Microbial bioconversion of metabolites from fermented succulent bamboo shoots into phytosterols

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Fermented succulent shoots of bamboo (*Bambusa balcooa* and *Dendrocalamus strictus*) are an enriched source of phytosterol. Microorganisms from the 'soibum exudate' involved in microbial bioconversion of phytosterol during fermentation of succulent bamboo shoots were isolated and identified as *Bacillus subtilis, B. licheniformis, B. coagulans* and *Micrococcus luteus.* Crude phytosterol was purified to isolate *b*-sitosterol by thin layer chromatography and identified by IR and mass spectral data. The isolated *b*-sitosterol was then subjected to microbial bioconversion using *B. subtilis* yielding a considerable amount of androstadienedione in the presence of metal chelate inhibitor (0.1% *a, a′*-dipyridyl).

BAMBOO cultivation is practised in many tropical countries. Bamboo is grown as a cash crop in the northeastern region of India. In Manipur, the fresh succulent bamboo shoots and the fermented preparation of bamboo shoot slices, locally called 'soibum' are a highly prized vegetable item. The soibum is manufactured by storing thin slices of fresh succulent and soft bamboo shoots in specialized containers/chambers for 2–3 month. The fermentation chambers are either made of bamboo planks or roasted earthen pots. The inner surface of bamboo chambers are lined with banana leaves and a thin polythene sheet. The presence of various ingredients of nutritional significance in succulent bamboo shoots has been described by many workers[1,2].

Phytosterols, which are the precursors of many pharmaceutically active steroids, are found in many plants[3,4]. Among the widely distributed phytosterols, sitosterols are the most abundant and have come into prominence because of their easy microbiological conversion into androstadienedione[5]. The increased demand for steroidal drugs in recent years has resulted in the depletion of natural resources such as *Dioscorea* and *Solanum* species[6]. Hence, an alternative source for a starting material of steroidal drugs is essential. In this regard, presence of phytosterols had been reported in bamboo[7,8]. However, no work had been done regarding the involvement of microorganisms in accumulating phytosterols in the fermenting process.

The present communication reports the microorganisms involved in higher accumulation of phytosterols in the fermenting bamboo shoot slices and their efficiency for bioconversion of phytosterols to androstadienedione (ADD).

Fresh succulent shoots of bamboo of *B. balcooa* (Figure 1 *a*) and *D. strictus* (Figure 1 *b*) were collected during the growing season (May to September). For enrichment of sterols, the fresh succulent bamboo shoot slices were subjected to fermentation by inoculating the thin shoot slices with the exudates obtained from already fermented samples of bamboo shoots (traditional way of fermentation), sold in the local market in the name of 'soibum'. After inoculation, the samples were kept in an incubator at 35 ± 2°C for a period of 60 days. The analyses in the content of total phytosterol were carried out both in the fresh, delicate, succulent bamboo shoot apex and after fermentation using Liebermann–Burchard reaction[9].

To study the microorganism involved in fermentation in succulent bamboo shoot slices, the exudates from the traditionally fermented bamboo shoots were used as inoculant and microbial study was conducted[10] and identification was done following *Bergey's Manual of Determinative Bacteriology*[11] and *Cowan and Steel's Manual for the Identification of the Medical Bacteria*[12].

To study the efficiency of the isolated bacterial strains in bioconversion of phytosterol, the four different isolated bacterial strains were then used in inoculating fresh shoot slices of *B. balcooa* and *D. strictus*. Fermentation was done in the laboratory using modified techniques under aseptic conditions[7].

For extraction of phytosterol, the fermented shoot slices (60-day-old) inoculated with *B. subtilis* were taken and 100 g of oven-dried (60°C), powdered, fermented material was refluxed with a solvent mixture of benzene, petroleum ether and 2*N*-ethanolic KOH (10 : 5 : 1) for 5 h in a 1000 ml Ca Clevenger apparatus[8]. Three fractions were obtained: a cream-coloured, crystalline precipitate floating at the surface of the solvent, light brown crystals sticking at the side wall of the beaker, and some sticky dark-brown residue at the bottom. The crystals sticking at the side wall of the beaker were collected and then further purified several times with acetonitrile till a pure white crystalline form was obtained. The white, crystalline, partially purified form so obtained was then subjected to thin layer chromatography[13] on silica gel-G (0.25 nm thick) plates (20 cm × 20 cm) using hexane : ethylacetate (3 : 1). The melting point of the crystals obtained after preparative TLC was determined. Analyses of IR and mass spectra were conducted and compared to that of the authentic sample (Sigma Chemicals, St. Louis, USA).

For the study of bioconversion of phytosterol extracted from the fermented bamboo shoots, *B. subtilis* isolated from the fermented bamboo shoot slices was used as the organism for conversion into ADD. The medium in which the bacterium was grown contained (g/l): $NaH_2PO_4.2H_2O$, 3; KH_2PO_4, 3.0; NaCl, 0.2; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, $2H_2O$, 0.1; $FeSO_4$, $7H_2O$,

Figure 1. Emerging fresh succulent bamboo shoots of ***a***, *B. balcooa* and ***b***, *D. strictus*.

Table 1. Level of total phytosterol in the succulent shoot samples of different species of bamboo

Bamboo species	Dry matter content (%)	Concentration of phytosterol (% dry wt.)	
		Fresh delicate shoot apex	Fermented shoot slices (60-day-old)
Bambusa balcooa	13.3	0.18 ± 0.01*	0.61 ± 0.05
Dendrocalamus strictus	10.7	0.14 ± 0.06	0.42 ± 0.02

*Standard error of the mean (n = 3).

Table 2. Total phytosterol level in the fermenting samples of *B. balcooa* and *D. strictus* inoculated with different isolated bacteria from the 'soibum exudate'

Bacterial isolate	Concentration of total phytosterol (% dry wt.)				
	Fermentation period (weeks)				
	0	2	5	7	9
Uninoculated	0.14 ± 0.01	0.02 ± 0.003	0.20 ± 0.01	0.20 ± 0.01	0.2 ± 10.01
Bacillus subtilis	0.14 ± 0.01	0.44 ± 0.01	0.58 ± 0.01	0.58 ± 0.01	0.56 ± 0.01
Bacillus licheniformis	0.12 ± 0.01	0.42 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.37 ± 0.01
Bacillus coagulans	0.13 ± 0.003	0.26 ± 0.001	0.31 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
Micrococcus luteus	0.14 ± 0.003	0.31 ± 0.01	0.34 ± 0.01	0.28 ± 0.01	0.30 ± 0.02

*Standard error of the mean (n = 3).

Table 3. Production of ADD from various phytosterols by bioconversion with *Bacillus subtilis* in the presence and absence of metabolic inhibitor (0.1% *a*, *a*′-dipyridyl)

Initial conc. of phytosterol (0.1%)	Conc. of inhibitor (0.1%)	Concentration of 17-ketosteroids (ADD) [mg/ml] Incubation period (h) 0	4	8	12	24	38	48	72	96
b-sitosterol (authentic sample)	Zero	0.16 ± 0.02	0.18 ± 0.04	0.20 ± 0.02	0.18 ± 0.03	0.22 ± 0.04	0.16 ± 0.03	0.08 ± 0.01	0.06 ± 0.02	0.04 ± 0.02
b-sitosterol (authentic sample)	Dipyridyl	0.15 ± 0.02	0.25 ± 0.03	0.26 ± 0.02	0.32 ± 0.04	0.34 ± 0.02	0.37 ± 0.01	0.29 ± 0.02	0.18 ± 0.06	0.09 ± 0.01
b-sitosterol (isolated from sample)	Zero	0.12 ± 0.04	0.19 ± 0.01	0.12 ± 0.21	0.14 ± 0.02	0.24 ± 0.02	0.08 ± 0.02	0.06 ± 0.11	0.06 ± 0.02	0.06 ± 0.02
b-sitosterol (isolated from sample)	Dipyridyl	0.20 ± 0.04	0.25 ± 0.04	0.25 ± 0.03	0.26 ± 0.04	0.28 ± 0.01	0.35 ± 0.04	0.28 ± 0.06	0.22 ± 0.04	0.12 ± 0.06

*Standard error of the mean (n = 3).

0.001; sodium molybdate, 0.001. pH was adjusted to 7.2 and a fine suspension of *b*-sitosterol (0.1%) was made in the above medium.

The bacterium was allowed to grow in a 250 ml flask containing 200 ml of the medium on a reciprocal shaker (80 strokes/min) at 30 ± 2°C for 24 h. A further addition of metal chelate inhibitor (0.1% *a*, *a*′-dipyridyl) was done. The incubation was then carried out for 96 h. Estimation of ADD by Zimmerman reaction[14] was done at weekly intervals.

Dry matter content was found to be higher in *B. balcooa* (13.3%) than in *D. strictus* (Table 1). The total phytosterol determined in 60-day-old fermented samples was much higher both in *D. strictus* (0.42% dry wt.) and *B. balcooa* (0.61% dry wt.) compared to their fresh samples (0.14 and 0.18% dry wt., respectively) as shown in Table 1. Fermentation increases the accumulation of certain by-products of small molecular weights as a result of breaking down of the raw organic molecules (polymers) by the activity of microorganisms. Acetyl-CoA is one of the by-products during fermentation and can be used for the biosynthesis of phytosterol[8].

The microorganisms involved in fermentation were found mostly to be *Bacillus* species. They were *B. subtilis*, *B. licheniformis*, *B. coagulans* and *M. luteus*. Among these four isolates, *B. subtilis* showed highest level of efficiency in accumulation of total phytosterol during fermentation (Table 2).

The co-chromatography with standard samples revealed that the side-wall sticking fraction was *b*-sitosterol. This was further identified by analysis of its melting point (140°C) and molecular weight (414, mass spectra). The IR spectral data and the mass spectra of the compound showed similarity with those obtained with the authentic sample of *b*-sitosterol (90% purity) obtained from Sigma Chemicals, St. Louis, USA.

The *b*-sitosterol (extracted from fermented shoot samples) was then used for microbial bioconversion into ADD using *B. subtilis* (isolated from the fermented bamboo shoots), which showed a gradual increase in the production of ADD with 38 h incubation and maintained for a period of 48 h after the inhibitor (0.1% *a*, *a*′-dipyridyl) was added, but decreased later (Table 3). This shows that the inhibitor protected the sterol nucleus from being used up by the bacterium, but the consumption of the side chain at C-17 position continued. As a result, ADD got accumulated in the cultural broth, which was determined by Zimmerman reaction[14].

In the present work, we attempt at utilizing succulent bamboo shoots as a non-conventional source of phytosterol. The microorganisms efficient in biotransforming phytosterol in fermented bamboo shoot were characterized and identified. These micro-organisms can be used to obtain a direct precursor of steroidal drugs by pharmaceutical industries to compete economically and commercially feasible with those obtained from other precursors and to make available in large quantities and within the reach of common man.

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Association of a badnavirus in black pepper (*Piper nigrum* L.) transmitted by mealybug (*Ferrisia virgata*) in India

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The association of a badnavirus with disease-affected black pepper leaf samples collected from Kozhikode (Calicut) and Wyanad districts of Kerala was established on the basis of symptomatology, vector transmission, electron microscopy and serology. The virus induces vein clearing, chlorotic flecks, chlorotic mottling along veins and characteristic curling of leaves leading to reduced vigour and yield. The virus was transmitted from diseased to healthy black pepper plants by grafting and mealybug (*Ferrisia virgata*). The virus could also be transmitted by mechanical means with difficulty to black pepper, but not to other hosts tested. The virus showed positive serological relationship with Banana streak virus (BSV) and Sugarcane bacilliform virus (ScBV) in direct antigen-coated enzyme-linked immunoassay (DAC-ELISA) using polyclonal antisera. The exact taxonomic identity of the virus remains to be determined.

BLACK pepper, obtained from dried berries of *Piper nigrum* L., is an important condiment of international commerce for India, earning around Rs 88 crores annually through export. India is a leading producer of black pepper in the world and the crop is grown in an area of 1.92 lakh hectares, with a production of 30.23 lakh tons annually[1]. The crop is mainly grown in Kerala and Karnataka. However, the productivity of the crop is considerably low due to many biotic stresses, including viruses. Viruses belonging to genera *Badna*, *Cucumo* and *Clostero* have been recorded on black pepper[2–6]. The disease caused by Cucumber mosaic virus (CMV) (genus: *Cucumo*) is characterized by small, crinkled, brittle, leathery leaves and chlorotic patches/streaks on leaves. In severe cases, the leaves become abnormally narrow with reduced internodal length, leading to typical stunting of plants[2,5,6]. The disease caused by Piper yellow mottle virus (PYMV) (genus: *Badna*) is characterized by chlorotic mottling, chlorosis, vein clearing, leaf distortion, reduced plant vigour and poor fruit set[2,4]. PYMV has been reported from Brazil, Malaysia, Thailand, Philippines and Sri Lanka[2,4].

In India, only the association of a CMV has been established with stunted disease-affected black pepper samples[5]. In addition, a mosaic disease on black pepper was observed in serious proportions in parts of Kerala for the past few years. Up to 100% incidence of this disease has been reported in certain black pepper plantations, especially in Kozhikode and Wyanad districts. The disease is characterized by vein clearing, scattered chlorotic flecks (Figure 1 *a*) followed by chlorotic mottling along veins leading to interveinal chlorosis and characteristic curling of the leaves (Figure 1 *b*). In a few cultivars, vein banding, vein thickening and green island-like symptoms are also seen. The infected vines had reduced vigour and yield. Though the disease has been noticed on all the cultivars, its incidence and severity was more on Karimunda. Since it has not been established so far, we report the results of our studies which revealed the association of a badnavirus based on its transmission, electron microscopy and serological characteristics.

The virus isolate was collected from black pepper vines from plantations in Kozhikode and Wyanad districts, including the experimental farm at the Indian Institute of Spices Research (IISR), Peruvannamuzhi during April–May 2002. The isolate was maintained on black pepper by vegetative propagation under insect-proof glasshouse conditions at 25–28°C. For mechanical inoculation, the inoculum was prepared by extracting the sap using chilled 0.1 M phosphate buffer (pH 7.2) containing 0.1% 2-mercaptoethanol poured in mortar kept in an ice tray. The inoculum was rubbed on the leaves of test plants dusted with celite or carborundum powder. For host-range studies, plants belonging to four families namely, Cucurbitaceae (*Cucumis sativus*, *C. pepo*), Fabaceae (*Cajanus cajan*, *Glycine max*, *Vigna mungo*, *V. radiata*, *V. unguiculata*), Poaceae (*Zea mays*) and Solanaceae (*Nicotiana benthamiana*, *N. glutinosa*, *N. tabacum*, *Physalis floridana*) were grown in pots raised under insect-proof glasshouse, and were rub-inoculated. Ten plants of each species were inoculated and kept under observation for two months. For graft transmission, scions from dis-

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