

Identification of spoilage yeasts in cashew apple (*Anacardium occidentale* L.) using MALDI-TOF MS



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Summary

Cashew (*Anacardium occidentale* L.) is a hardy and golden crop of arid regions, and the pseudo fruit, cashew apple is a vital source of sugars and vitamins that is discarded as a waste during cashew nut processing. Cashew apples start fermenting soon after the harvest, and due to the presence of a wide spectrum of polyphenols and tannins, the change of colour of cashew apples leads to an unappealing marketability and storage stability. The present study is aimed to rapid identification of different spoilage yeasts and to identify them using MALDI-TOF Mass Spectroscopy. Spoilage yeasts like *Candida krusei*, *C. tropicalis*, *Pichia norvegensis*, *Brettanomyces bruxellensis* were the spoilage yeasts found to have high confidence score values of 2.00 - 3.00 with high consistency.

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Introduction

The Cashew apple is an edible sugary rich golden crop of arid regions, and its a pseudo-fruit that can be eaten raw or mixed with fruit salads and is exploited commercially for ready-to-serve beverages, drinks, sweets, jams, pickles, candies, vinegar, wine, cajuina in Nigerian states such as Bissau, Canchungo, Ingore and Quinhame¹, Fenny in Goa, India and several other cashews growing countries across the world. But in the international market, cashew apple-based products are less widespread. Cashew apple starts to ferment within a few hours after harvest. The fleshy fruit is thin-skinned, which may be red or yellow coloured depending upon variety, spongy, fleshy, juicy with a sweet smell, slightly astringent and acidic in taste². Even the juice from cashew apples darkens quickly after extraction due to the rapid oxidation of some of the flavonoids, polyphenols and organic acids, making it unfavourable

for commercialization by making it into an unappealing dark brown colour. It has been challenging to preserve cashew apple juice in bottles and serve it fresh without adding any preservatives.



Figure 1. Cashew apples with nuts from variety VRI 3, Tamil Nadu, India

Saccharomyces cerevisiae, the beneficial brewer's yeast, is used in the mass production of cashew wine and renders good prospects for the alcoholic beverage sector. Fermented alcoholic beverage production with certain strains of yeast, such as *S. cerevisiae* and *Hansenula guilliermondii* with cashew apple juice as the fermentative substrate, possess the

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capability to metabolize polyphenols and certain aroma compounds, such as acetate ester and β -phenyl ethanol during the process of fermentation³.

Identification of spoilage yeasts in cashew apples by MALDI TOF MS

Rapid analysis of bio-molecules/microorganisms based on the protein patterns present in any live sample can be carried out through Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS). It is a high throughput and rapid tool for species classification because of its, minimal pre-sample preparation, and it displays high dynamic ranges for the organism identified. Most importantly, MS allows for detecting a wide microbial protein spectrum without using standard-specific DNA primers or antibodies. These protein patterns serve as fingerprint regions that could be constructed as yeast/bacteria/fungi or mould for genus and species classification of microorganisms. Intact cell mass spectrometry was also developed to discriminate bacteria without sample pretreatment. Most published studies of the direct mass spectrometric analysis of microorganisms are based on MALDI techniques because of their speed and simplicity. There is only a handful of studies for identifying yeasts in cashew apple fruit samples by MALDI-TOF MS.

Cashew apple juice extraction and isolation of spoilage yeasts

Cashew apples from varieties such as VRI 2&3 were collected from the old farm of the Regional Research Station, Vridhachalam, Tamil Nadu, India. Cashew apple juice was obtained through a mechanical press in which the peeled cashew apples were compressed and squeezed of the juice and filtered using a cheese cloth. From this juice sample, one ml was inoculated into a sterilised Yeast Extract Malt Extract (YM) broth. Surface of the broth in the tubes was sealed with one cm layer of sterile paraffin and incubated at 28°C for 48 h⁴. After incubation, single yeast colonies, based on the well-separated, distinctness of colony morphology, isolates were selected, labelled, and purified by cross streak method on the YPD agar plates and incubated at 28°C for 48 h⁴. One ml of incubated YM broth was taken and serially diluted upto 10⁻⁶ dilution in sterile water and one ml of 10⁻⁶

diluted suspension was spread over Yeast Extract Peptone Dextrose (YPD at pH 3.5) agar plates and incubated at 28°C until yeast colonies appeared⁵.

Characterization of spoilage yeasts in cashew apples

Purification was repeated until to get morphologically identical distinct colonies. After incubation, the cultures were stored inside the refrigerator at 4°C. The isolated yeast cultures were identified based on their cell morphology, colony characters and biochemical characters^{6,7}. The yeast isolates were activated by inoculating each strain in a tube containing 25 ml of YPD broth containing yeast extract 1% (w/v), peptone 2% (w/v), glucose 1% (w/v) and agar (20 g/L) and incubated at 28°C for 24 h. After 24 h, physiologically active cells were used as inoculum for biochemical/physiological and MALDI TOF MS identification tests.

Preparation of culture samples for MALDI-TOF MS identification

The yeast cultures isolated from the cashew apple extract were prepared as per the method developed by Schulthess et al. (2013)⁸. Direct colony transfer - formic acid method was adopted for preparing the isolated yeast cultures in feeding the MALDI-TOF MS. Young 24 hours grown yeast isolates (CAP 1 to CAP 9) were smeared separately on a polished steel MSP 96 target plate using a tooth pick on which 1 μ L of 70% formic acid was added, and thin smear was prepared allowed for air drying. Followed by it, saturated-cyano-4-hydroxycinnamic acid (HCCA) matrix (1 μ L) prepared using 50% acetonitrile and 2.5% trifluoroacetic acids solution was overlaid onto the air-dried yeast isolates on the target plate at room temperature. The target plate was placed into the plating chamber of the MALDI-TOF MS and shuttered for performing the identification of the yeasts.

Results and Discussion

Based on the serial dilution technique, the isolation of various yeast strains of cashew apple extract proved that there were nine different isolates (CAP1 to CAP 9). The details of the biochemical tests performed are presented in Table 1.

Table 1. Biochemical characterization of yeast isolates from cashew apple

Biochemical tests for yeast identification	CAP1	CAP2	CAP3	CAP4	CAP5	CAP6	CAP7	CAP8	CAP9
Glucose Fermentation	+	++	++	++	++	++	++	+	+++
Sucrose Fermentation	++	+	+	++	++	+	+	+	++
Maltose Fermentation	++	++	+	+	+	+	++	+	++
Lactose Fermentation	-	-	-	-	-	-	-	-	-
D-ribose	-	++	-	+	-	-	-	++	+
D-mannitol	+	+	+	++	+	+	++	++	+
Urease activity	-	-	-	-	+	-	+	+	+++
Ester production	+	+	+	+	+	+	++	+	++
Acid production	-	-	-	-	-	-	-	-	-

Tolerance to acetic acid	-	-	-	-	-	-	-	-	-
Gelatinase activity	-	-	-	-	-	-	-	-	-

Rapid identification of yeast cultures using MALDI-TOF MS

The MALDI-TOF mass spectra measurements of the target plate loaded with yeast culture samples were performed by using Bruker MALDI TOF Biotyper 4.1.70 (PYTH) at Anbil Dharmalingam Agricultural College and Research Institute (TNAU), Trichy, Tamil Nadu, India. As per the instructions of the MALDI-TOF MS manufacturer,

Table 2. Identification of spoilage yeasts in cashew apples by MALDI-TOF MS

S. No.	Spoilage yeasts in cashew apple	Confidence of Identification	Consistency score	Average Score value
1.	<i>Brettanomyces bruxellensis</i>	+++	A	1.93
2.	<i>Candida krusei</i>	+++	A	2.59
3.	<i>Candida tropicalis</i>	+++	A	2.12
4.	<i>Pichia norvegensis</i>	+++	A	1.99

Score values denotes

Brettanomyces bruxellensis is a ubiquitous yeast that possesses the ability to form various volatile phenols and biofilm formation that confers unpleasant organoleptic characteristics of wine and provoke economic loss for the wine industry. Due to the production of volatile phenols, a stable or sweat odour of horse is liberated by *B. bruxellensis* deters the quality of wine^{9,10}. In the present study, similar types of yeasts were identified, witnessing an unpleasant odour of the cashew apple heaps just a day after harvest.



Figure 2. Colonies of *Brettanomyces bruxellensis* CAP 9 in YPD agar plates

Conclusion

The results proved that there is inherent yeast colonization inside the fruits, which proliferates upon time when sufficient nutrients are provided in the laboratory. Furthermore, spoilage yeasts such as *Candida*, *Pichia* and *Brettanomyces* are the contaminant candidate in the preparation of value-added products from cashew apples rendering to early spoilage of fruits.

calibration was carried out using test standards initially. Analyses of all the isolates were run in triplicates. The identification results were expressed by log score values which denote, 2.3 to 3.0: highly probable species identification, 0 to 2.3: secure genus identification, probable species identification, 2.1.7 to 2.0: probable genus identification, 0 to 1.7: not reliable identification and 'A' stands for high consistency

Cashew apples should be processed immediately after harvest and can be valorised as a renewable and sustainable sugary-rich source for the development various fermentative-derived cashew apple byproducts. The spoilage yeast *Brettanomyces bruxellensis* identified from cashew apples may be exploited for the development of high-quality wine or other yeast-based fermented foods.

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