Diversity of yeast with β-glycosidase activity in vanilla (*Vanilla planifolia*) plant

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Microbial contribution to vanilla flavour development has not been fully investigated. The precursor molecules of vanillin are converted to vanillic acid by β-glycosidase enzyme. Contribution of β-glycosidase enzyme from plant and microbial sources during the process of curing is yet to be fully understood. In the present work the diversity of yeast from rhizosphere soil in curing and storing of bean has been studied. Yeast with β-glycosidase activity was 0.47% in the field, which increased to 1.75% during curing of the total diversity. A total of 4655 isolates with 86 morphological different colonies were screened, of which 35 forms belonging to 17 genera were found to be β-glycosidase producers. Further testing for their ability to convert ferulic acid to vanillic acid proved all the 35 forms utilized ferulic acid. *Bullera* sp. (MVY22) was found to be the most promising, which was further investigated for its conversion ability. HPLC analysis proved the conversion of ferulic acid to vanillic acid by this isolate.

Keywords: Aroma, curing, β-glycosidase, vanilla, yeast diversity.

MICROBIAL contribution to natural vanilla flavour has been suggested but never investigated[1]. The differences in microbial abundance, communities and strain characteristics between batches could differ, resulting in difference in flavour[1]. Green vanilla beans contain aroma precursors, primarily vanillin β-D-glycosidase (or glucovanillin) and minor glycosides of *p*-hydroxyl benzaldehyde, *p*-hydroxy benzyl alcohol and vanillic acid[2], which are hydrolysed by β-D-glucosidase activity upon ripening on the vine or during the curing process[3,4]. This results in the release of aromatic glucons and the generation of a strong vanilla flavour. β-Glycosidase (β-glucan glucohydrolase) EC.3.2.1.21 has been used in the industry for production of fuel alcohol[5] and for animal feed due to its cellulolytic activity. Ferulic acid is one of the major phenolic lignin monomers to be degraded by β-glycosidase[6]. Microbial activities on vanilla bean cell compounds release ferulic acid[7], which can be transformed by a large variety of bacteria and fungi into flavour compound, like vanillin and guaiacol[8]. Ferulic acid, eugenol, isoeugenol, vanillic acid and coniferyl aldehyde have been the most frequently studied biochemical precursors of vanillin[9]. Exploiting microbial and enzymatic transformation of renewable natural materials like ferulic acid is a means of generating value-added products. The present work was undertaken to study the succession of yeast flora with β-D-glucosidase activity during bean maturation and curing.

Three vanilla plants (*Vanilla planifolia*) were identified and tagged at a plantation in Puttur, Karnataka (India). Samples were collected from November to March 2006–07. Vanilla flowers and beans are harvested from February to April and November to January respectively. During this period stem, leaves, flowers, young beans and rhizosphere soil were collected from the tagged plants.

Samples from various stages of curing, starting from freshly harvested beans to fully cured and stored beans were collected from four locations. Location I at Puttur from where plant samples were collected. Locations II and III from Kasargod, Kerala and location IV from Kodagu, Karnataka. Samples from different stages of processing were collected as listed in Table 1. The samples were then transported in an ice box (4°C) to the laboratory and stored at –30°C till further use.

For the isolation of surface microflora, samples were swabbed using sterile cotton swabs from leaf, stem and bean (10 cm area). Outer and inner surface of the flower was swabbed and used. Swabs were resuspended in 10 ml saline solution and serially diluted to appropriate dilutions. Soil rhizosphere microflora was isolated by serial dilution method using 1 g of soil. Component plating methods were employed to isolate yeast from various components of the bean, like outer skin, pulp, papillae and seeds. Whole undamaged pods were surface-sterilized with 30% hydrogen peroxide (5 min) and washed with sterile distilled water twice and the components were dissected using sterile forceps and plated. Yeast count was performed on Sabourauds Dextrose Agar (SDA) medium containing 12 μg ml^{-1} streptomycin and incubated at 37°C for 7 days. All analyses were done in triplicate. Colonies with different morphology were selected from the plates and subcultured. Purity of the isolates was checked by streaking on fresh SDA plates, followed by microscopic examination. Isolated strains of yeast were preserved as slants with 15% glycerol and stored at –20°C. Yeast isolates were identified based on morphological and biochemical test, according to Barnett *et al.*[10].

Using the agar plate method β-glycosidase activity was checked with esculin, as described by Rosi *et al.*[11]. The medium contained yeast extract 6.7 g, esculin 5 g, agar 20 g in 1000 ml distilled water at pH 5.0.

In the microtitre plate assay, sterile microtiter plate with wells of 200 μl capacity was loaded with 100 μl esculin broth and 5 μl of culture. The plates were incubated for 96 h at 22°C. Appropriate control was maintained. The change in colour was noted visually and the development of fluorescence was confirmed under UV.

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Table 1. Vanilla samples from various locations

Location I	Location II	Location III	Location IV
1. FH	1. FH	1. FH	1. FH
2. One-day SD	2. Six-day SD	2. Kelled	2. Nine-day SD
3. Two-day SD	3. Rack-dried	3. One-day SD	3. One-year old
4. Three-day SD	4. Conditioned	4. Two-day SD	
5. Four-day SD		5. Seven-day SD	
6. Five-day SD		6. Nine-day SD	
7. Rack-dried		7. Twelve-day SD	
8. Conditioned		8. Fourteen-day SD	
		9. Rack-dried	
		10. Cured bean	

FH, Freshly harvested; SD, Sun-dried.

Table 2. Yeast isolates from various parts of the vanilla plant

Month	Soil ($\times 10^4$/g)	Stem	Leaf	Bean	Flower
November	470 ± 55	483 ± 69	518 ± 49	463 ± 67	–
January	398 ± 49	382 ± 91	427 ± 5	388 ± 34	–
March	325 ± 23	261 ± 38	400 ± 27	–	140 ± 8

Counts are average of triplicates with standard deviation. Total count expressed as CFU. –, Not done.

Selected yeast isolates which exhibited β-glycosidase activity were grown in 250 ml basal medium and incubated on a rotary shaker at 250 rpm, 22°C for 48 h. The medium contained (g/l) maltose 20, ammonium nitrate 1.842, potassium dihydrogen phosphate 0.2, calcium chloride 0.0132, magnesium sulphate 0.5, yeast extract 0.3, ferulic acid 0.3, pH 5.6. Samples were withdrawn at 0, 24 and 48 h and centrifuged at 8000 *g* at 4°C and the supernatant was collected for the assay. A modified method of Anthony and Crowford[12] was followed to assess the bioconversion of ferulic acid to vanillin. Supernatant (100 μl) was diluted to 2 ml with distilled water and subjected to spectrophotometer analysis between 225 and 375 nm. The rate of vanillic acid production was measured by monitoring an increase of absorbance at 282 and 250 nm by one unit.

The concentrations of individual polyphenols were determined using Shimadzu LC-6A HPLC system. A reversed phase C-18 column (15 cm × 150 mm with a pore size of 5 μm) was used with a flow rate of 1 ml min^{-1}. Water, methanol and acetic acid were used as eluents in the ratio of 80 : 15 : 5. A SLC-6A system controller and a CR4A data processor were used. Compound detection was done through a UV detector, SPD-AV at 280 nm. The standard mixtures of phenolics were prepared in the concentration of 1 mg/ml. Twenty microlitres of standard mixture and culture supernatant was injected into the column after filtering through 0.2 μm filter. Peaks were identified by comparison of retention times of the standards like gallic, tannic, protocatecholic, vanillic, caffeic, syringic, gentisic, *p*-coumaric, ferulic and cinnamic acids.

A total of 4655 yeast isolates with 86 morphologically different colony characters (size, shape and budding) were isolated from the soil, leaves, stem, bean and flower (Table 2). Yeast colonization was better in November in all the samples. This may be due to the high moisture prevalent in the samples at the end of the rainy season. Among the field samples, rhizosphere soil had the maximum population. On testing for β-glycosidase activity, only 22 were found positive.

Fresh beans at the curing unit had more microbial count compared to the freshly harvested samples (Table 3). This may be due to the proliferation of yeast during transport from field to curing units. The beans are normally bundled together and wrapped as soon as they are harvested (each bundle of approximately 1–1.5 kg, i.e. 150–200 beans) and are stored under shade till they are transported to the curing units at the end of the day. Results revealed that there was a decrease in the yeast population during curing. This may be due to the large temperature increase faced by the microbes during curing, as the pods are packed in airtight boxes in the evening after sun-drying everyday and temperature normally ranges between 40°C and 47°C. A total of 299 morphologically different colonies were isolated from curing unit 1, 118 from unit 2, 196 from unit 3 and 53 from unit 4 during the whole period of the investigation. Of these, five were found to be positive for β-glycosidase activity.

Component plating is regularly used to isolate intracellular microflora[13]. Component plating indicated very low colonization of yeast inside the pods. Only 76 isolates were isolated from various components, even though representative samples from all the groups during curing from all four locations were plated. No sample had more than one type of colony, with a large number of samples having no yeast microflora. Among the yeast isolates, only eight were found to be positive for β-glycosidase activity. They were further tested and confirmed for enzyme activity.

Table 3. Yeast microflora during curing

Sample*	1	2	3	4
Freshly harvested	560 ± 49	380 ± 78	420 ± 49	–
Kelled			210 ± 59	–
One-day SD	460 ± 36	400 ± 39	230 ± 82	–
Two-day SD	620 ± 89	–	230 ± 60	–
Three-day SD	460 ± 66	–	–	170 ± 59
Four-day SD	390 ± 31	–	–	–
Five-day SD	230 ± 50	–	–	–
Six-day SD	–	400 ± 84	–	100 ± 68
Seven-day SD	–	–	260 ± 55	–
Nine-day SD	–	–	300 ± 36	70 ± 33
Twelve-day SD	–	–	260 ± 51	–
Fourteen-day SD	–	–	80 ± 31	–
Rack-drying	140 ± 19	240 ± 39	90 ± 22	–
Conditioning	130 ± 8	160 ± 19	–	–
Cured bean	–	–	10 ± 4	–
One year stored bean	–	–	–	100 ± 13

*Readings are averages of triplicates. Total count expressed as CFU.

Table 4. Samples showing positive β-glycosidase activity

Sample	Month	No. of isolates with β-glucosidase activity
Soil	November	–
	January	2
	March	1
Stem	November	–
	January	1
	March	1
Leaves	November	2
	January	4
	March	5
Beans	November	1
	January	3
Flower	March	2
Total		22

Morphologically different yeast cultures were isolated, purified and tested for β-glycosidase activity. Samples collected in the month of November had the least number of positive isolates (3), whereas samples of January and March had 10 and 9 respectively (Table 4). This result is contrary to the total population, where there was a slightly higher population in November. Among the parts, leaf had maximum of 11 isolates with β-glycosidase activity and flower the least number. Bean had four isolates positive for β-glycosidase. During curing, bean swab sample had maximum isolates (5) with β-glycosidase activity. Whereas component plating indicated the presence of three isolates each in seed and pulp.

The yeast isolates producing β-glycosidase yeast were identified based on morphological and biochemical tests[10] (Table 5). The forms identified were *Cryptococcus* (2 isolates), *Stephanoascus* (1), *Kockovaella* (1), *Brettanomyces* (1), *Rhodotorula* (1), *Trichosporon* (1), *Dekkara* (2), *Candida* (6), *Lipomyces* (3), *Sacchoromycopsis* (1), *Pichia* (4), *Debaryomyces* (1), *Bullera* (4), *Bensingtonia* (1), *Nadsonia* (3), *Kodamaceae* (1) and *Saccharomyces* (1).

Yeast isolates with β-glycosidase activity hydrolyse the substrate and a dark brown colour develops in the agar around the colony. Plates when observed under UV light exhibited a fluorescent halo around the culture. Isolates with β-glycosidase-positive forms were tested for their ability to convert ferulic acid to vallinic acid by UV absorption method.

Decrease in ferulic specific absorption at 350 nm and increase in vanillic acid specific maxima at 282 and 250 nm indicated conversion by the isolates[12]. Upon aeration and incubation at 22°C, bioconversion of ferulic acid to vanillic acid was observed. All the β-glycosidase-positive yeast isolates depicted bioconversion of ferulic acid into vanillic acid.

Among the isolates, *Bullera* MVY 22 exhibited very high conversion of ferulic acid to vanillic acid. The culture exhibited strong vanillin flavour and this could be detected without opening the cotton plug. Hence, it was incubated in darkness in basal medium containing ferulic acid. The UV spectral analysis of *Bullera* MVY 22 drawn at 0, 24 and 48 h of incubation indicated that the biotransformation takes place from ferulic acid to vanillic acid (Figure 1). Ferulic acid concentration in the culture broth decreased as the growth progressed. During the same period absorbance between 220 and 230 nm increased, indicating accumulation of vanillic acid. HPLC analysis of the culture supernatant from this isolate indicated the utilization of ferulic acid and its conversion to vanillic acid (Figure 2). Along with vanillic acid, tannic and genetic acids were also synthesized by the organism.

Presence of β-glycosidase in vanilla beans and its role in the synthesis of aroma have been well-documented[14]. However, most of the enzymes are degraded during curing due to the high temperatures attained[15]. The thermal process along with plant and microbial enzyme activity

Table 5. Yeast isolates from vanillia plant exhibiting β-glucosidase

Isolate no.	Identified as	Sample	Month/days
MVY 6	*Cryptococcus*	Rhizosphere soil	March
MVY 7	*Stephanoascus*	Rhizosphere soil	January
MVY 9	*Kockovaella*	Stem swab sample	March
MVY 14	*Brettanomyces*	Leaf swab sample	November
MVY 15	*Bullera*	Leaf swab sample	January
MVY 17	*Bensingtonia*	Leaf swab sample	January
MVY 22	*Bullera*	Leaf swab sample	March
MVY 28	*Rhodotorula*	Bean swab sample	November
MVY 30	*Trichosporon*	Bean swab sample	January
MVY 37	*Bullera*	Stem swab sample	January
MVY 44	*Cryptococcus*	Leaf swab sample	January
MVY 47	*Dekkara*	Bean swab sample	January
MVY 52	*Candida*	Leaf swab sample	March
MVY 55	*Bullera*	Bean swab sample	January
MVY 57	*Lipomyces*	Rhizosphere sample	January
MVY 59	*Lipomyces*	Leaf swab sample	November
MVY 60	*Candida*	Leaf swab sample	January
MVY 62	*Sacchoromycopsis*	Leaf swab sample	March
MVY 63	*Pichia*	Flower swab sample	March
MVY 64	*Debromyces*	Leaf swab sample	March
MVY 65	*Pichia*	Flower swab sample	March
MVY 66	*Candida*	Leaf swab sample	March
DVY 2	*Lipomyces*	Outer skin (location II)	Six-day SD
DVY 5	*Candida*	Pulp (location II)	Rack-dried
DVY 5	*Nadosonia*	Surface wash (location II)	
DVY 18	*Pichia*	Outer skin (location IV)	Nine-day SD
DVY 37	*Dekkera*	Seed (location I)	Four-day SD
DVY 47	*Kodamaceae*	Seed (location I)	Four-day SD
DVY 61	*Pichia*	Pulp (location I)	Rack-dried
DVY 81	*Saccharomyces*	Surface wash (location III)	Kelled
DVY 89	*Nadsonia*	Surface wash (location III)	Seven-day SD
DVY 90	*Candida*	Surface wash (location III)	Twelve-day SD
DVY 98	*Candida*	Pulp (location III)	Rack-dried
DVY 99	*Nadsonia*	Seed (location III)	Rack-dried
DVY 100	*Debaryomyces*	Surface wash (location III)	Cured bean

Identified based on Barnett *et al.*[10].

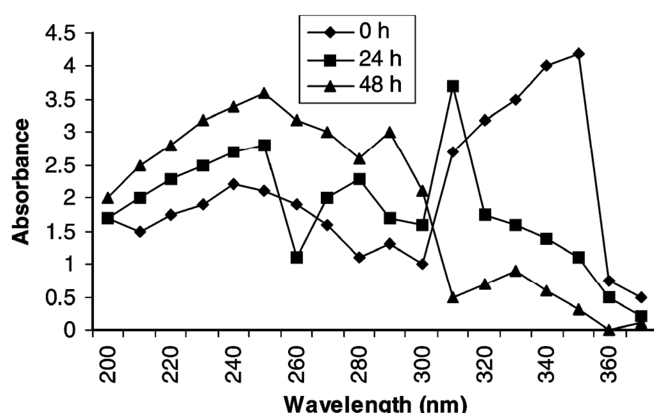

Figure 1. Bioconversion of ferulic acid to vanillic acid by MVY 22.

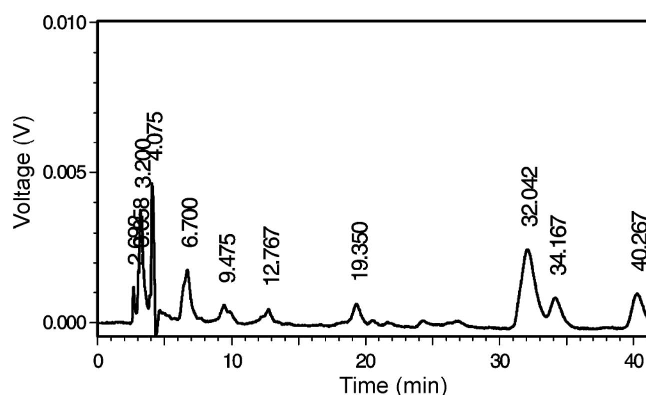

Figure 2. HPLC profile of culture filtrate of MVY 22 (retention time (min) – gallic acid 3.2, tannic acid 4.0, protocatecholic acid 9.4, gentic acid 6.7, vanillic acid 32 and ferulic acid 40.7).

lead to the conversion of glycoside into aroma compounds[1]. Microbial degradation of cellulose and hemicellulose along with lignin yields aromatic compounds[16] as a consortium of microbes can attack different substrates to synthesize these compounds. Production of aromatic compounds in wine has been attributed to yeast[17]. The present observations indicate that only a few yeast isolates from the total microflora possess β-glycosidase activity and they may be involved in the synthesis of vanillin. Even though the percentage of yeast with β-glycosidase

activity was 0.4 before curing, it increased to 1.75 during curing. This may be due to the conducive atmosphere created during curing for these forms to prolifer. During the post-harvest process, 0.75% of the external and 10.52% of the intracellular yeast flora were positive for *β*-glycosidase activity, indicating the role in flavour development of the intracellular flora. These organisms probably help in maintaining the enzyme activity even after the plant enzymes are killed. Earlier, Digum *et al.*[14] had tried to localize the enzyme activity in various components of the bean and had confirmed the distribution of the activity throughout the bean. On the contrary, the present result indicates the presence of a maximum number of organisms with *β*-glycosidase activity as intracellular microflora which may contribute to enzyme activity.

Flavour development occurs during curing[14,18]. But if plant enzymes are sensitive to heat and get killed during curing, the conversion of glycoside to aromatic compounds needs to be carried out by enzymes from sources other than the plant[19]. We postulate that the enzymes required are contributed by the microbial population (both bacteria and yeast). Conversion of ferulic acid to vanillic acid by yeast and bacteria is well documented[20,21]. The present investigation reports the bioconversion of ferulic acid to vanillic acid by all the *β*-glycosidase positive isolates present in the bean. Further investigation with MVY 22 showed the conversion of ferulic acid to vanillic and other aromatic acids.

The importance of the present result is the wide spectrum of yeast forms present in vanilla plant. There were 17 genera of yeast with *β*-glycosidase activity, of which six isolates belong to *Candida* and four each are of *Bullera* and *Pichia*. Even though most of the work on the synthesis of aromatic compounds in wine is reported from *Saccharomyces*[17], our investigation showed that their numbers are low. Earlier Digum *et al.*[14] had concluded that there are more than one type of *β*-glycosidase enzymes involved in the development of aroma in vanilla. The presence of a number of genera would help in the development of a wide spectra of aroma compounds from various precursor molecules. Further work on the enhancement of flavour by artificial inoculation of these organisms needs to be done.

Even though the number of isolates positive for *β*-glycosidase was low compared to the total yeast isolates, the results indicate the contribution of yeast microflora in the synthesis of vanillic acid. Further, work on the production of vanilla fermented with the isolated cultures needs to be done for the improvement of its quality and quantity.

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ACKNOWLEDGEMENTS. We thank Chairman, Microbiology Department; Vice-Chancellor, Mangalore University and Director, CFTRI for support and encouragement.

Received 27 August 2008; accepted 6 April 2009