Microbial characteristics of freshly tapped Palmyra Palm (*Borassus flabellifer*) sap

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Abstract— Microbial flora of the freshly tapped popular palmyara palm sap was investigated from coastal Karnataka, India. ITS region of rDNA OF yeast isolates was successfully amplified using fungal primer ITS4 & ITS5 and 16S ribosomal RNA of bacterial isolates were amplified by fDD2 & rPP2 primer. Two dominant yeast species identified in the palm sap was *Saccharomyces cerevisiae isolate* YN3, and *Lachancea fermentati* isolate UCLM 17A, and dominant Lactic acid bacteria are *Leuconostoc mesenteroides* ATCC 8293(T) and *Fructobacillus fructosus* KCTC 3544(T). Present study is the first to give comprehensive microbial data of palm sap and provides crucial data for future intervention of the palm sap from fermentation.

Index Terms— Palmyra Palm, Yeast, Lactic Acid Bacteria, *Borassus flabellifer*, Restriction Pattern Analysis, Toddy, Palm sap.

1 INTRODUCTION

The Palmyra palm (*Borassus flabellifer*) sap, an alcoholic beverage available along coastal Karnataka, India. Fresh palm sap tapped from the tip of the inflorescence of Palmyra palm tree is sweet, but gets contaminated by microorganisms by the sap collection vessel and the insects that are attracted towards it [1],[2],[3]. Nutritionally rich palm sap is very good source of medium for the microorganisms to grow. Freshly tapped sweet palm sap contains 11.36% (w/v) of Total sugar, 0.96% (w/v) of reducing sugar, 0.35% (w/v) of protein, 0.056% (w/v) of nitrogen, 0.14% (w/v) of phosphorus, 0.54% (w/v) of mineral ash, 0.4% (w/v) of iron, 13.25% (w/v) of Vitamin C, 3.9 IU of Vitamin B_1 and pH of 7.25[4]. Even though reducing sugar is found in traces in fresh palm sap, rapid fermentation by microbes hydrolyses half of sucrose to glucose and fructose within 24 hours resulting increase in the reducing sugar and production of lactic acid of 0.05-4.78% (w/v) and acetic acid of 0.01-0.24% (w/v) along with the ethanol of 0.21-5.28% (w/v) reduces the pH of the palm sap to about 5, might makes palm sap unacceptable to consumers [5],[6],[7],[8]. Microorganisms *like Schizosacharomyces pombe*, *Saccharomyces cerevisiae*, *Saccaromyces chevalieri*, *Debaryomyces hansenii*, *Geotrichum lactis*, *Zygosaccharomyces rouxiiin, Kloeckra apiculata*, *Bacillus cereus*, *Bacillus Sphaericus, Leuconostoc palmae*, *Fructobacillus fructosus* and *Bacillus firmu*s were isolated in Palm vine [9],[10],[11]. Since microorganism associated with palm sap is known to pay a major role in fermentation, identification and characterization of the palm sap could give crucial information to plan a strategy to intervene the fermentation of sweet palm sap to bitter palm "toddy".

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2 MATERIALS AND METHODS

2.1 Sample Collection

Fresh palm sap samples were collected from seven palm trees (*Borassus flabellifer* L.) at around 6.00 AM professional tappers Sajipa of Dakshina Kannada District (Karnataka, India) over 14 tapping process in the month of December. Each day 50 mL of the samples are collected directly from the Palm sap collection earthen pot into a sterile 50 mL sample collection tubes under sanitary conditions. The ambient temperature recorded during this month was ranging between 18 to 24°C. The Freshly tapped palm sap samples were collected in sterile plastic containers and immediately stored in an ice box (4°C) to avoid fermentation during transportation and transported to the department of Biotechnology, P. A. College of Engineering, Mangalore within 30 min of collecting the samples to reduce fermentation rate considerably [12], [13]. On reaching the laboratory, the samples were filtered by sterile muslin cloth and kept at 4^0C until analysis.

2.2 Chemicals

Analytical grade and were manufactured by Merck Limited (Mumbai, India) were used, and solution were prepared using chemicals supplied by Durga Lab Pvt. Ltd, Mangalore as per the current American Chemical Society specifications[14]. Utensils and Glassware manufactured by Borosil (Mumbai, India) were used for the current research. Yeast Extract Potato dextrose Agar (YEPDA) was prepared using 10g/L of Yeast extract, 20g/L of peptone, 20g/L of dextrose, 20g/L of agar in deionised water was used for microbial growth. The deMan, Rogosa and Sharpe (MRS) Agar was incorporated with 10 g/L of peptone, 10 g/L of Beef extract, 5 g/L Yeast extract, 20 g/L of dextrose, 1 g/L of Polysorbate 80, 2 g/L of $C_6H_{17}N_3O_7$, 5 g/L of CH_3COONa, 0.1 g/L of MgSO4, 0.05 g/L of $MnSO_4$, 2 g/L of K_2HPO_4 and 12 g/L of agar with final pH (at 25°C) 6.5±0.2. Breaking buffer was prepared using 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 m*M* Tris pH 8, 1 m*M* EDTA at pH 8.

2.3 Proximate analysis

Palm sap samples were collected in quadruplicates at different intervals of experiment. Portable Glass electrode pH meter manufactured by Systronics, Mumbai was used to measure the pH of the palm sap. Total protein content of the palm sap was estimated as per Lowry and others, [15] using bovine serum albumin (BSA) as a standard and values were expressed as mg/mL. In this Folin-Ciocalteau method 0.4 mL of samples were incubated with 0.5 mL of 4 *M* NaOH at 100°C for 5 min, and assayed after cooling the homogenate. Total lipid content was estimated by extraction with chloroform ethanol method followed by reaction with sulfuric acid and vanillin phosphoric acid reagent and values were expressed in percentage [16]. Vitamin C in the sample was estimated by Redox Titration methods using 2, 4-dinitrophenyl hydrazine (DNPH) dye and standard ascorbic acid in Systronics Double-beam UV-spectrophotometer, and values were expressed as mg/mL [17] Reducing sugar was measured by dinitrosalicylic acid reagent, and values were expressed in percentage (v/v) [18]. Glucose and Sucrose was estimated using High sensitive Glucose and Sucrose Assay kit supplied by EMerck, India. Ethanol content in the palm sap was estimates using Colorimetric method consist of color reaction of ethanol with sodium dichromate, and values were expressed in percentage (v/v) [19]. Total microbial count on YEPDA and MRS agar was performed as per APHA method and values were expressed in cfu/mL [20]. All the samples from each palm tree were analyzed in quatriplate and data was subjected to One way-analysis of variance (ANOVA) using the Fisher's least significant difference (LSD) test to estimate the significant differences between each palm tree sample ($P \leq 0.05$) using Statgraphics Centurion XV software (Statpoint Technologies Inc., Warrenton, VA, USA).

2.4 Phylogenetic analysis of fungal isolate

Total number of microbial isolates analyses in this work is 400. These isolates were cultures in 5 mL of YEPDB at 28°C in an BOD incubator (Rotek, Cochin) at 200rpm for 18 hours and 1.5 mL each of these culture broths were clarified at Relative Centrifugal Force(RCF) of 20,000×*g* for 5 min at 8°C (Systronics, Mumbai). Supernatant were decanted and pellets were used for DNA extraction as per the standard method with following modifications [21], [22] Breaking buffer was prepared using 2% Triton X-100, 1% SDS, 100 m*M* NaCl, 10 m*M* Tris pH 8, 1 m*M* EDTA at pH 8. The pellets were resuspended in 200 µL of breaking buffer, 0.3 g of glass beads of diameter 0.42-06 mm and 200 µL of buffered phenol, chloroform, isoamyl alcohol at the ratio of 50:48:2, mixed for 1 min in Vortex (Rotech, Cochin). The homogenate was centrifuged for 10 min at 16 000×*g* at 4°C after mixing with 200 µL of 10 m*M* Tris buffer with 1 m*M* EDTA of pH 7.6. The aqueous phase is collected and DNA is precipitated using 2.5 volumes of absolute ethanol and again centrifuged at 16 000×*g* at 4°C for 10 min. Then pellets were washed with 70% ethanol and after drying resuspended in 50 µL of sterile demineralised water with 2 IU RNase. These samples were incubated at 37°C for 30 min and then stored at −20°C. The nuclear rDNA regions including the internal transcribed spacer regions such as ITS 1, 5.8 rRNA gene, and ITS 2 (5.8S-ITS) was amplified using thermal cycler (Corbett Research, Australia) under the PCR conditions[23]. Here, initial denaturation was done at 95°C for 5 min; 35 cycles of denaturation was carried at 94°C for 1 min, primers annealing was carried at 55.5°C for 2 min, extension was done at 72°C for 2 min and final elongation was carried out at 72°C for 10 min. The assays were carried out in 50 mL of solution containing 1x PCR buffer with 2.25 m*M* of $MgCl_2$,100 µ*M* of deoxyribonucleotide phosphate (dNTP) mix, 0.5 µ*M* each of Forward primer ITS5(5' TCCTCCGCTTATTGATATGC 3') and reverse primer ITS4 (5' GGAAGTAAAAGTCGTAACAAGG 3'), 1.25 U of Taq DNA polymerase, and 2 µL of DNA solution. Amplified products were analysed by 1.5 % agarose gel electrophoresis. The products of PCR, at 10 µL of each amplicon with 1.5 µL of 6x loading buffer were separated electrophoresis in a 1.5 % (w/v) agarose (Invitrogen) gel containing 0.7 µg/mL of ethidium bromide, in 0.5 x TBE buffer (44.5 m*M* Tris), 44.5 *mM* of boric acid, 1 mM of Na_2-EDTA for 90 min at 100 V. A standard molecular weight marker (100-bp DNA ladder; Invitrogen) was used to determine the approximate sizes of the amplicons. The gel was photographed under transilluminated Ultra-violet (UV) light. Kodak Molecular Imaging Software version 5.0 (Carestream Health, Inc,Rochester, NY, USA) was used to determine band size by comparison against the DNA ladders. Sequence was performed on ABI Prism model 3100 automated DNA sequencer by the termination method using ABI-BigDye^R Terminator 3.1Cycle sequencing kit. GenBank database at http://www.ncbi.nlm.nih.gov/nucleotide was referred to compare the sequence using the Basic Local Alignment Search Tool (BLAST). The sequences have been deposited in GenBank under NCBI accession numbers KJ5026662.1, KC621077.1, KC515355.1, JQ418546.1, JN403044.1, GQ340445.1, GQ340438.1, KJ451620.1, GQ340439.1, and GQ340440.1.

2.5 Phylogenetic analysis of bacterial isolate

Genomic DNA was isolated using GenElute™ Bacterial Genomic DNA Kit according to manufacturer's instructions. An overnight bacterial culture of volume 1.5 mL was centrifuged at 16,000×*g* to isolate cells into pellets. Pellets were resuspended in 200 µL of 2.115 x106 lysozyme solution and incubated at 37°C for 30 min. To this mixture 20 µL of the Proteinase K solution, and then 200 µL of Lysin solution C was added. The resulting mixture is thoroughly mixed in the vertex for 15 min and then incubated at 55 °C for 10 min. Homogeneous mixture was loaded to GenElute Miniprep Column

and centrifuged at 12,000×*g* to remove separate lysate. The lysate was washed with 200 µL of ethanol and thoroughly mixed in vertex for 10 min. The entire lysate was loaded to binding column using wide bore pipette and centrifuged at 7000×*g* for 1 min. The ethanol free column was washed using wash solution 1 and centrifuged at 7000×*g* for 1 min. The alcohol free column was again washed using wash solution 1 and centrifuged at 16,000×*g* for 3 min. Column was eluted with 200 µL of Elution solution and centrifuged at 7000×*g* for 1 min. The eluate containing genomic DNA was stored at -20°C. The 16S rRNA gene was sequenced over a continuous stretch of 1531 bp to determine the phylogenetic position of the isolates. Amplification of the 16S ribosomal RNA(rRNA) conserved region was performed using the universal primers fDD2 (5′ CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG 3′) and rPP2 (5′ CCAAGCTTCTAGACGGITACCTTGTTACGACTT 3′) [24]. PCR was set up using 12 µL of double distilled water, 2 µL of 10×PCR buffer, 2 µL deoxynucleoside triphosphate (dNTP) mixture, 0.4 µL of each primer, 0.2 µL of 1.5 U Taq DNA polymerase and 2 µL of template DNA. Amplification was performed using the thermal cycler (Corbett Research, Australia) and the PCR cycling parameters consisted of initial denaturation was done at 95°C for 5 min; 35 cycles of denaturation was carried at 94°C for 1 min, primers annealing was carried at 55.5°C for 2 min, extension was done at 72°C for 2 min and final elongation was carried out at 72°C for 10 min. The PCR product obtained from the above reactions were then processed for Cycle Sequencing reaction. The amplified fragments were sequenced by ABI 3730 XL with BigDye® Terminatorv3.1 Cycle Sequencing kit. The analysis of the sequenced fragments was done by Avant 3100 Gene Analyzer. DNA sequences were determined by the chain termination method using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems). Gene sequence of closely related species retrieved from GenBank is used to compare the 16S rRNA gene sequence of strain. A phylogenetic tree was constructed based on the multiple alignment-based similarity matrixes by the neighbor-joining method using the software package Bionumerics, version 3.50 (AppliedMaths)[25].

3 RESULTS

3.1 Physico chemical characteristics of palm sap

Freshly tapped palm sap collected from inflorescence of *Borrasus flabellifer* was transparent without any colour and less viscous. The pH of the fresh samples was ranging between 7-7.4 and near to neutral. Fresh palm sap samples were estimated for total sugar, reducing sugars, non-reducing sugars, glucose and non-glucose reducing sugar content in cell free medium. Non-reducing sugar in the palm sap is sucrose. Content of reducing sugars is subtracted from the content of total sugar to calculate the content of non-reducing sugar. Total sugar content in the fresh palm sap was ranging between 09.88±0.08% (w/v) and 17.32±0.04% (w/v). Here, One way ANOVA with *post hoc* Tukey's test was able to establish a significant(p<0.05) difference in the total sugar content amongst the samples collected from seven different palm trees. Non-reducing sugar content in the palm sap estimated immediately after the tapping was ranging between 8.49±0.06% (w/v) and 2.64±0.01% (w/v), wich is mainly sucrose. The overall variation of non-reducing sugar levels amongst the samples collected from seven different palm trees remained at 5% level of significance, as indicated by One way ANOVA with *post hoc* Tukey's test. Here, of the total sugar content of palm sap 86.41±0.99% of content of is non-reducing sugar. Content of reducing sugar in the palm sap was varing between 1.38±0.03% (w/v) and 2.64±0.01% (w/v), of which percentage of glucose varied between 0.69±0.01% (w/v) and 01.32±0.06% (w/v), and non-glucose reducing sugar varied from 0.53±0.02% (w/v) to 1.32±0.06% (w/v). We have recorded a significant (p<0.05) variation in the level of reducing sugars collected from seven palm trees. Here, percentage of reducing sugar is 13.38±0.98% of the total sugar content of the palm sap. Of the total reducing sugar content of the palm sap samples, 49.84±0.14% of content was glucose and 40.46±3.08% of content is non-glucose. Moreover, it very interesting here to record that eventhough there was a significant (p<0.05) variation in the content of given type of the sugar between the samples collected from seven palm trees, One way ANOVA with *post hoc* Tukey's test was not able to establish any significant (p>0.05) variation the the ratio of the reducing sugar or non-reducing sugars to total sugar content of the palm sap. Protein content of palm sap collected from seven different trees varied significantly(p<0.05) between 0.99±0.76 mg/mL and 2.90±0.45 mg/mL, and lipid content varied significantly (p<0.05) between 0.027±0.002% and 0.09±0.002%. Similarly, Vitamin C in palm sap collected from seven different trees varied significantly(p<0.05) between 0.04 ±0.005 mg/mL and 0.11 ±0.005 mg/mL. Total bacterial count reported between palm sap collected from seven different palm trees varied from $2X10^3$ cfu/mL to $3X10^6$ cfu/mL, and total mould count reported between palm sap collected from seven different palm trees varied from $8X10^2$ cfu/mL to $9X10^5$ cfu/mL. Bacterial or mould polpulation of the freshly collected palm sap varied significantly amongst the samples of seven different palm trees. Freshly tapped palm sap did not record significant (p<0.05) level of alcohol, as indicated by One way ANOVA with *post hoc* Tukey's test. Two types of yeast were isolated from YAPDA at 30°C. Isolate with smooth colonies, round colony margins, with budding sperical cells were identified as *Saccharomyces.* The other yeas colony was creamish, smooth, glucose, spindle shaped cell with ascospores, galactose and sucrose assimilating isolate was identified as *Lachancea sp.* The two types of bacterial colonies were isolated on MRS medium

incubated at 30°C. Small, whitish, convex, circular, and smooth colonies were Gram positive cocci with the chain of up to 40 cells, non-motile, non-spore forming, facultaive anaerobic, catalase negative, resistant upto to 3% of Nacl, that produced lactic acids and ethanol were identified as *Leuconostoc sp.*, marked as MRS1. Where as Small, whitish, convex, circular, and smooth colonies were Gram positive bacilli that produced acetic acids were identified as *Fructobacillus sp,* marked as LAC1.

3.2 Phylogenetic analysis of bacterial isolate

Pure cultures were obtained from the palm sap and genomic DNA from such pure cultures was used to optimize the amplification conditions. Polymerase chain reaction (PCR) amplification and analysis of restriction of the complex Internal Transcribed Spacer (ITS) regions, both non-coding and variable and 5.8S rRNA gene, both coding and conserved provides phylogenitic relationships. Fungal isolates of palm sap are grouped by amplification of the 5.8S-ITS region of the yeast, and bacterial isolates of palm sap are grouped by amplification of the 16S rDNA ITS region of the bacteria. Phenotypically characterised yeasts were further grouped using ITS-PCR into 2 different groups, and bacteria were clustered into 2 different groups using ITS-PCR. To identify the yeast isolate of palm sap, 5.8S-ITS region of the yeast was amplified using ITS4 and ITS5 primers. GenBank database was referred to compare the sequence using the BLAST. The sequences have been deposited in GenBank under NCBI accession numbers KJ5026662.1, KC621077.1, KC515355.1, JQ418546.1, JN403044.1, with the data of *Saccharomyces cerevisiae* isolate YN3, *Saccharomyces cerevisiae* strain DQY2, *Saccharomyces cerevisiae* strain Sc01, *Saccharomyces cerevisiae* strain BAPY3, *and Saccharomyces sp.* BTPJ1 with the Alignment statistics given in the Table 1.

Table 1 BLAST analysis of sequence of fungal isolates.

Gene Bank Accession number	Description	Max score	Query cover	E value	Identity
KJ5026662.1	*Saccharomyces cerevisiae* isolate YN3	807	807	0.0	99%
KC621077.1	*Saccharomyces cerevisiae* strain DQY2	807	807	0.0	99%
KC515355.1	*Saccharomyces cerevisiae* strain Sc01	807	807	0.0	99%
JQ418546.1	*Saccharomyces cerevisiae* strain BAPY3	807	807	0.0	99%
JN403044.1	*Saccharomyces sp.* BTPJ1	807	807	0.0	99%

Analysis of the resulting 16S rDNA sequences was carried out to confirm the genetic homogeneity of the selected target among the subjected isolates and to estimate identity with the type of the isolates. The yeast isolate showed 99% sequence similarity with *Saccharomyces cerevisiae*. Sequence analysis with NCBI accession number KJ502662.1 *Saccharomyces cerevisiae* isolate YN3 showed that the query length was 454, score was 807 bits(894), expect was 0.0, identities were 452/454 (99%), Gaps were 1/452(0%), and Strand was Plus/Plus.

The 5.8S-ITS region *Lachancea sp* was amplified using ITS4 and ITS5 primer. The sequence of the *Lachancea sp.* was fed to GenBank to compare the sequence using the BLAST as indicated in the Table 2. The sequences have been deposited in GenBank under NCBI accession numbers GQ340445.1, GQ340438.1, KJ451620.1, GQ340439.1, GQ340440.1, with the data of *Lachancea sp.* UCLM 88.3, *Lachancea fermentati* isolate UCLM 1aA, *Lachancea sp* Y309, *Lachancea fermentati* isolate UCLM 55A, *Lachancea fermentati* isolate UCLM 17A with the Alignment statistics given in the Table 2.

Table 2 BLAST analysis of sequence of fungal isolates.

Gene Bank Accession number	Description	Max score	Query cover	E value	Identity
GQ340445.1	*Lachancea sp.* UCLM 88.3	1074	1074	0.0	99%
GQ340438.1	*Lachancea fermentati* isolate UCLM 1aA	1074	1074	0.0	99%
KJ451620.1	*Lachancea sp* Y309	1058	1058	0.0	99%
GQ340439.1	*Lachancea fermentati* isolate UCLM 55A	1054	1054	0.0	99%
GQ340440.1	*Lachancea fermentati* isolate UCLM 17A	1047	1047	0.0	99%

Analysis of the resulting 16S rDNA sequences was carried out to confirm the genetic homogeneity of the selected target among the subjected isolates and to estimate identity with the type of the isolates. The *Lachancea sp* showed 99% sequence similarity with *Lachancea fermentati*. Sequence analysis with NCBI accession number GQ340438.1 *Lachancea fermentati* isolate UCLM 1aA showed that the query length was 604, score was 1074 bits(1190), expect was 0.0, identities were 599/600 (99%), Gaps were 1/452(0%), and Strand Plus/Plus.

To identify the *Leuconostoc* spp. of palm sap, 16S rDNA ITS region of the bacteria amplified using fDD2 and rPP2 primers. Bioinformatical Analysis of the resulting 16S rDNA sequences was carried out to confirm the genetic homogeneity of the selected target among the subjected isolates and to esti-

mate identity with the type of the isolates. Genetic homogeneity of 16S rDNA among different strains of the isolate was confirmed. Dendrogram of the tested isolates and the type strain was constructed based on these data. The alignment of the 16S rDNA sequences confirms the presence of highly homogeneous region consisting of 387 bp. This analysis of *Leuconostoc* spp. is in distinct line of descent within the genus *Leuconostoc*, with the closest neighbors being *Leuconostoc mesenteroids* subspecies *mesenteroids* ATCC 8293(T) ribosomal RNA gene, partial sequence (CPOOO414) with 99.45% similarity(Fig 1).

Fig. 1. Analysis of alignment of the 16S rDNA sequences of *Leuconostoc* spp (MRS 1).

Similarly, analysis of alignment of the 16S rDNA sequences of *Fructobacilli* confirms within the genus *Fructobacilli*, with the closest phylogenetic affiliation with *Fructobacillus fructosus* KCTC 3544(T) ribosomal RNA gene, partial sequence (AEOPO1000025) with 98.31% similarity(Fig 2).

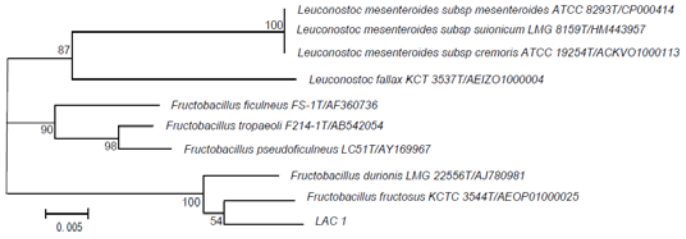

Fig. 2. Analysis of alignment of the 16S rDNA sequences of *Leuconostoc* spp (LAC 1).

5 DISCUSSION

The freshly tapped palm sap is transparent, clear, less viscous, sweet, with a sugar content of approximately 9-17% w/v [26],[27],[1],[28],[29],[7]. Of the total sugars estimated in the freshly tapped palm sap, around 86% is non-reducing sugar of which majority is sucrose and around 13% is reducing sugar of which is equally shared by glucose and non-glucose reducing sugars. Proportion of the sugars of the sap of *Phoenix dactylifera* reported to have of 95.27% of sucrose, 2.51% glucose and 1.61% fructose on dry weight basis [30]. Freshly tapped palm sap samples were recorded with pH near to neutral, which indicates the freshness of palm sap [31],[1],[32],[7]. Palm sap changes the colour, taste, and appearance during collection period and transport from colourless, transparent, sweet and less viscous to whitish, translucent, sour, and more viscous palm wine due to the initial activity of Lactic acid bacteria that are reported to produce gum like dextranes [33],[29]. Subsequently, bacterial and yeast cell loads is also responsible for giving a milky-white appearance of the palm sap [34]. The levels of composition of palm sap depends on the state of the fermentation taken place by Lactic acid producing bacteria, followed by alcohol producing yeast, subsequently by acetic acid producing microbial flora [35],[26],[1],[5],[6],[7].

In the initial stage of fermentation palm sap is sweet and does not contain alcohol [31],[1],[32],[7]. On the first day of tapping Raphia palm wine was reported to contain sucrose, maltose, glucose and fructose sugars, on the middle of the tapping period the wince was recorded to possess xylose and cellobiose, and after few days of tapping palm wine was reported have the irregular appearance of galacturonic acid, arabinose and rhamnose sugars[37],[38]. Members of microbial consortium of each stage mutually communicate to trade metabolites, respond to the presence of other and help in metabolic activities of their counterparts of next stage [26],[39]. One the first day of sampling palm wine of *Borassus flabellifer* reported to have to 9.29 to 17.44% of sucrose, 0.50 and 1.85% of glucose, and 0.050 and 1.81% of fructose [29]. Total sugar content of palm wine of *Elaeis guineensis* reported to have reduced to more than half during every week of tapping, but level of sugar in freshly tapped palm sap is maintained through continuous oozing of the sweet sap by the plant [1],[32],[3]. During the early stages of tapping, low level of sugar content in the palm sap is a clear indication that a major portion of the sugars is fermented as a result of the microbial metabolic activity. Variation in the sugar content amongst palm trees are due to the cutting of trees, leaves those effects the photosynthesis [3],[37],[38].

Initial microbial activity decreases the pH that inturn enhances the invertase activity of the yeast of the next stage [26],[29], and subsequent ethanol produced by the yeast is used as substrate by the acetic acid producing bacteria to produce acetic acid [26],[1]. In our study pH of the freshly tapped palm tap was approximately neutral, and with the microbial activity pH falls down due to the production of lactic acid by lactic acid producing bacterial during the initial period and latter by the production of acetic acid acetic acid producing bacteria that inturn gives particular aroma of palm wine [27],[31], [1][5],[6],[32],[7]. However, level of these constituents depends on the several factors such as types of the microbes involved, composition of the palm sap, species of palm, environmental temperature, wind velocity, type of tapping, flow rate of the sap, time of the tapping, and time lag period between sap collection and analysis of the sample wind [7]. Even

though Palm sap tapper slice off the walls of the receptacle that reduces the microbial load, palm sap collected directly from the palm tree early in the morning in our study was not sterile probably due to some microbiota that has colonized the walls of the receptacle got into the flowing palm sap into clean collecting pot [1], [7]. Characteristics aroma of the palm wine is due to varieties volatile components such as alcohols, esters, acids, aldehydes, ketones [40],[41],[42].

Total bacterial count reported between palm sap collected from seven different palm trees varied between $2X10^3$ cfu/mL and $3X10^6$ cfu/mL, and Total mould count reported between palm sap collected from seven different palm trees varied between $8X10^2$ cfu/mL and $9X10^5$ cfu/mL. Total bacterial count varied between $2X10^3$ cfu/mL and $3X10^6$ cfu/mL, and Total mould count varied between $8X10^2$ cfu/mL and $9X10^5$ cfu/mL, which is much less than the earlier reported microbial loads in other palm wines. Elarlier report recorded the yeast populations palm wine of between 10^4 to 10^7 cfu/mL, and Total aerobic mesophiles varied between 10^6 and 10^9 cfu/mL [26],[1] [5],[34],[6],[7]. Predominant Lactic acid bacteria reported in palm wine fermentation are *Lactobacillus plantarum* and *Leuconostoc mesenteroides,* and are responsible for the sour taste of palm wine and are responsible for the pH decrease during the taping through the organic acids production [1],[6]. Yeast isolated from the palm sap was identified phenotypically by cultural characteristics on the YEPDA and microscopic characteristics and the Lactic acid bacteria on isolated from the palm sap was identified base on the cultural characteristics on MRS agar, Gram staining, Catalase test and carbohydrate assimilation [45], [46]. Lactic acid bacteria was phenotypically characterized by microscopic morphology, Gram staining, catalase test, and carbohydrate assimilation pattern and the yeasts was identified using standard morphological and physiological tests such as morphology, colony characteristic, pseudohyphae, ascospore, vegetative reproduction sugar assimilation [46]. Two dominant yeast species identified in the palm sap was *Saccharomyces sp.*, and *Lachancea sp.*, and dominant Lactic acid bacteria are *Leuconostoc sp.* and *Fructobacillus sp.* However, identification of microbial flora through phenotypic characterisation is inaccurate and time consuming [47]. Pure cultures were obtained from the palm sap and genomic DNA from such pure cultures was used to optimize the amplification conditions. Yeasts isolated from palm sap were grouped by amplification of the 5.8S-ITS region of the yeast, and Lactic acid bacteria isolated from palm sap are grouped by amplification of the 16S rDNA ITS region of the bacteria. The *Saccharomyces* isolated from palm sap showed 99% sequence similarities with *Saccharomyces cerevisiae* isolate YN3 and the *Lachancea sp.* showed 99% sequence similarity with *Lachancea fermentati* isolate UCLM 1aA. Similarly, analysis of alignment of the 16S rDNA sequences of analysis of *Leuconostoc sp.* confirms affiliation with *Leuconostoc mesenteroids* subspecies *mesenteroids* ATCC 8293(T) and *Fructobacilli sp.* confirms affiliation with *Fructobacillus fructosus* KCTC 3544(T).

6 CONCLUSION

The chemical composition of the freshly tapped palm sap from different parts of Mangalore district varied, but ratio of the reducing sugar and non-reducing sugar remained almost same. Glucose is the main reducing sugar and sucrose is the major non-reducing sugar of palm sap, and are the substrate for the alcohol, lactic acid and acetic acid producing microbial flora in the palm sap, making the palm vine an interesting source to isolate microbes of potential industrial importance. Traditional microbial techniques along with phylogenetic identification to identify the microbial flora of the palm sap are very useful. Dominant microbial flora isolated from the palm sap collected for the seven palm tree around Mangalore were *Saccharomyces cerevisiae* isolate YN3, *Lachancea fermentati* isolate UCLM 1aA, *Leuconostoc mesenteroids* subspecies *mesenteroids* ATCC 8293(T) and *Fructobacillus fructosus* KCTC 3544(T). Information on source of these diverse microbial flora in palm sap and change in microbial population during fermentation of palm sap and associated changed taking place in the chemical composition during this period has to be studied in detail.

REFERENCES

[1] W.K. Amoa-Awua, E. Sampson and K. Tano-Debrah, "Growth of yeasts, lactic and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm Elaeis guineensis in Ghana," *J Appl Microbiol*, vol. 102, no. 1, pp 599-606, 2007.

[2] P. Naknean and M. Meenune, "Impact of Clarification of Palm Sap and Processing Method on the Quality of Palm Sugar Syrup (*Borassus Flabellifer Linn.*)," *Sugar Tech*, vol. 17, no. 1, pp. 195, 2015.

[3] R. Poché, R. Garlapati, K. Dixon, A. Gendernalik, D. Poché, D. Perry, "The role of Palmyra palm trees (*Borassus flabellifer*) and sand fly distribution in northeastern India, *Journal of Vector Ecology*, vol. 37, no. 1, pp. 148–153, 2012.

[4] D. Barh and B.C. Mazumdar, "Comparative nutritive values of palm saps before and after their partial fermentation and effective use of wild date (*Phoenix sylvestris Roxb.*) sap in treatment of Anemia," *Res. J. Medicine & Med. Sci.*, vol. 3, no. 2, pp. 173-176, 2008

[5] M. Stringini, F. Comitini, M. Taccari and M. Ciani, "Yeast diversity during tapping and fermentation of palm wine from Cameroon," *Food Microbiol*, vol. 26, no. 1, pp. 415–420, 2009

[6] L. Ouoba, C. Kando, C. Parkouda, H. Sawadogo-Lingani, B. Diawara and J.P. Sutherland, " The microbiology of Bandji, palm wine of *Borassus akeassii* from Burkina Faso: identification and genotypicdiversity of yeasts, lactic acid and acetic acid bacteria,'' *J Appl Microbiol,* vol. 113, no.1, pp. 1428–1441, 2012

[7] J.A. Santiago-Urbina, A.G. Verdugo-Valdez, F. Ruíz-Terán, "Physicochemical and microbiological changes during tapping of palm sap to produce an alcoholic beverage called "Taberna", which is produced in the south east of Mexico," *Food Control*, vol. 33, no. 1, pp. 58–62, 2013

[8] S.Ghosh, R. Chakraborty, A. Chatterjee, and U. Raychaudhuri, "Optimization of media components for the production of palm vinegar using

response surface methodology", vol. 120, no. 4, pp. 550–558, 2014
[9] B.Boboye, I.Dayo-Owoyemi, and F.Akinyosoye, "Organoleptic Analysis of Doughs Fermented with Yeasts from A Nigerian Palm Wine (Elaeis guineensis) and Certain Commercial Yeasts," *The Open Microbiology Journal*, vol. 2, no. 1, pp. 115-119, 2008
[10] I. Nwachukwu, V. Ibekwe, R. Nwabueze and B Anyanwu, "Characterisation of palm wine microbial isolates for industrial utilization," *Afr J Biotechnol*, vol. 5, no. 9, pp. 1725-1728, 2008
[11] R. K. chrystopher and K. Theivendirarajah," Palmyrah palm wine 1: Microbial and Biochemical changes," *J Natn Sci Coun SriLanka*, vol. 16, no. 1, pp.131-141, 1988
[12] O. Bassir, "Observation on the fermentation of Palm wine," *West Afr J Biol Appl Chem*, vol. 6,no.1, pp 20-25, 1962
[13] O. Obire, "Activity of Zymomonas species in palm sap obtained from three areas in Edo state, Nigeria," *J. Appl. Sci. Environ. Manage*, vol. 9, no.1, pp. 25-30, 2005
[14] American Chemical Society (ACS), Lab Guide, Washington D.C., American Chemical Society, pp. 98-99,1999
[15] O.H. Lowry, N.J., Rosebrough, A.L. Farr and R.J. Randall, "Protein measurement with the folin phenol reagent," J Biol Chem, vol. 193, no. 1, pp. 265-275, 1951
[16] E.V. Handel, "Rapid Determination Of Total Lipids In Mosquitoe," *J Aru Mosq. Conrnol Assoc*, vol.1, no.3, pp. 302-304, 1985
[17] S. B. Mussa and I. Sharaa, "Analysis of Vitamin C (ascorbic acid) Contents packed fruit juice by UV -spectrophotometry and Redox Titration Methods", *IOSR Journal of Applied Physics*,vol. 6, no. 5, pp. 46-52, 2014
[18] G.L.Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.*, vol. 31, no. pp. 426, 1959
[19] S.V Sumbhate, Satish Nayak, Damodar Goupale, Ajay Tiwari, Rajesh S Jadon, Journal of Analytical Techniques, Vol 1, No 1 (2012).
[20] APHA, Recommended Procedure for the Examination of Seawater and Shellfish, 2nd ed., American Public Health Association, Washington, pp. 12-13, 1982
[21] F.M. Ausubel, R.Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl, "Current Protocols in Molecular Biology," Wiley, New York. 1995
[22] L. Cocolin, L.F. Bisson and D.A. Mills, "Direct profiling of the yeast dynamics in wine fermentations," vol. 189, no. 1, pp. 81–87, 2000
[23] B.Esteve-Zarzoso, C.Belloch, F.Uruburu and A.Querol, "Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers," *Int J Syst Bacteriol*, vol. 49, no. 1, pp. 329–337, 1999
[24] D.E. Rawlings, "Biohydrometallurgical Processing", University of Chile, Santiago, vol. 2, pp. 9–17, 1995
[25] N. Saitou and M. Nei, "The neighbor-joining method: a new method for reconstructing phylogenetic trees". *Mol Biol E*, vol. 4, no. 1, pp. 406–425, 1987
[26]. J. D. Atputharajah, S. Widanapathirana and U. Samarajeewa, "Microbiology and biochemistry of natural fermentation of coconut palm sap", Food Microbiol, vol. 3, no. 4, pp. 273-280, 1986
[27]. M.O. Eze and O.Uzoechi, "Sugars of the unfermented sap and the wine from the oil palm, *Elaeis guineensis*, tree," *Plant Foods for Human Nutrition*, vol. 38, no.1 , pp. 121-126, 1988
[28]. F.I. Obahiagbon and P. Oviasogie, Changes in the physico-chemical characteristics of processed and stored *Raphia hookeri* palm sap (shelf life studies)," *American Journal of Food Technology*, vol. 2, no. 4, pp. 323-326, 2007
[29]. P.Naknean, M. Meenune and G. Roudaut, "Characterization of palm sap harvested in Songkhla province, Southern Thailand," *International Food Research Journal*, vol. 17, no.4, pp. 977-986, 2010
[30]. I. B. Thabet, S. Besbes, H. Attia, C. Deroanne, F. Francis, N. Drira and C.Blecker, "Physicochemical characteristics of date sap "Lagmi" from deglet nour palm (*Phoenix dactylifera* L," *Int J Food Prop*, vol. 12, no. 1, pp.659–670, 2009
[31]. I.E. Ezeagu, M.A. Fafunso and F.E. Ejezie, "Biochemical constituents of palmwine," *Ecology of Food and Nutrition*, vol. 42, no. 3, pp. 213-222,. 2003
[32]. D. Karamoko, N.T. Djeni, K.F. N'guessan, K.M.J Bouatenin and K.M. Dje, "The biochemical and microbiological quality of palm wine samples produced at different periods during tapping and changes which occurred during their storage," *Food Control*, vol. 26, no. 2, pp. 504-511, 2012
[33]. R.J. Alcántara-Hernández, J. A. Rodríguez-Álvarez, F.A. Valenzuela-Encinas, F.A. Gutiérrez-Miceli, H. Castañón-González, R. Marsch, T. Ayora-Talavera and L. Dendooven, "The bacterial community in "taberna" a traditional beverage of Southern Mexico,". *Letters in Applied Microbiology*, vol. 51, no. 5, pp. 558-563, 2010
[34]. O. Lasekan, A. Buettner and M.Christlbauer, "Investigation of important odorants of palm wine (*Elaeis guineensis*," *Food Chemistry*, vol. 105, no.1, pp. 15-23, 2007
[35] N. Okafor, "Microbiology and biochemistry of oil-palm wine", *Advances in Applied Microbiology*, vol. 24, no. 1, pp. 237-25, 1978
[36]. L. Ouoba, C.Kando, C.Parkouda, H. Sawadogo-Lingani, B. Diawara and J. P. Sutherland," The microbiology of Bandji, palm wine of *Borassus akeassii* from Burkina Faso: identification and genotypic diversity of yeasts, lactic acid and acetic acid bacteria", *Journal of Applied Microbiology*, vol. 113, no. 6, pp. 1428-144, 2012
[37]. S.I. Faparusi and O. Bassir, "Factors affecting the quality of palm wine. l. Period of tapping a palm tree", West *African journal of Biological and Applied Chemistry*, vol. 15, no. 1, pp. 17-23, 1972
[38]. S.I. Faparusi, "Sugar identified in Raphia palm wine," *Food Chemistry*, vol. 7, no. 2, pp. 81-86, 1981
[39]. E.J. Smid and C. Lacroix, "Microbe-microbe interactions in mixed culture food fermentations," *Current Opinion in Biotechnology*, vol. 24, no. 2, pp. 148-154, 2013
[40]. S.V.A Uzochukwu, E. Balogh, O.Tucknott, M.J. Lewis and P. O. Ngoddy, "Volatiles of palm wine using solvent extracts," *Journal of Food Quality*, vol. 20, no.6, pp. 483-494, 1997.
[41]. O. Lasekan, A. Buettner and M. Christlbauer, "Investigation of important odorants of palm wine (*Elaeis guineensis*)," *Food Chemistry*, vol.105, no. 1, pp. 15-23, 2007.
[42]. R. Nur Aimi, F. Abu Bakar and M.H. Dzulkifly, "Determination of volatile compounds in fresh and fermented Nipa sap (*Nypa fructicans*) using static headspace gas chromatography-mass spectrometry (GC-MS)", *International Food Research Journal*, vol. 20, no. 1, pp. 369-376, 2013