV electron accelerator (Mukherjee, 1997) (Forward Industries Ltd., UK) delivering 50 ns single electron pulses at a dose rate of ~10 Gy/pulse. A 450 W Xe-arc lamp, a UV-visible kinetic spectrophotometer, a Larsen & Toubro digital oscilloscope (model 4072) and a computer were used for data acquisition and analysis. Dosimetry was performed by pulse radiolysis of an air-saturated 5×10$^{-2}$ mol dm$^{-3}$ KSCN solution with $\lambda_{\text{max}}$ at 500 nm using $\epsilon_{500\text{nm}}$ D 21,520 dm$^{-3}$ mol$^{-1}$ cm$^{-1}$ (Fielden, 1982). Electrochemical studies for the determination of reduction potential of vanillin were performed using a cyclic voltammeter (Princeton Applied Research Universal Potentiostat-model 273). A three-electrode glass cell comprising a glassy carbon electrode (0.4 cm diameter), a saturated calomel electrode (SCE) and a Pt auxiliary electrode were used for all measurements. Sample solutions were prepared using nanopure water and contained 10$^{-3}$ mol dm$^{-3}$ vanillin, 10$^{-3}$ moldm$^{-3}$ phosphate buffer and 0.1 moldm$^{-3}$ KCl as a supporting electrolyte. The solution pH was adjusted by adding dilute acid/alkali. Prior to any measurements the glassy carbon electrode was polished using standard technique (Mahal et al., 1999). All sample solutions were maintained at 25±2°C and purged with purified N$_2$ gas for five minutes prior to scan runs. The observed potential values were against SCE and converted to those versus the normal hydrogen electrode (NHE) by adding 0.24 V to the observed values (Mahal et al., 2001).