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Changes in Quality Profile and Flavour Components of Coconut Sap during Natural Fermentation

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ABSTRACT

The study focused on the changes in physico-chemical quality parameters as well as flavor components of coconut sap during natural fermentation at different time intervals. pH of the coconut sap was found to reduce from 7.3 to 5.9 at the incubation period of 4 h followed by the drastic reduction to 3.3, a clear indication of acid production at the final stages of storage period. At the same time total titrable acidity was found to be increased from 15 to 177.9 mg/L. Fresh coconut sap contains 14.35% sucrose, 0.32% fructose and 0.28% glucose but within the first 12 h of storage sucrose content decreased up to 1.04% and fructose and glucose content increased to 5.75 and 8.72%, respectively. The alcohol content recorded maximum increase to 5.17% in the 40th hour of storage when compared with the 0.013% alcohol at 0th hour. Microbiological analysis showed high amount of microbial load (total plate count and yeast and mold count) in the *Neera* samples. The absence of E. coli colonies shows the clear indication of absence of faecal contamination. GC-MS analysis of the volatile isolates showed nineteen compounds from the fresh *Neera* and twelve compounds from fermented *Neera* samples. The prominent volatile components were 1-Pentanol, 2-Methyl Pyrazine and 2-Methoxy-4 Vinylphenol in fresh *Neera*; these could be major flavor character impact compounds. The quantity of 3-Methyl 1-Butanol, increased fifty-fold, n-Hexadecanoic acid, Phenyl ethyl alcohol and 2, 2-diethoxy Propane, was found in higher concentrations in fermented *Neera*, while the amount of other compounds increased marginally.

Keywords: Coconut sap, GC-MS, Volatile compounds, Fermentation, *Neera*, Alcohol content

INTRODUCTION

Coconut palm (*Cocos nucifera* L.) is an important member of the monocotyledons. Coco is a Portuguese word for 'fruit' and nucifera is a Latin word for nut bearing and this tree mainly grows in tropical coastal areas. Every part of the tree is useful and therefore, coconut palm is an important economic crop of the local people. Coconut sap (*Neera*) is a sweet juice, obtained by tapping the unopened spadix of the coconut palm. *Neera* is usually consumed by people in Southeast Asia and is also used as raw material for the production of sugar, alcoholic beverages, vinegar and acetic acid [1]. Coconut sap has gained increasing popularity due to its sweet taste, nutritive values and inherent flavor.

Neera is rich in sugar (10-15%) when it is collected under hygienic conditions and possesses neutral pH. It is rapidly ferments and gets converted to toddy as the sugar is transformed to alcohol (upto 5.8%) during the fermentation [2]). It contains sixteen different amino acids and various vitamins such as vitamin C, vitamin B complex, especially nicotinic acid. However, it is highly susceptible to spontaneous fermentation, initially alcoholic, followed by acidic fermentation due to the presence of native microflora consisting of yeast and bacteria [1,3]. This process is reported to be rapid under sunlight and the fermented *Neera* is known as toddy. *Neera* is susceptible to spontaneous fermentation even during the process of harvesting, especially in sunlight. This process will take place from the time when sap come out and contact with the air [1,4]. Sources of the fermenting organisms are gourds, tapping implements and air [5]. The fermenting organisms are dominated by yeasts, particularly *Saccharomyces cerevisiae* [6,7].

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The composition and quality of *Neera* is found to vary with the place, time and duration of tapping. *Neera*, unless it is collected under hygienic conditions, rapidly ferments and gets converted to toddy as the sugar is transformed to alcohol during the fermentation. Further, the toddy, through a process of acetic fermentation, yields 'coconut vinegar' containing 4-7% acetic acid. This poses a preservation problem, leading to harsh aroma and taste, because of the highly fermentable nature of the sap. *Neera*, when tapped fresh, possesses a pleasant odour which turns harsh on fermentation to toddy and makes it unpalatable, despite being nutritious [8,9].

Neera is nutritive as well as healthful and it is low on cholesterol and fats. The *Neera* beverage promotes blood circulation and helps to promote digestive system. *Neera* was found to be laced with minerals which boost immune system and helps body to fight against antibodies and kidney stones. The purges out the waste from intestine and helps to detoxify on a cellular level. Balanced administration of fresh and fermented date sap was found to improvise the treatment of hemoglobin deficient anemic patients and to supplement vitamin-B12 level in the vitamin deficient patients [10].

Many researchers studied the volatile components of fresh and fermented *Neera* and their composition. Atputharajah et al. [3] studied the distribution of microorganisms during fermentation of coconut sap and isolated 166 yeast species and 17 bacterial species. However, reports on the changes of quality profile and flavour components profile of coconut sap during the course of natural fermentation are scanty. Hence, this paper is aimed to evaluate the changes in volatile components along with physico-chemical and microbial parameters of fresh coconut sap during natural fermentation.

MATERIALS AND METHODS

Materials

Fresh *Neera* was collected from Palakkad Coconut Producers Company Limited, Kerala, India. Potato dextrose agar (PDA), Eosin-methylene blue (EMB) agar, Plate count agar (PCA) (Himedia, Mumbai, India) were procured. All other reagents and chemicals were of analytical grade.

Methods

***Neera* collection and preservation:** *Neera* was collected by tapping the unopened spadix of the palm of the healthy coconut trees (*Cocos nucifera* L.) under hygienic conditions during the night (6 PM to 6 AM), to avoid exposure to sunlight, in polythene covers which were immersed in insulated ice box. Collected *Neera* was transported at low temperatures (<4°C) to prevent fermentation and processed immediately.

Quality analysis of fresh *Neera*: Fresh *Neera* was kept in room temperature (27 ± 5°C) in PET bottles. Stored Samples were analyzed for different quality parameters different intervals namely 0, 4, 8, 12, 24, 28 and 40 h. The parameters include pH, titrable acidity (TTA), Brix, carbohydrate concentration, TPC, E. coli count as well as yeast and mold count.

pH of the fresh *Neera* was detected by a digital laboratory pH meter. Fresh *Neera* with pH 7 only was used for the study. Brix of the *Neera* samples were measured using a refractometer (HTA instruments Private Limited). Total titrable acidity (TTA) was analyzed using automated titrable acidity machine (Hanna Instruments, USA) against standard solution of NaOH and the obtained TTA was expressed in mg/L.

Microbiological quality of *Neera* was analyzed using standard microbiological methods. Total plate count was determined by counting the colonies formed in the PCA plates by serial dilution technique. Escherichia coli present in the samples were detected by EMB agar. Sample (0.1 ml) was plated on PCA and EMB plates using spread plate method. Yeast and mold count was detected by checking the colony formation in PDA plates (Lab Manual, FSSAI, 2012 [11]).

Analysis of carbohydrates using HPLC: HPLC system containing a LC-10AT pump, RID-10A RI detector (Shimadzu, Japan) and UBondapakTMNH2 10 µm (3.9 × 300 mm) column (Water, Ireland) was used to analyze the sucrose concentration. The clear solution (20 µl) obtained after filtration (membrane filter, 0.45 µm pore) (Whatman Inc., USA) was injected onto the column using a mixture of acetonitrile and water (7:3) as the mobile phase at a flow rate of 1 ml/min. The standard used was a mixture includes D-glucose, D-fructose and sucrose.

Estimation of alcohol in the *Neera*: Alcohol content was determined by measuring the generation of chromic ions formed by alcohol oxidation, which was measured at 600 nm using a Spectrophotometer (Model: UV 160A, M/s. Shimadzu, Japan) as per the procedure provided by Chanukya et al. [5] 2013. The standard calibration graph of known alcohol concentrations was graphically plotted (R2=0.99) and used for identification of the concentration of alcohol in the samples.

Flavor isolation from *Neera*: Fresh *Neera* (1 L) was placed in a 2 L round-bottom flask, along with an internal standard (4 µg ethyl caproate). In another 250 ml round-bottom flask, dichloromethane (100 ml) was placed. The two round-bottom flasks were placed on heating mantles and attached to the two arms of Likens-Nickerson simultaneous distillation-cum-solvent extraction (SDE) apparatus, equipped with a condenser and cryogenic liquid for circulation. The temperature of coolant was maintained at 10°C and the extraction carried out for 3 h by heating both the solutions at its boiling temperatures. After the flavor extraction, the solvent was removed from the extract using a Vigreux column on a water bath with the chilled water circulation. After removing the solvent, 2 ml of extract was

collected and further concentrated to 1 ml by flushing with nitrogen. The experiment was repeated for fermented *Neera*. Isolated volatiles of fresh and fermented *Neera* (40 h) were preserved at 4°C for further analysis.

Gas chromatographic-mass spectrometric analysis: The volatile concentrates were analysed, using Gas chromatograph (GC) (Perkin Elmer instruments, Auto system XL), equipped with mass spectrometer (Perkin Elmer instruments, Turbomass Gold). The GC was fitted with Elite-5 column (length 30 m, film thickness 0.25 µ, internal diameter 0.25 µ). Helium was the carrier gas at a flow rate of 1 ml/min. The injector port temperature was 220°C, the detector temperature was 230°C and the oven temperature was maintained at 35°C for 2 min and then increased to 90°C at the rate of 3°C/min and further increased to 220°C at the rate of 2°C/min. Two µL of the sample was injected by split-less injection mode and the ionization voltage was 70 eV. Retention indices for all the compounds were determined according to the Kovats method, using n-alkanes as standards [8]. Flavour compounds were identified by comparing retention times of the GC peaks with those of reference compounds run under identical conditions and by comparison of retention indices with literature data [1,6,8]. Fragmentation patterns in mass spectra were matched with those of the NIST62-LIB library and published mass spectra [1,18]. Compounds were quantified using the internal standard method.

RESULTS AND DISCUSSION

Quality parameters of *Neera*

Quality parameters of *Neera* during the course natural fermentation at different time intervals of storage at room temperature presented in **Table 1**.

Table 1. Quality parameters of fresh *Neera.*

Parameters/storage period	0 h	4 h	8 h	12 h	24 h	28 h	40 h
pH	7.3 ± 0.2	5.9 ± 0.1	3.8 ± 0.06	3.6 ± 0.1	3.5 ± 0.3	3.3 ± 0.1	3.3 ± 0.3
TTA (mg/L)	15.0 ± 0.8	18.0 ± 0.5	35.0 ± 2.2	73.0 ± 4.6	140.0 ± 6.3	154.0 ± 8.7	177.0 ± 9.1
Brix	16.4 ± 0.1	17.0 ± 0.1	16.8 ± 0.2	16.2 ± 0.2	13.6 ± 0.1	12.8 ± 0	11.6 ± 0.1
TPC (cfu/ml) × 10^5	1 ± 0	6 ± 0	15 ± 1	31 ± 2.1	107 ± 11	61 ± 5	2 ± 0
E. coli (cfu/ml)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Yeast and Mold (cfu/ml) × 10^5	2 ± 0	7 ± 0	66 ± 4	108 ± 8.5	251 ± 18	281 ± 25	293 ± 22

Potential hydrogen and total titrable acidity: The potential hydrogen values of fresh as well as stored samples were determined in terms of pH at regular intervals. pH was found to reduce from 7.3 to 3.3 during the storage period (40 h). At 4 h of incubation pH was reduced to 5.9, after that pH was drastically decreased to 3.3. This may due to the production of acid during the fermentation in the medium. At the same time TTA was increased from 15 to 177.9 mg/L. The total titrable acidity of a solution is measured by reacting the acids present with a base such as sodium hydroxide (NaOH) to a chosen end point, close to neutrality, as indicated by an acid sensitive colour indicator. The total titrable acidity of a solution is an approximation of the solution's total acidity. The increase in TTA value in the *Neera* sample shows the increase in acid production, this may be due to the microbial fermentation of carbohydrates present in the *Neera*. The same trend was observed by Konan et al. [10] wherein the TTA increased from 0.26 g/100 g to 1.85 g/100 g during the storage of 120 h.

Carbohydrates and alcohol content: Fresh coconut sap from the cut inflorescence has sucrose (14.35%), fructose (0.32%) and glucose (0.28%), by weight as depicted in **Figure 1**. HPLC analysis revealed that concentration of sucrose decreased gradually whereas fructose and glucose increased and as the time increases **(Figure 1)**; this also supports the acid production in the medium and utilization of

sucrose by different microorganisms including yeast. Within the first 12 h of storage the conversion of sucrose to fructose and glucose is faster, i.e., sucrose content decreased to 1.04% and fructose and glucose content increased to 5.75 and 8.72%, respectively. At the later stages of storage (after 24 h), the conversion rate of sucrose to the monomers is significantly lower. Kalaiyarasi et al. [9] reported that fresh coconut sap from the cut inflorescence has 12-15% of sucrose (by weight) and trace amount of glucose, fructose, maltose and raffinose. The sap contains approximately 0.23% protein, 0.02% fat and is rich in Na and K content. Half of the total sugars are fermented during first 24 h and ethanol content of the fermented palm sap reaches maximum of 5.0-5.28% (v/v) after 48 h.

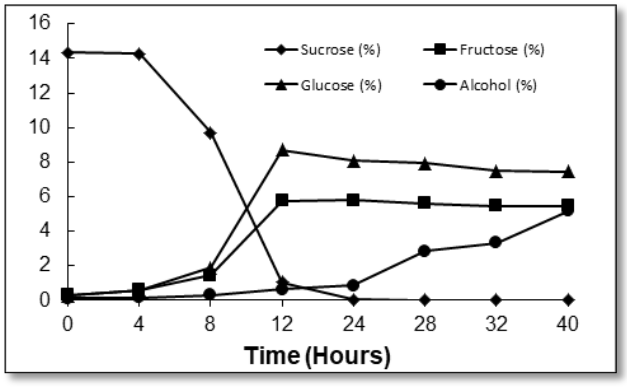

Figure 1. Changes in carbohydrates and alcohol content of coconut sap during natural fermentation.

Brix, sugar content of an aqueous solution, also found to reduce during the time period from 16.4 to 11.6. One degree Brix is 1 g of sucrose in 100 g of solution and represents the strength of the solution as percentage by weight (% w/w). If the solution contains dissolved solids other than pure sucrose, then the °Bx only approximates the dissolved solid content. The °Bx is traditionally used in the wine, sugar, fruit juice and honey industries.

As seen in **Figure 1** at 0 h, the % alcohol in *Neera* was negligible (0.036%), showing no signs of fermentation. The fermentation and hence, production of alcohol is not observed until 5 h of storage. At the 8th hour of storage, the sample showed 0.13% alcohol, indicating production of alcohol by the fermenting microorganisms. At the 24th hour of storage the % alcohol content increases to 0.3% indicating higher production of alcohol. At the 32nd hour and beyond, the % alcohol in the *Neera* substantially increases and reaches 2.3% alcohol in the 40th hour of storage. This trend is confirmed with the changes in the pH of the *Neera*, which turns acidic at 32nd hour.

The major physical, chemical and microbiological changes occurring in the fermenting sap indicated that a natural fermentation of coconut sap consist of an initial lactic acid fermentation, a middle alcoholic fermentation and a final acetic acid fermentation. It also appeared that activities brought about by micro-organisms of early phase helped the activities of the micro-organisms in each of the later phases [3].

The rate and degree of fermentation of the *Neera* depends on the practice of *Neera* collection and the environmental conditions of the region. Hygiene collection of the samples preserves the *Neera* in the fresh form for comparatively longer durations. However, owing to the high sucrose content in the fresh *Neera*, the microorganisms initiate the fermentation of sucrose, glucose, followed by the fermentation of trace sugars and hence production of alcohol [18,19].

Microbiological analysis of *Neera*: All the TPC and PDA plates showed more than high amount of microbial load in the *Neera* samples. At the same time EMB plates showed the absence of *E. coli*, a clear indication of absence of faecal contamination. When time increases bacterial count was found to decrease and yeast and mold count found to increase along with the time. This may be due to the production of acid and alcohol in the medium. Yeast and mold are best suited to survive on acidic and alcohol condition than bacteria.

Atputharajah et al. [3] reported a total of 166 isolates of yeasts and 39 isolates of bacteria from coconut sap (*Neera*) during natural fermentation. Seventeen species of yeasts was found to belonging to eight genera. The largest number of isolates (72%) belonged to genera Candida, Pichia and Saccharomyces. *Saccharomyces chevalieri* was the most dominant yeast species and accounted for 35% of the total isolates. Seven genera of bacteria were isolated. The predominant Genera were Bacillus and other included Enterobacter, Leuconostoc, Micrococcus and Lactobacillus.

Comparison of volatile components of fresh and fermented *Neera*: Fresh *Neera* samples of 1 L each were stored in plastic bottles and allowed to naturally ferment. Volatile components were isolated from fresh and fermented (40 h) *Neera*. The volatile isolates were subjected to GC-MS analysis for the identification of chemical constituents. Nineteen compounds from the fresh *Neera* were characterised, which constitutes more than 98% of the volatiles. The details of these compounds are shown in **Table 2**. The prominent volatile compounds may be broadly classified as aliphatic hydrocarbons, carbonyls, esters, carboxylic acids, aliphatic and aromatic alcohols, phenols and heterocycles. The changes of these compounds are given in **Table 3**. In general, there is an increase in the concentration of carbonyl, esters and carboxylic acids during the fermentation. However, the heterocyclic compounds, which are responsible for the overall flavour is totally degraded in the fermented sap. The prominent volatile components were 1-Pentanol, Octane, 2-methyl Pyrazine, and 2-methoxy 4-Vinyl Phenol. 2-methoxy 4-Vinyl Phenol is major flavour impact compound in *Neera*. Majority of the volatile compounds are different from the report of Borse et

al. [8]. Presence of heterocyclic compounds is not reported earlier. The reasons for differences in the profile could be: (i) *Neera* samples are from two different regions; (ii) Height from sea levels differs with different environmental conditions. Twelve compounds from fermented *Neera* were identified **(Table 2)**, which comprised more than 95% of the volatiles. Of these, only two compounds viz., 3-methyl Butanol and n-Hexadecanoic acid were found in fresh *Neera*. The major contributing compounds were 1-Butanol, 3-methyl Propane, 2,2-diethoxy Propanoic acid, 2-hydroxy Ethyl ester, 2-Pentanone, 4-hydroxy-4-methyl n-Hexadecanoic acid, Octanoic acid, n-Decanoic acid and Phenyl ethyl alcohol. The astringency and harsh note of the fermented *Neera* could be due to the increased amounts of acids as well as 1-Butanol, 2,2-diethoxy 3-methyl Propane along with higher concentrations of ethyl alcohol and ethyl esters. The quantity of 3-Methyl 1-Butanol, increased fifty-fold, n-Hexadecanoic acid and 2, 2-diethoxy Propane, was found in higher concentrations, while the concentration of other compounds increased marginally.

Table 2. The compounds and its concentration (µg/l) in the fresh and fermented *Neera* as identified by GC-MS.

RT	KI	Compound name	(µg/l)	
			0 h	40 h
3.25	634	1-Pentanol	349.9	-
3.40	655	3-methyl-1-Butanol	51.28	2562.01
3.92	718	2,2-diethoxy Propane	-	1748.31
4.44	773	Octane	365.78	-
5.17	818	2-hydroxy-ethyl ester Propanoic acid,	-	965.23
5.30	823	2-methyl Pyrazine,	370.34	-
5.99	848	4-hydroxy-4-methyl-2-Pentanone	-	436.73
8.44	918	2,5-dimethyl Pyrazine	35.34	-
8.48	919	2,6-dimethyl Pyrazine	125.02	-
10.57	943	Dimethyl Trisulfide	1.484	-
11.98	997	Decane	20.9	-
12.34	1004	2,3-Octanedione	-	64.61
12.49	1005	Butanoic acid, 3-hydroxy-, ethyl ester	-	67.53
13.17	1064	2-acetyl Thiazole	1.68	-
14.87	1036	3-methyl 4-Heptanone	3.34	-
15.68	1045	Propanoic acid, 2-methyl-, 3-methylbutyl ester	2.72	-
16.05	1049	2,3-Butanediol, diacetate	18.12	-
16.29	1052	2-Amino-6-methylheptane	0.86	-
16.36	1052	3-methyl 4-Heptanone	0.78	-
16.74	1056	3-ethyl 3-methyl Heptane	10.9	-
17.79	1067	Phenyl ethyl alcohol	8.90	196.20
21.54	1202	Propanedioic acid, propyl-	-	35.49
22.03	1214	Octanoic acid	-	241.05
25.28	1281	Undecane 4,7, Dimethyl	13.746	-
27.44	1323	2 Methoxy 4 Vinyl Phenol	145.38	-
31.70	1395	n-Decanoic acid	-	149.04
60.95	1864	1-Hexadecanol	-	74.32
62.12	1984	n-Hexadecanoic acid	83.08	219.85

Table 3. Volatile compounds in fresh and fermented coconut sap.

S. No.	Compound	Number	µg/l	
			0 h	40 h
1.	Carbonyl compounds	05	4.12 (0.26%)	2249.65 (33.28%)
2.	Esters	04	20.84 (1.30%)	1032.76 (15.28%)
3.	Aliphatic Alcohols	03	401.18 (25.07%)	2636.33 (38.99%)
4.	Aromatic Alcohols	01	--	196.20 (2.90%)
5.	Heterocyclic compounds	06	534.724 (33.41%)	--
6.	Phenolic compounds	01	145.38 (9.08%)	--
7.	Carboxylic acids	04	83.08 (5.19%)	645.43 (9.55%)
8.	Aliphatic Hydrocarbons	04	411.326 (25.69%)	--

CONCLUSION

Neera is a highly nutritive and a good digestive agent, however its highly fermentable nature makes difficulties in large scale production and storage. The present experimental study is focused on investigating the development of alcohol and volatiles in the *Neera* over a period of 40 h. The hygienically collected fresh *Neera* was stored at different time intervals and changes occurring in terms of the physico-chemical parameters, microbiological quality and volatiles development was analysed. The development of alcohol in the *Neera* samples stored at different time intervals were evaluated using dichromate-oxidation method. The alcohol content recorded maximum of 5.17% in the 40th hour of storage when comparing with the 0.013% alcohol at 0th hour. Microbiological analysis showed high amount of microbial load (total plate count and yeast and mold count) in the *Neera* samples. Simultaneous distillation and solvent extraction technique was adopted for the extraction of volatiles form *Neera*. Thus, recovered volatiles were further subjected to GC-MS analysis and major compounds were identified. Many heterocycles are found to present, which could be characteristic of fresh *Neera* of this region. However, all heterocycles are degraded during the course of fermentation and not observed in fermented (40 h) sample.

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