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Research Article

Study of Fermentation Kinetics of Palm Sap from *Cocos nucifera*

Premalatha Shetty[1]*, Avila D'Souza[2], Sangeetha Poojari[3], Jayadurgi Narayana[3], Priya Rajeeva[3]

[1]Biotechnology and Microbiology Department, Shri Dharmasthala Manjunatheshwara Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi-574118, Karnataka, India;

[2]Euchem Biologicals, Research and Analytical Laboratory, Shivalli Industrial Estate, Manipal-576104, Karnataka, India;

[3]Department of Applied Bio-sciences, Bhandarkars' Arts and Science College, Kundapura-576201, India

Abstract

Palm wine plays an important role as an alcoholic beverage in traditional practices. It is important to study the biochemical characteristics and microbiological aspects to understand the fermentation kinetics of palm saps. In the present investigation an elaborate study was carried out to study the fermentation kinetics of coconut palm sap. Total sugar, reducing sugar content and glucose concentration was estimated periodically during fermentation for 16h. Microbial load and invertase assay results were related to the changes in sugar concentration. Initial predominance of lactic acid bacteria was followed by dominance of yeasts. Hydrolysis of non reducing sugar occured at a faster rate between 3-9h of fermentation. During this period, the multiplication of yeasts began and reached its peak at 11h fermentation. Ethanol concentration was around 4.0 and 4.1% at 11h and 13h of fermentation respectively.

Keywords: Palm sap; Cocos nucifera; Fermentation; Lactic acid bacteria; Yeast.

Introduction

Saps extracted from inflorescence of various species of palm trees are rich sources of sugar and minerals and provide optimal conditions for the growth of yeasts and lactic acid bacteria. The fermentation starts soon after the sap is collected and within few hours, the sap becomes reasonably high in alcohol up to 4-5% (Naknean *et al.*, 2010; Singaravadivel, 2012; Falegan and Akoja, 2014). Continued fermentation turns the sap into vinegar. Apart from acetic acid, lactic acid production by lactic acid bacteria is been reported (Chooklin *et al.*, 2011; Chandrasekhar *et al.*, 2012). Palm saps are common in parts of Africa, India, Myanmar and Mexico. *Borassus* (Palmyra Palm), *Phoenix sylvestris, Cocos nucifera, Arenga saccharifera*, *Nypa fruticans* and *Corypha elata* are some of the palm species which have been extensively exploited for harvesting the sugary phloem fluid (Shamala and Sreekantiah. 1988; Santiago-Urbina *et al.*, 2013). In India Palmyrah, coconut and date palm are the major contenders. Geo- seasonal variations affect the chemical composition and microbial population leading to concomitant alterations

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[1]***Corresponding author**
Premalatha Shetty,
Biotechnology and Microbiology Department, Shri Dharmasthala Manjunatheshwara Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi-574118, Karnataka, India
Email: premalathashetty@gmail.com

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in the fermentation kinetics of plant saps. Although a rich source of vitamins and minerals, these products are losing their market share due to the inconsistency in their quality. Optimization and standardization of fermentation conditions is needed to improve the reproducibility of the product characteristics and to increase the market value of these products (Ghosh *et al.*, 2012; Kapilan *et al.*, 2015; Nwaiwu *et al.*, 2016). It would be interesting to study the profile of hydrolytic enzymes and their effect on sugars as results obtained from such studies may be indicative of changing microbial dominance and stages of fermentation. Yeasts isolated from plant products have been assessed for ethanol production from palm juices. Literature is replete with information on the chemical composition of the palm sap products (Eze and Uzoechi, 1988; Singaravadivel, 2012; Ngoc *et al.*, 2013; Francois *et al.*, 2016). Several yeasts and lactic acid bacteria have been identified in palm saps. However, an elaborate study involving the enzymatic kinetics of the fermentation correlating it to the changing microbial populations may explain the chemical changes that occur in the sap at different stages of fermentation. High amount of sugars in the sap has impeded the study of enzymes in these saps. Palm sap finds application as an acidulant in addition to its use as a rich source of sugars and alcoholic beverage. Study of fermentation kinetics may provide clues to modulate the fermentation conditions as per the required end use.

Sap tapped from *Cocos nucifera* (coconut) is consumed extensively in fresh as well as its fermented form. Nutraceutical values have been ascribed to various products derived from the palms (Akpa and Gunorubon, 2012; Kalaiyarisi *et al.*, 2013; Hebbar *et al.*, 2015). Fermented beverage obtained from palm juice has the potential to be used as biofuel (Joseph *et al.*, 2014). There is a need to understand natural fermentation of the sap through quantitative analyses of various biomolecules and enzymes responsible for the change along with the microbial dynamics. In the present investigation, coconut palm sap was studied at various stages of fermentation. Profiling of total sugars, reducing sugars and glucose in the sap and, invertase and pectinase content of the cellular fraction in the sap was done. Microbial dominance was correlated to the biochemical changes during fermentation.

Materials and Methods

Materials

The coconut palm sap was collected from local coconut trees. All the chemicals and reagents used for the study are of analytical grade. Glocose oxidase-peroxidase kit for glucose estimation was procured from Agappe Diagnostics Ltd, India.

Collection of fluid

Tapping was done at 7.00a.m in the morning. The samples were incubated at 30±0.5°C. Aliquots were removed aseptically at periodic intervals over a period of 3 days. Each aliquot was divided into two parts. One part was immediately processed for microbial studies. The other part was subjected to centrifugation at 7000 rpm for 20min at 4°C. Cell fraction was washed with sterile saline and subjected to analyses. The pH of the cell free fraction was noted down. The cell free fractions and cell suspensions were stored at -20°C when not in use.

Estimation of Sugars

Reducing sugars in the cell free sap were measured by DNSA method. Total sugars were measured by Phenol-Sulfuric acid method. To 0.5ml of the sample to be analysed, 0.5ml of 5% phenol was added and mixed, followed by gentle addition of 2ml Sulphuric acid followed by incubation of 10min. The contents were mixed and absorbance was read at 490 min after 20min. Glucose was estimated using glucose oxidase-peroxidase kit.

Estimation of Alcohol Content

To five ml of the sap, 45ml of distilled water was added and distilled. Distillate around 20ml was collected, volume was made to 25ml and estimated for alcohol content based on oxidation by dichromate reagent (Sumbhate *et al.*, 2012)

Enzyme Assays

Invertase assay

Activity in the cellular fractions was carried out by incubating the cell suspension with 0.25% sucrose in 10mM phosphate buffer, pH 5.5 at 37°C for 20min in shaker water bath at 50 rpm. Reducing sugars produced were measured by DNSA method.

Pectinase assay

Pectinase activity in the cellular fractions was monitored by incubating the cell suspension with 0.3% pectin in 10mM phosphate buffer, pH 5.5 at 37°C for 20min shaker water bath at 50 rpm. Reducing sugars produced were measured by DNSA method.

Enzyme units were expressed as micromoles of reducing sugars equivalent to glucose produced per minute under the defined assay conditions.

Microbial count

Haemocytometric method for yeast cell count

The sap was diluted 10 times in Saline containing 4% sucrose and the suspension was immediately loaded into haemocytometer chamber. Yeast cells were counted under 40x magnification in six RBC chambers. The morphology was noted and cell count was calculated per ml.

Viable count:

Serial dilutions of 0h, 6h, 9h, 14h and 18h were made in sterile saline containing 0.01% tween-80 and 4% sucrose. Hundred micro litres of each dilution was spread onto MRS agar. The plates were incubated under airtight conditions for 48h.

Results and Discussion

The sap collected was incubated at 29±1˚. Variation in the composition of sugars indicates the stages of fermentation (Rokosu and Nwisienyi, 1980). Samples were collected at periodic intervals and stored at 0˚C. The cell free broth was subjected to analyses. The total sugars, reducing sugars and glucose content in the cell free broth were estimated (Table 1). Sucrose is the non-reducing sugar found in palm saps. The non-reducing sugar content was calculated by subtraction of reducing content from total sugars. Total sugar content in the fresh sap was around 15.6%. Sucrose content in the sap immediately after collection was found to be around 11% (Fig. 1). Palm saps are known to contain 10-18% of total sugars and 9-11% sucrose (Eze and Uzoechi, 1988; Santiago-Urbina and Ruíz-Terán, 2014). Profile of non-reducing sugar (sucrose) content showed that sucrose hydrolysis occurred at a faster rate after 3h of incubation and continued till 9th hour of fermentation. Thereafter the rate of hydrolysis slowed down.

Table 1: Fermentation of sugars in Coconut palm sap

Incubation period (h)	Total sugars (TS)	Reducing sugars (RS)	Glucose (G)	Non-reducing sugar (TS-RS)	Non-glucose reducing sugars (RS-G)
0	15.56±0.56	4.76±0.9	2.65±0.11	10.80	2.11
1	15.3±2.13	5±0.48	3.18±0.44	10.30	1.83
3	15.4±1.25	5.1±0.77	2.88±0.69	10.31	2.24
5	13.1±0.38	6.1±0.5	3.12±0.5	6.97	2.96
7	13.2±0.56	7.7±0.69	3.47±0.35	5.54	4.19
9	14.8±1.49	11.2±2.94	7.47±1.64	3.69	3.7
11	11.7±1.48	7.96±1.8	3.53±0.63	3.73	4.43
13	10.2±1.35	7.6±1.22	2.63±1.03	2.64	4.95
16	9.5±0.61	7.39±0.87	2.7±1.12	2.1	4.69

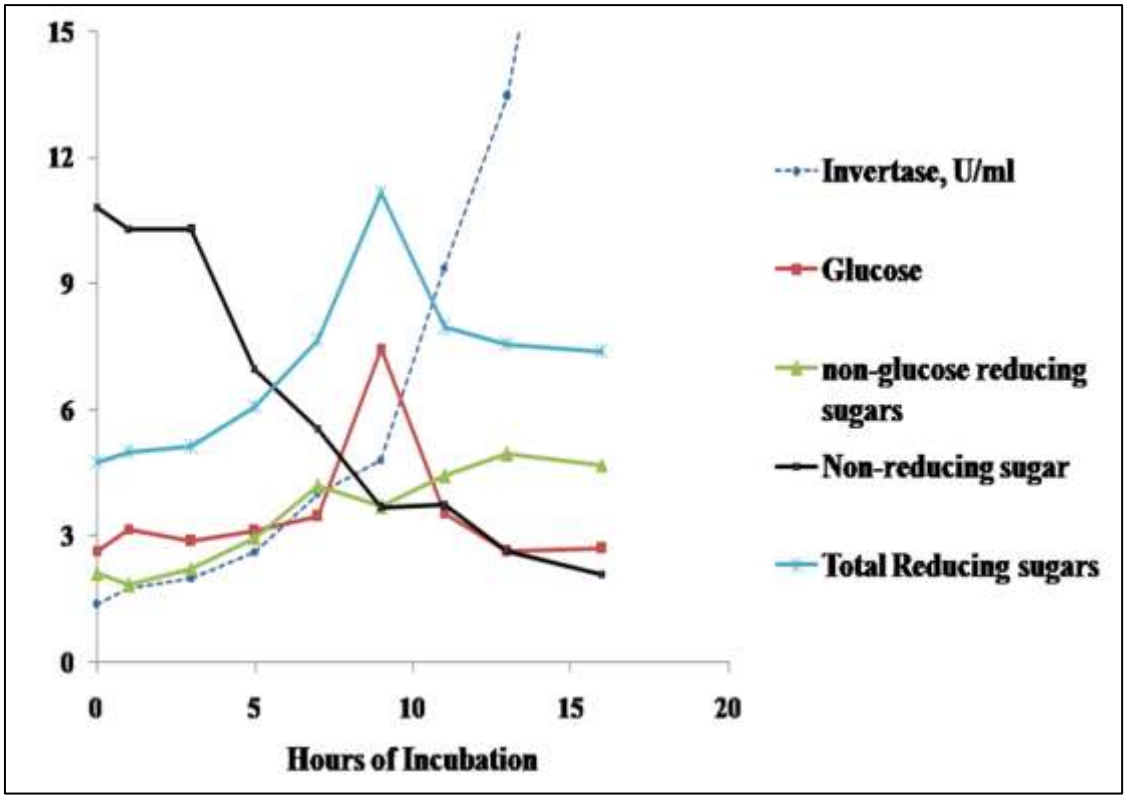

Fig. 1: Kinetics of sugar hydrolysis during fermentation of coconut palm sap Sugar concentration expressed as g%.

Total reducing sugars was measured by DNSA method. At the time of collection the reducing sugar content was found to be around 4.8±0.9%. Most of the reducing sugars may be arising from hydrolysis of sucrose. The resulting sugars arising due to invertase action on sucrose yields glucose and fructose. These sugars are utilized by the microbial population for their proliferation. Lactic acid bacteria (LAB) and yeasts are the major players in the fermentation of palm saps. Invertase is expressed by many LAB and yeast species. In addition to production of invertase, yeasts are known to express the enzymes responsible for production of alcohol from the reducing sugars (Theivendirarajah and chrystopher, 1987; Ngoc *et al*., 2013).

Enzyme activity in the cell free broth was found to be negligible and, it was likely that the high concentration of the reducing sugars in the samples were interfering in the assays. Therefore, each sample was subjected to salting out with ammonium sulfate at 80% saturation and the salted out fraction was assayed for activity after dialysis. Invertase activity in the 0 and 5h samples found to be 0.014 and 0.027 U/ml of the sample respectively. Pectinase activity was found to be around 0.012 and 0.028 U/ml at 0 and 5h respectively. The salted out fractions therefore, exhibited negligible activity. Since invertase activity is one of the major contributors in fermentation of palm saps, it is unlikely that the invertase activity would be so low. Invertase is often found to be an intracellular enzyme. In the yeast cells, invertase is likely to be localized in the periplasmic space of the cells. Hence, the enzyme activities in the cellular fractions were assayed. Cellular fractions showed high invertase activity. Invertase activity gradually increased from 1.4U/ml at 0h to 4.8 U/ml at 9^{th} hour of incubation. Thereafter a sharp increase in the activity was seen reaching 26 U/ml at the end of 16^{th} hour.

As shown in Fig. 2, two kinds of colonies were prominent in MRS agar. Bright creamish opaque colonies which were large (>1.5-2mm), and whitish colonies which were smaller (<1.5mm) and less opaque. The large colonies were few in number in comparison to the small colonies. Five colonies each of the small and large size were picked up and subjected to gram staining. The large and the small colonies were found to be comprised of yeast cells and lactic acid bacteria (LAB) respectively. All the LAB species were rod shaped indicating the dominance of *Lactobacillus* species. Large colonies were excluded and the lactic acid bacteria (LAB) count was carried out considering the small colony forming units. LAB were the predominant species till 7^{th} hour of incubation (8-12 $x10^7$ cfu/ml). By the end of 13^{th} hour of fermentation the LAB count decreased to $2.5x10^7$ cfu/ml (Fig. 3). Yeast cells were counted in the Neubar chamber. Each buddng cell was counted as one. The cell count remained in the range of 3.4-4.4 x 10^7 till the 7^{th} hour of fermentation. Thereafter, the proliferation of the yeast population was at a much faster rate reaching its peak at the 11^{th} hour to a cell count of 8.75 x 10^7/ml. The cell count decreased 11^{th} hour onwards reaching 5.8 x 10^7/ml. Budding yeast cells were predominant in the three sap samples collected from 5-9^{th} hour of incubation. Invertase content which had increased at slow rate till 5h of incubation started increasing gradually till 9^{th} and thereafter a sharp increase in the activity indicated that yeast population in the sap have the ability to express invertase in large amounts while LAB population were not rich sources of invertase. Increase in glucose concentration especially between 7^{th} and 11^{th} hour of fermentation was in tandem with the total amount of reducing sugars. However, non-glucose reducing sugars did not increase in proportion with glucose indicating its utilization by the microbial flora. Fig. 3 shows that there was a sharp increase in yeast population during this period. It appears that the yeast population utilized the non-glucose reducing sugar (mainly fructose) for its proliferation. Although yeast cell count decreased after 13h of fermentation, it was interesting to find that invertase activity increased to 18U/ml at the end of 16h of incubation. Although the increase in invertase activity was observed, the non-reducing sugar (sucrose) concentration had decreased to around 2.1%. It is known that diverse bacterial and yeast populations are involved in natural fermentation of palm saps (Atputharajah *et al.*, 1986; Stringini *et al.*, 2009; Ouoba *et al.*, 2012). Alcohol tolerant yeasts are likely to be present in palm wine and therefore, possibility of a diverse population of alcohol tolerant yeast population expressing high amount of invertase taking over can't be ruled out. Pectinase activity was comparatively much lower than the invertase activity. Pectinase is a complex of pectin esterases (PE) and pectin depolymerases. The three most widely occurring enzymes are polygalacturonase (PG), polygalacturonate lyase (PGL) and PE. These enzymes catalyze breakdown of high molecular weight pectin to its smaller units (kawano, 1999). The enzymes which act on high molecular substrates are often secreted into the extracellular environment and, the enzymes acting on resulting products of low molecular weight may be intracellular or extracellular. Although the cellular fraction exhibited low activity in the cellular fractions, it is possible that it represents a much lower value than in its natural environment.

Alcohol content in the 9h, 11h and 13h samples was estimated to be 3.5, 4.0 and 4.1% respectively. Thus there was not much increase in alcohol content between 11-13h of incubation. The possibility of initiation of conversion of alcohol to acetic acid during this period by acetic acid bacteria cannot be ruled out. While counting yeast cells in Neubar chamber it was observed that apart from yeast cells, rest of the microbial population comprised predominantly of short rods. Majority of small rods were sluggishly motile. However, in the 16h sample actively motile short rods were observed. The sample was allowed to ferment for 24h and in this sample a significant population of short bacillary

species were observed which were actively motile. While most of the LAB species are known to be immotile/sluggishly motile, the acetic acid bacteria are reportedly actively motile. Thus it appears that 16h onwards, the acetic acid bacteria may have started proliferating, setting the stage for conversion of alcohol to acetic acid.

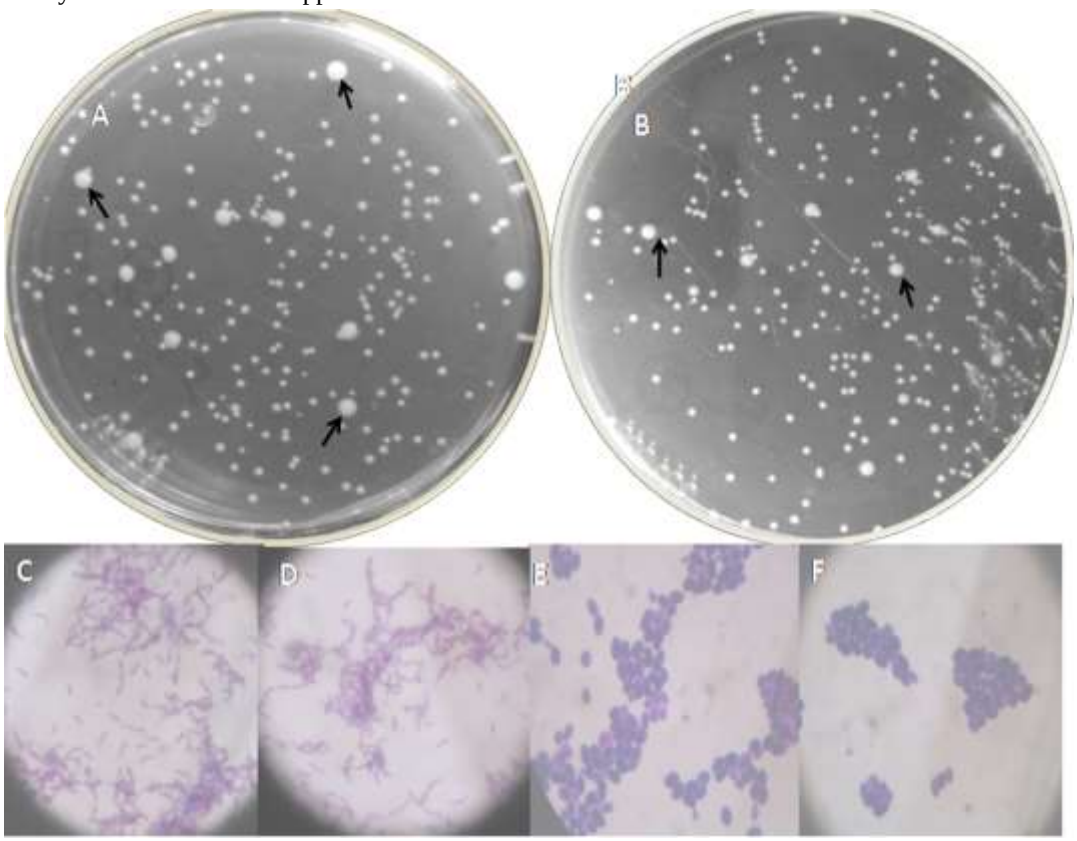

Fig. 2: Microbial study in the coconut palm sap Plates A & B:: Viable count of lactic acid bacteria on MRS agar- Large colonies representing yeast cells (few areindicated with arrow) and small colonies represent lactic acid bacteria; Staining of, Plate C & D: small colonies; E & F: large colonies

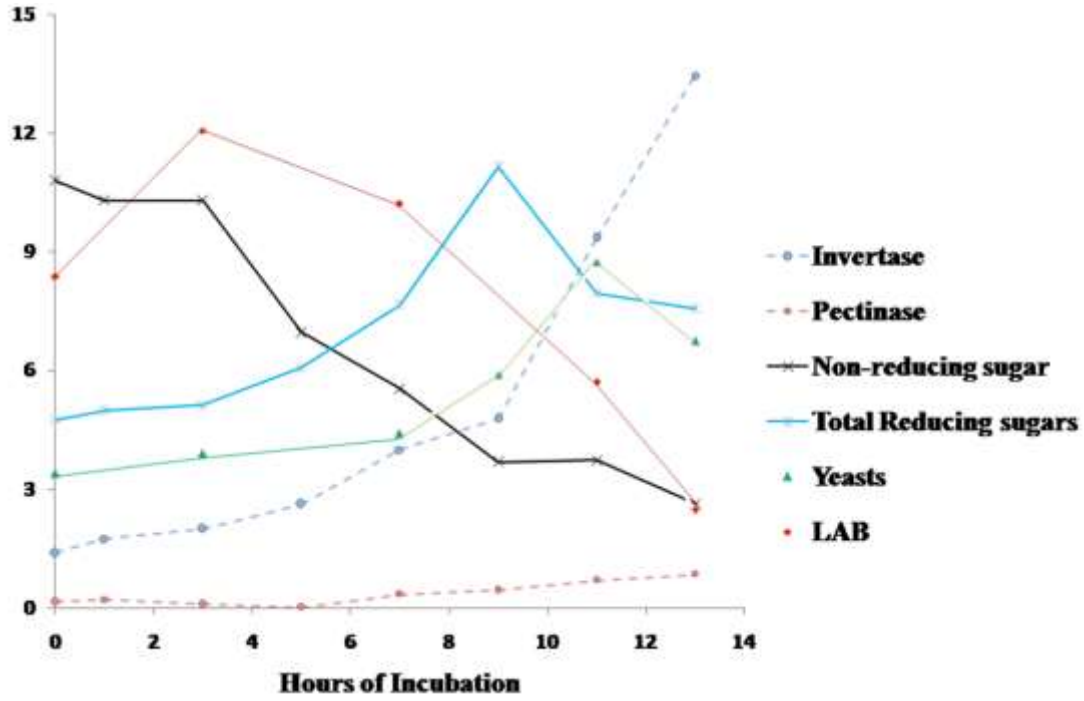

Fig. 3: Fermentation kinetics in relation to yeast and lactic acid bacterial count Sugar concentration expressed as g%; Invertase and pectinase activity in U/ml; Yeast and LAB count - x10^7 cfu/ml

Conclusion

Lactic acid bacteria were the predominant flora in coconut palm sap until 7h of incubation. LAB count decreased thereafter with simultaneous increase in yeast population. Increase in yeast population was accompanied by increasing concentration of invertase and concomitant increase in the reducing sugars. Alcohol content at 13h of fermentation was found to be around 4.1%.

References

Akpa and Gunorubon J (2012) Modeling of a bioreactor for the fermentation of palm wine by *Saccaharomyce cerevisiae* (yeast) and lactobacillus (bacteria). *American Journal of Scientific and Industrial Research* **3**(4): 231-240. DOI: 10.5251/ajsir.2012.3.4

Atputharajah JD, Widanapathirana S, and Samarajeewa U (1986) Microbiology and biochemistry of natural fermentation of coconut palm sap. *Food Microbiology* **3**(4): 273-280. DOI: 10.1016/0740-0020(86)90009-2

Chandrasekhar S, Sreevani P, Seshapani J and Pramodhakumari (2012) A Review on palm wine. *International Journal of Research in Biological Sciences* **2**(1): 33-38.

Chooklin, S. Kaewsichan L and Kaewsrichan, J (2011) Potential use of *Lactobacillus casei* TISTR 1500 for the bioconversion from palmyra sap and oil palm sap to lactic acid. *Electronic Journal of Biotechnology* **14**(5). DOI: 10.2225/vol14-issue5-fulltext-11

Eze, MO and Uzoechi O (1988) Sugars of the unfermented sap and the wine from the oil palm, *Elaeis guineensis*, tree. *Plant Foods for Human Nutrition* **38**(2): 121-126. DOI: 10.1007/BF01091716

Falegan CR and Akoja SO (2014) Microbiological and physicochemical studies of two Nigerian fermented alcoholic drinks (palmwine and burukutu) in ekiti state, Nigeria. *European Journal of Food Science and Technology* **2**(2): 13-22.

François T, Aly S, Cheikna, Z, Alfred TS (2016) Improvement of *Bborassus akeassii* wine quality by controlled fermentation using *Saccharomyces cerevisiae* strains. *The Journal of Microbiology, Biotechnology and Food Science*. **5/6**: 589-592. DOI: 10.15414/jmbfs.2016.5.6.589-592

Ghosh S, Chakraborty R, Chatterjee G and Raychaudhuri U (2012) Study on fermentation conditions of palm juice vinegar by response surface methodology and development of a kinetic model. *Brazilian Journal of Chemical Engineering* **29**(3): 461–472. DOI: 10.1590/S0104-66322012000300003

Hebbar M, Arivalagan MR, Manikantan AC, Thamban C. Thomas, George V and Chowdappa, P (2015) Coconut inflorescence sap and its value addition as sugar – collection techniques, yield, properties and market perspective. *Current Science* **109**(8): 1411-1417. DOI: 10.18520/v109/i8

Joseph P, Gabriel O, Richard A and Godwin A (2014) Bioethanol potential from oil palm sap in Ghana. *International Journal of Renewable Energy Research* **4**(1): 54-60.

Kalaiyarasi K, Sangeetha K and Rajarajan SA(2013) Comparative study on the microbial flora of the fresh sap from cut inflorescence and fermented sap (toddy) of *Borrassus flabellifer* Linn (Palmyrah tree) and of *Cocos nucifera* Linn (Coconut tree) to identify the microbial fermenters. *International Journal of Research in Pure and Applied Microbiology* **3**(3): 43-47

Kapilan R, Kailayalingam R, Mahilrajan S, Srivijeindran S (2015) Efficient fermentation inhibitor of sweet phloem sap of Palmyrah (Borassus flabellifer L.) in Sri Lanka. *Int J Adv Res Biol Sci* **2**(10): 89–95

Naknean P, Meenune M and Roudaut G (2010) Characterization of palm sap harvested in Songkhla province, Southern Thailand. *International Food Research Journal* **17**: 977-986.

Ngọc NTM, Minh NP, Dao DTA (2013) Isolation and Characterization of Yeast Strains for Palm (*Borassus Flabelliffer*) wine. fermentation. *International Journal of Engineering Research Technology* **2**(11): 3602-3615.

Nwaiwu O, Ibekwe VI, Amadi ES, Udebuani AC, Nwanebu FC, Oguoma OI and Nnokwe JC (2016) Evaluation of fermentation products of palm wine yeasts and role of *Sacoglottis gabonensis* supplement on product abundance. *Beverages* **2**(9). DOI: 10.3390/beverages2020009

Ouoba L, Kando C, Parkouda C, Sawadogo-Lingani H, Diawara B and Sutherland JP (2012) 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* **113**(6): 1428-144. DOI: 10.1111/jam.12014

Rokosu AA and Nwisienyi JJ (1980) Variations in the components of Palm-wine during fermentation. Enzyme and Microbial Technology **2**(1): 63-65. DOI: 10.1016/0141-0229(80)90011-3

Santiago-Urbina JA, and Ruíz-Terán F (2014) Microbiology and biochemistry of traditional palm wine produced around the world. *International Food Research Journal* **21**(4):1261-1269

Santiago-Urbina JA, Verdugo-Valdez AG, and Ruíz-Terán, F (2013) 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* **33**: 58-62. DOI: 10.1016/j.foodcont.2013.02.010

Shamala TR and Sreekantiah KR (1988) Microbiological and biochemical studies on traditional Indian palm wine fermentation. *Food Microbiology* **5**(3): 157-162. DOI: 10.1016/0740-0020(88)90014-7

Singaravadivel K, Alagusundaram K and Hariharan B (2012) Physicochemical properties of fresh and stored coconut palm toddy. *Open Access Scientific Reports* **1**: 8.

Stringini M, Comitini F, Taccari M and Ciani M. (2009) Yeast diversity during tapping and fermentation of palm wine from Cameroon. *Food Microbiology* **26**(4): 415-420. DOI: 10.1016/j.fm.2009.02.006

Sumbhate S, Nayak S, Goupale D, Tiwari A and Jadon RS (2012) Colorimetric Method for the Estimation of Ethanol in Alcoholic-Drinks. *Journal of Analytical Techniques*. 1(1):1-6

Theivendirarajah, K and Chrystopher, RK (1987) Microflora and microbial activity in palmirah (*Borassus flabellifer*) palm wine in Sri Lanka. *MIRCEN Journal* **3**(1): 23-31. DOI: 10.1007/BF01090492