Vidyodaya J. of Sci, 8 (1999) Vol 91- 108

Biochemical and microbiological changes of

Caryota urens (Kithul palm) phloem sap.

A.N. Jayathilake and S.C. Wijeyaratne
Department of Botany,
University of Sri Jayewardenepura, Nugegoda.

Receivd on 99.02.18
Accepted on 99.03,08

Abstract

Biochemical and microbiological changes that occur during fermentation of *Caryota urens* phloem sap were studied with a view to conducting basic research for later upgrading the local fermentation industry and to developing new biotechnological processes by recognizing yeasts suitable to local conditions. Sap, which was taken after keeping a sterilized pot for 10 hours was free of alcohol, showing that fermentation has not taken place yet. Paper chromatographic studies done with this unfermented sap revealed that the main type of sugar was sucrose while glucose and fructose were in trace amounts. The content of reducing sugar of phloem sap at the time of collection was very low (0.14%). However this started to increase after 24 hours and reached a maximum value of (4.1%w/v) after 24 hours. The alcohol concentration, measured by ebulliometer, increased only after appreciable quantities of reducing sugars was formed. Maximum alcohol concentration of 7% v/v was obtained on the fifth day. The unfermented sap had a pH around 7. The pH value decreased substantially and reached a constant value around 4 after the fourth day of fermentation. Decrease in pH coincided well with increase of acidity. Microbial analysis of fermenting sap revealed that the type of orgnisms present in the sap changed with time. At the beginning of fermentation the sap contained mainly bacteria with a colony count in the order of 10^7 cells/ ml. They were found to be mainly *Lactobacilli* and *Leuconostoc.*

The yeast cells were very few in number initially but increased after 24 hours and reached a maximum of 8×10^7 cell/ml after the third day. During the latter stages of fermentation, there was a drop in the viable yeast cell count, while the bacterial count increased again to 10^6 cells/ml. A total of eleven yeast strains were isolated from fermenting phloem sap. *Saccharomyces cerevisiae* was found to be the most predominant yeast species. In the early stages *Candida tropicalis* and *Pichia membranifaciens* were found to be abundant.

Key words : *Caryota urens,* palm phloem sap, Kithul palm sap.

1. Introduction

Caryota urens (Kitul palm) grows wild in tropical rain forests of Sri Lanka and is considered to be indigenous to India, Sri Lanka and Malaya. It is also grown in some home gardens due its commercial importance and multipurpose nature. This palm yields a plentiful supply of sweet toddy from the immature spadices. The phloem sap thus obtained by tapping the inflorescence of this palm is allowed to ferment naturally to produce toddy or wine. The unfermented sap or sweet toddy which contains mainly sucrose (about 12-14%) is used in the preparation of jaggery and treacle. The naturally fermented phloem sap which, results from an uncontrolled spontaneous process is considered as a nutritive alcoholic beverage. Fermentation that takes place in the collecting pot is due to the growth and activity of wild yeast coming from various sources. As the same earthenwater pot is used for several sucessive days for sap collection, its inner surface gets coated with a layer of yeast and bacteria. These microorganisms bring about certain biochemical changes in the sap, mainly fermentation of sugars to produce ethyl alcohol.

Although much work has been done on the biochemical and microbiological aspects of phloem saps of coconut *(Cocos nucifera)* [1,2,3] and *Borassus fabellifer*[4], no previous work has been reported on *Caryota urens* phloem sap. This paper describes some of the microbiological and biochemical chang that take place in the phloem sap of *Caryota urens* during natural fermentation.

2. Materials and methods

Collection of sap:

Caryota urens sap, tapped in the traditional way was collected for 12 hr. in a flame sterilised clay pot and brought to the laboratory in sterilised flasks. The time at which the pot was removed from tree was taken as the zero time.

Isolation of yeast:

Samples from the fermenting sap were taken out at regular intervals of time. The first sample was taken immediately after the pot was removed from the tree. Serial dilutions were prepared and plated on different culture media. To isolate yeast, aliquots (0.1 ml) of dilutions were plated in

duplicates using several media, such as malt extract, yeast extract, glucose, peptone (MYGP) medium, potato dextrose agar (PDA), yeast extract, peptone, dextrose (YPD), synthetic medium[5] and sweet toddy agar. On the first day of fermentation 10^3 and 10^4 dilutions were used to isolate yeast, while 10^5 and 10^6 dilutions were used on the second day of fermentation. Streptomycin was used in all media at a concentration of 50 µg/ml, to suppress the bacterial growth. The plates were then incubated at 30 °C for 3-4 days. Yeast colonies that appeared on different media were transferred on to new plates containing the same medium on which the colonies originally appeared. Separate single colonies that appeared on the plates were then transferred on to YPD slants and stored at 4 °C.

Isolation of bacteria:

Serial dilutions prepared for yeast cell isolation were also used for bacteria isolation. Samples (0.1 ml) were taken from 10^5 and 10^6 dilutions and plated in duplicate on petri dishes containing nutrient agar medium on the first day of fermentation, while 10^2 and 10^3 dilutions were used on the second day of fermentation. The plates were incubated at 30° C for 3-4 days. Bacterial colonies that appeared on these plates were later transferred to plates containing fresh nutrient agar medium to get single cell colonies. These were transferred to nutrient agar slants and stored at 40 °C.

Measurement of yeast cell number during fermentation of sap:

Direct microscopic count:

Samples were withdrawn from fermenting sap at different time intervals and prepared separate dilution series for each sample. Using these dilutions, total cell number in each saple was counted using an improved bright lined Neubaue-Haemocytometer. (Area: 0,100mm, Depth: 0.0025mm^2).

Plate count of yeast and bacteria:

Culture media used for isolation of yeast and bacteria were also used for plate count. Yeast colonies that appeared on maltose, yeast extract, glucose, peptone (MYGP), yeast extract, peptone, dextrose, (YPD), potato, dextrose agar (PDA), synthetic and sweet toddy media were counted to enumerate viable yeast cell number. Bacterial colonies that appeared on nutrient agar plates were counted to enumerate the viable number of bacteria in different samples.

Identification of microorganisms:

Indentification of yeast:

Methods described in "The Yeast" by Lodder[6] and "The Yeast, a Taxonomic Study." by Kreger-van Rij[7] were followed for taxonomically identify yeast strains isolated.

Identification of bacteria:

Identification of bacteria was done using Bergey's Manual of Determinative Bacteriology[8].

Determination of percentage of alcohol v/v:

Samples (50 ml), which were taken at different intervals of time from the flasks containing fermenting phloem sap, were checked for alcohol content using a Durardin-Salleron ebulliometer.

Measurement of total acidity:

The total acidity of samples of sap were taken at intervals by titiration against 0.1 N sodium hydroxide. Phenolpthalein was used as the indicator. Acidity was calculated as mg of acetic acid/100 ml of sample.

Measurement of pH:

Using a Jenway 3020 pH meter with hydrogen-calomel electrodes, *pH* values of samples were recorded

Determination of sugar content:

For the determination of sugars in the phloem sap followed Lane and Eynon method as described in Official Methods of Analysis[9].

Total sugar content:

Sample of sap that was hydrolysed with HCI was neutralised with approximately 1 M NaOH and diluted to a desired concentration. This solution was titrated with the 10-ml standard Soxhlet solution.

Reducing sugar content:

10-ml standard Soxhlet solution was titrated with samples of unhydrolysed sap that were diluted to desired concentration.

Identification of sugars:

Samples of sap (25 ml) were neutralised with approximately 1 M sodium hydroxide and filtered through a sintered glass funnel, then concentrated on a water bath at 70 °C for the assay of sugars by chromatography. A mixture of Analar grade authentic sugars (sucrose, fructose and glucose) were dissolved in distilled water and spotted along with the concentrated phloem sap samples on Whatman No.01 chromatographic paper. Descending paper chromatography was performed with the solvent system, isopropanol water (90:10 by volume)after equilibrating the loaded paper for 10 - 12 hr[10].

Detection of spots:

The air dried chromatograms were sprayed with a mixture of aniline 1% and diphenyl amine in acetone (1%) and 85% phosphoric acid containing a few drops of water mixed in the ratio of 10 : 1 respectively. After spraying, the chromatograms were heated at 100-105 °C for about 5 minutes [10]. Sugars were identified by comparing their Rf and Rg values with the R_f and R_g values of authentic sugars which were allowed to run along with samples.

3. Results

Microbial changes:

Yeast cell count:

During the first six hours (beginning of the first day) of fementation the yeast counts were less than 105 cells/ml. Thereafter, it increased gradually. After 24 hours of fermentation, the yeast cell number was as high as 10^7 cells/ml with vigorous evolution of carbon dioxide. After 96 hours of fermentation, plate count results showed that the viable yeast cell number in the fermenting sap started to decrease gradually. (Fig. 1 & Table 1)

Bacteria cell number:

During the first eight hours of fermentation bacterial cell count in the fermenting sap was found to be in the order of 10^7 cells/ml. Thereafter it decreased gradually and on the second day (48 hours) of fermentation, it was about 10^3 cells/ml. However, after fourth day (96 hours) of fermentation bacterial number started to increase again and on the sixth day (144 hours) of fermentation it was about 10^6 cells/ml. (Fig. 1, Table 1)

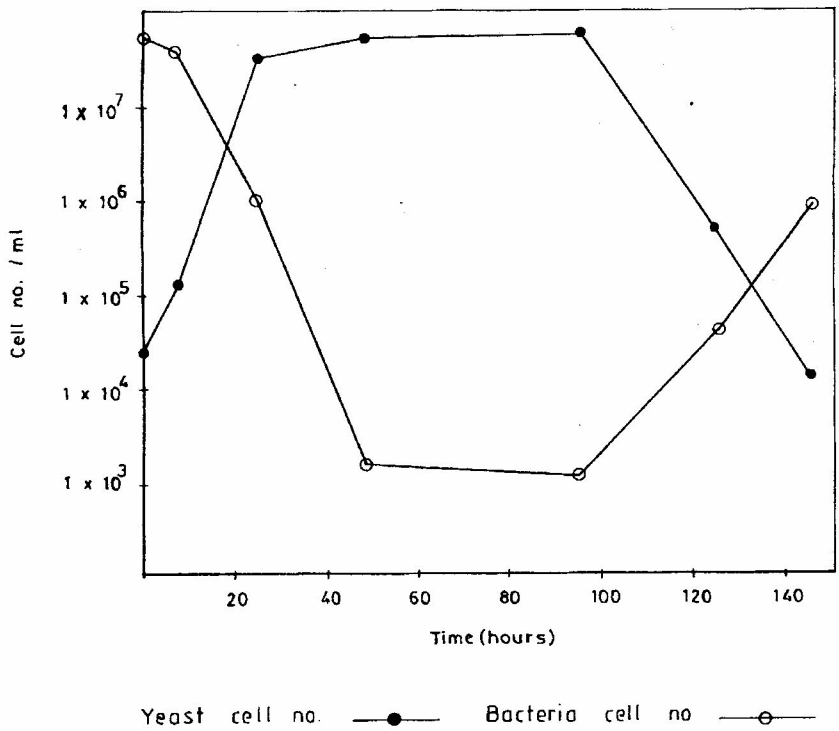

Fig - 1 Change in yeast and bacteria cell number with time in fermeting *Caryota urens* phloem sap

Table I Change in the number of yeast and bacterial cells with time in fermenting *Caryota urens* phloem sap.

Time (hours)	Yeast Cell No.	Bacterial Cell No.
0	4.0×10^4	7.6×10^7
8	4.5×10^4	6.5×10^7
24	5.0×10^5	1.1×10^6
48	6.0×10^7	1.6×10^3
96	9.0×10^7	1.1×10^6
144	5.0×10^4	1.1×10^6
152	2.0×10^4	1.3×10^6

Identification of yeast and bacteria:

At different stages of fermentation, eleven yeast strains with different colony characters were isolated from twelve separate fermenting sap samples (Table II). The majority of yeast strains isolated during first 24 hours of fermentation, had oval to long oval cells (size ranging from (4-6) x (5-7) μm). The vegetative cells reproduced by multilateral budding. Filamentous growth was observed on potato-glucose-agar but sporulation was not observed. Based on the morhological and physiological characters (Table 2 & 3) the yeast strains isolated during first 24 hours of fermentation were identified as *Candida tropicalis and Pichia membranifaciens.*

Most of the yeast strains isolated after 24 hours of fermentation had round to oval cells (Size (3-6) x (4-6.5) μm. The vegetative cells reproduced by multilateral budding. Pseudomycelium was formed on potato-glucose-agar. Sporulation was observed on acetate agar and 2-4 ascospores were produced per ascus. Based on the morphological and physiological characters strains isolated after 24 hours of fermentation were identified as different strains of *Saccharomyces cerevisiae* (Table II & III).

Table II. Morphological characters of some yeast strains isolated from fermenting sap of *Caryota urens*

Morphology on YM medium

Broth	***C.tropicalis***	***P. membranefaciens***	***S.cerevisiae***
Cells after 48 h.	Oval-long oval (3-5)x(4x10) μm	Oval-long to Oval (3-4)x(6-8) μm	Oval-long oval (4-8)x(6-9) μm
Culture after 21 d	Cream, Non-flocculent deposit	Cream Non-flocculent deposit, pellicle present	Cream Non-flocculent deposit
Agar			
Cells after 48 h	Round-long oval (2-8)x(2-9) μm	Short oval-long oval (2-7)x(3-11) μm	Round to long oval (3-8)x(5-10) μm
Culture after 21 d	White, Shiny & smooth	Cream white, smooth shiny	Cream/white smooth slightly shiny

Pseudo/true mycelium

CMA			
Aerobic	True mycelium	No mycelium	Poorly developed mycelium with irregular branches
Anaerobic	Ture mycelium	Pseudomycelium	Pseudomycelium with blastospore
PDA			
Aerobic	*Candida* type Pseudmycelium	No mycelium Pseudomycelium	Poorly developed Pseudomycelium

Anaerobic	Pseudomycelium	No mycelium	Poorly developed Pseudomycelium
Vegetative spores			
Balistospores	Negative	Negative	Negative
Arthrospores	Negative	Negative	Negative
Endospores	Negative	Negative	Negative
Chlamydospores	Negative	Negative	Negative
Sexual spores			
CMA	Negative	1-4 ascospores / ascus	Negative 2-4 ascospores/
KAC & Gorodkowa	Negative	Hart-shaped,dehiscent	ascus, round and smooth

YM - Malt Extract, Yeast extract; **PDA** - Potato Dextrose agar; **CMA** - Corn meal agar; **KAC** - Potassium acetate agar.

Table III. Physiological characters of *Candida tropicalis*, *Pichia membranefaciens*, and *S. cerevisiae* isolated at different stages from fermenting sap of *Caryota urens*.

	C. tropicalis		***P. membranefaciens***		***S. cerevisia***	
	Ferment.	**Assimi.**	**Ferment.**	**Assimi.**	**Ferment.**	**Assimi**
D-Glucose	+	+	-	+	+	+
D-Galactose	+	+	-	-	+	+
Sucrose	+	+	-	-	+	+
Maltose	+	+	-	-	-	-
Cellobiose	-	+	-	-	-	-
Trehalose	+	+	-	-	-	+(s)
Lactose	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-
Raffinose	+	+	-		-	-
Melzitose	+	+	-	-	-	+
Methyl-D Glucopyranoside	-	+	-	-	+	+
Inulin	-	-	-	-	-	-
Sol. Starch	+	+	-	-	-	-

Contd...

	C. tropicalis Assimilation	*P. membranefaciens* Assimilation	*S. cerevisea* Assimilatin.
Xylose	+	-	-
L-Arabinose	-	-	-
D-Arabinose	-	-	-
D-Ribos	-	-	-
L-Rhamnose	-	-	
Ethanol	+	+	+
Glycerol	-	+	+
Sorbose	+	-	-
Erythritol	-	-	+
Ribitol	+	-	+
Galctitol	-	-	-
D-Mannitol	+	-	+
Glucitol	+	-	-
Salicilin	-	-	-
Lactic cid	+	+	+
Succinic acid	+	+	+
Citric acid	+	-	-
myo-inositol	-	-	-
D-Glucono 1,5 lactone	+	-	-
D-Glucosamine	-	-	-
Methanol	-	-	-

Contd..

Xylitol	+	-	-
Ammonium sulphate	+	+	+
Potassium nitrate	-	-	-
Ethyl amine	+	+	+
Cadaverine	+	+	-
L-Lysine	+	-	-
Cycloheximide (0.01% and 0.1%)	+	-	-
Glucose growth(50%)	+	+(w)	+
Glucose growth (60%)	+	-	+
1% Acetic acid	-	+	-
Acid Production	-	-	-
Growth at 40 °c	+		+
Arbutin hydrolysis	-	-	-
Urease activity	-	-	-
Salt tolerant 10%	+	-	+

s = slow w =weak

The predominant bacteria at the initial stages of fermentation were species of *Lactobacilli* and *Leuconostoc.* During later stages of fermentation species of *Staphylococcus, Bacilli* and *Acetobacter* were found to be dominant.

Biochemical changes:

At the time of collection, alcohol content of sap was zero. Alcohol was detected in the sap only after 48 hours of fermentation. Thereafter, the amount of alcohol in the fermenting sap increased gradually and a maximum alcohol concentration of 7.4 %, v/v was observed on the fifth day. Then, it started to decrease gradually and reached a value of 5.8%, v/v after the sixth day of fermentation (Fig. 2).

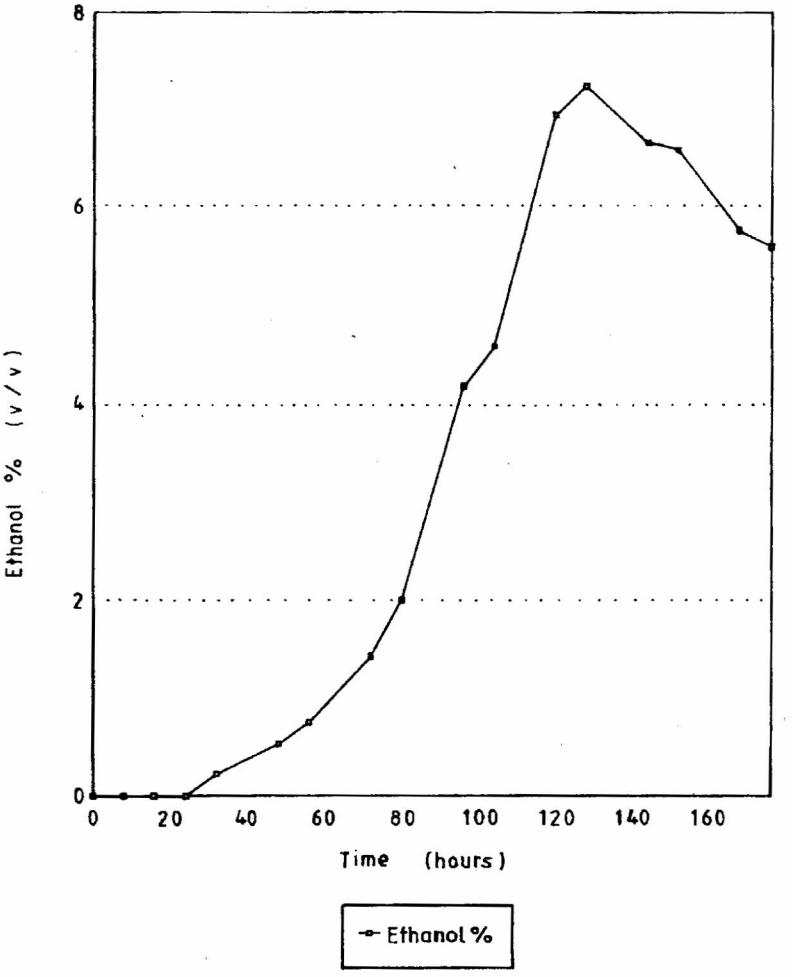

Fig - 2 Change in alcohol concentration with time in fermenting *Caryota urens* sap

Change in alcohol concentration:

The acid content (acetic acid) of sap was about 0.02 mg/100 ml at the initial stages of fermentation. This value then increased gradually and reached a value of 0.8 mg of acetic acid / 100 ml of sap, at 72 hours of fermentation. After the third day it increased only slightly and reached up to 0.9 mg of acetic acid/100ml of sap on the sixth day of fermentation. (Fig. 3)

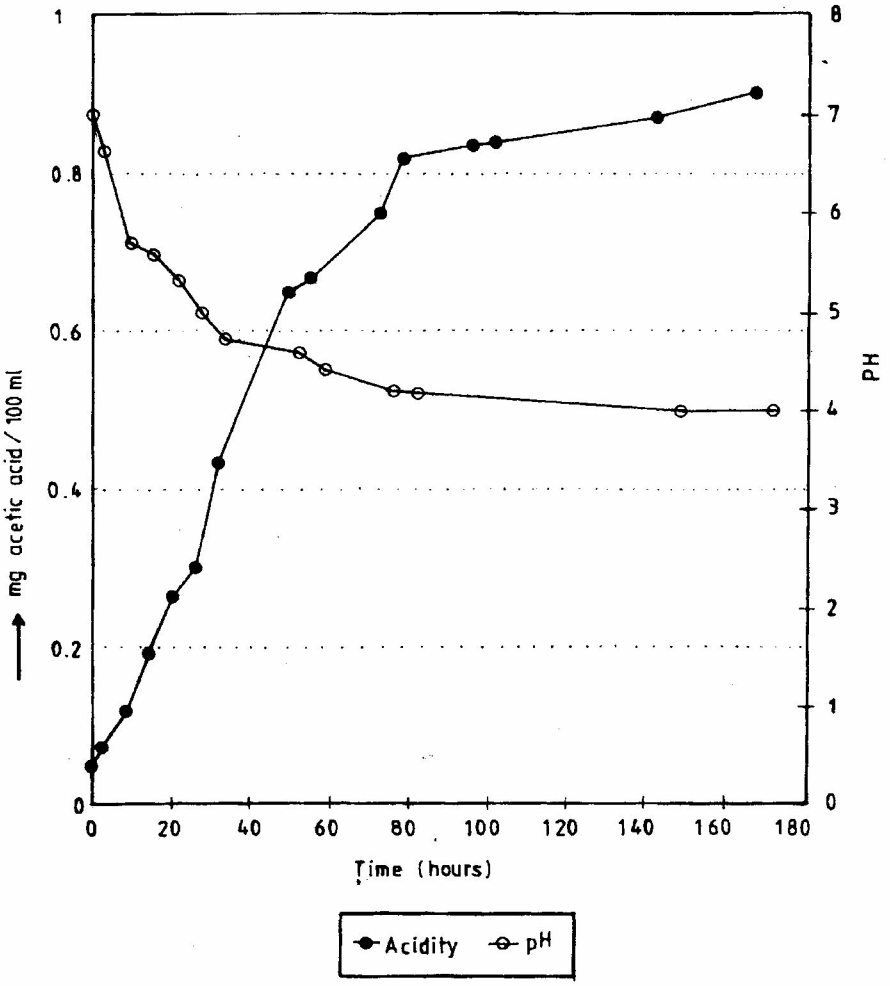

Fig - 3 Changes in alidity and pH with time of fermenting *Caryota urens* sap

Change in acidity of sap:

Initial pH of the unfermented sap was around 7.00. This value gradually decreased and reached a constant value of 4, after the third day of fermentation. (Fig. 3)

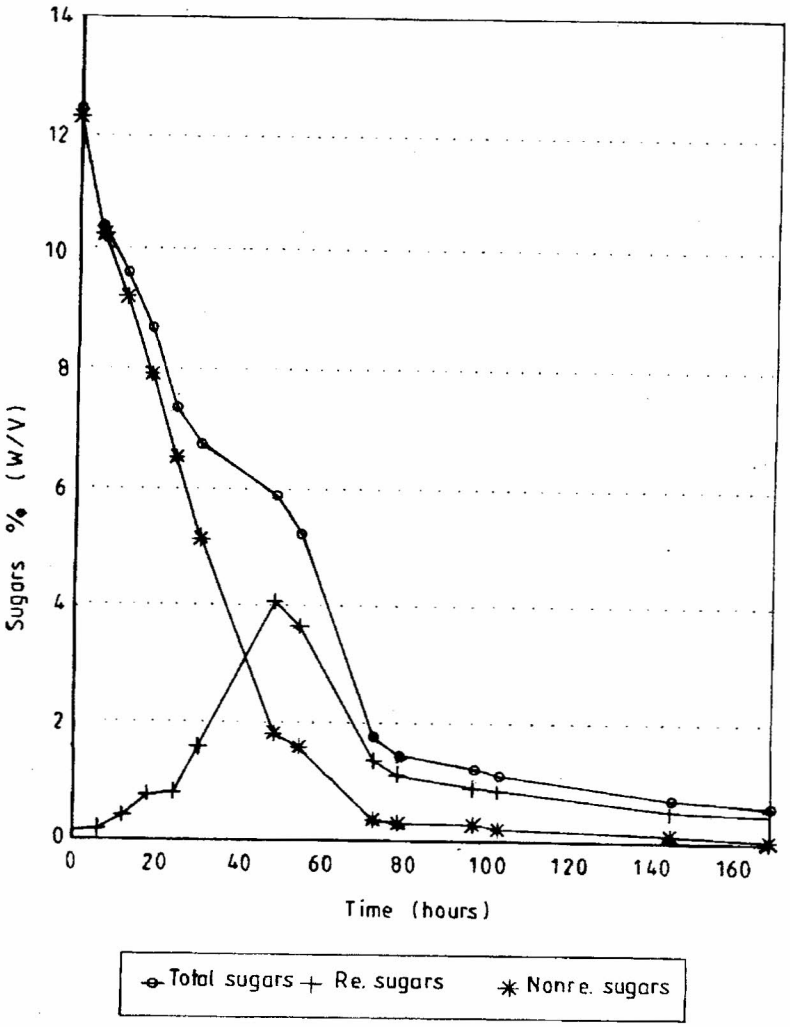

Fig - 4 Change in tolal reducing and non-erducing sugars with time of fermenting *Caryota urens* sap

Change in pH:

The total sugar content of the sap at the time of collection was around 12% while the reducing sugar content was negligible. The non-reducing sugar content of the sap decreased gradually from 12% to 2% while there was an increase of reducing sugar content during this period. Reducing sugar reached its maximum of 4.2% (w/v), after second day (48 hours) of fermentation. At final stages of fermentation the amount of reducing sugar in the sap was more than the amount of non-reducing sugar (Fig.4).

Changes in sugar composition:

Sucrose was the major sugar present in the unfermented phloem sap of *Caryota urens*. After 48 hours of fermentation, in addition to sucrose, glucose and fructose were also present in the fermenting sap. After six days of fermentation, fructose was the major sugar present in the fermenting sap (Fig. 5 and 6).

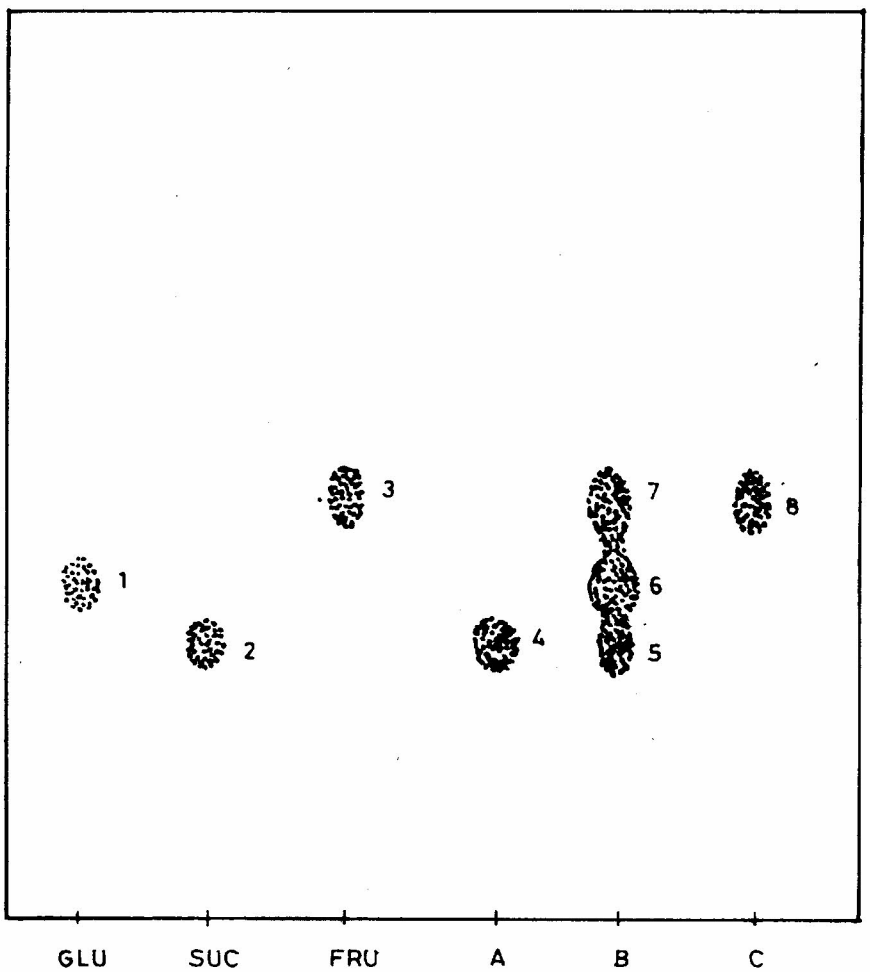

Fig. - 5 Paper chromatogram shoving major sugars present in fermenting Caryota urens sap taken at aifferent stages of fermentation A-unfermented sap, B-atter 48 hrs of fermentation, C-after six daus of fermentation Glu-glucose suc-sucrose, FRU, fructose

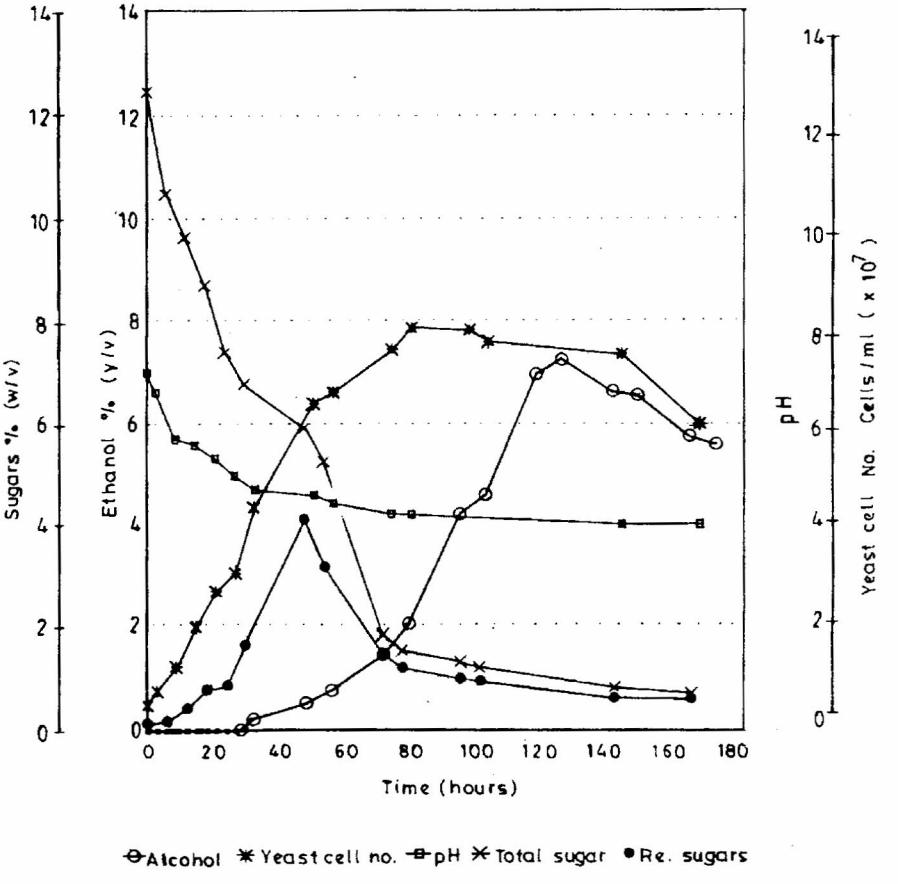

Fig - 6 Changes take place during the time course of fermentation of *Caryota urens* sap.

Overall changes observed in fermenting sap:

Changes taking place during the time course of fermentation of *Caryota urens* phloem sap are shown in Fig.6. It is evident that change in pH level, which is brought about by lactic acid bacteria, favours the conversion of non-reducing to reducing sugar by yeast cells. Further, the alcohol concentration increased only when appreciable amount of glucose was found in the sap (Fig 6). Alcohol production decreased although there was sugar in the medium indicating that yeast strains that bring about alcohol fermentatiion are affected by alcohol.

4. Discussion:

The phloem sap of *Caryota urens* contains much nutrients and hence it is an ideal medium for the growth of microorgansms. Its neutral pH favours the growth and multiplication of bacteria. Initially the fermenting phloem sap had a bacteria count around 10^7 cellsml, while the yeast cell count was less than 10^5 cells/ml. After the first 24 hours of fermentation, the yeast cell count increased up to 10^7 cells/ml while the bacteria count decreased to about 10^3 cells/ml. Thus, there is a shift in dominance between the bacterial and yeast populations as the fermentation progressed. Similar changes had been observed in the microflora of palmyrah sap[4], cocount sap[3] and in other palm wines [11] during natural fermentation.

Similar to other phloem saps, the unfermented phloem sap of *Caryota urens* also contained predominantly non-reducing sugar. After some time (10 hrs) the reducing sugar content of this sap started to increase while the non-reducing sugar content decreased concomitantly (Fig. 4.) Thus, it is clear that the initial step of the alcoholic fermentation process of this palm sap is the hydrolysis of sucrose to reducing sugars. This hydrolysis is brought about by the invertase enzyme produced by yeast cells, which too has increased during this period. (Fig 6). The reducing sugar content gradually increased and reached its maximum around 48 hours and this coincided with the initiation of alcohol production. Further, it is clear that ethanol production begins only after the formation of substantial quantities of reducing sugars. A maximum alcohol content around 7.4% v/v was recorded only after the third day. Similar changes had been reported in palmyrah and coconut phloem sap fermentations[3].

The decrease in pH value, which also occurs in all palm wines [3, 11] allows the growth of yeast but the growth of bacteria is suppressed at such lower pH levels. The initial pH dropped in the fermenting sap is due to the activity of lactic acid bacteria. This lowering of pH favours the activity of invertase enzyme produced by yeast cells, which in turn increases the multiplication of yeast. The invertase enzyme converts sucrose to reducing sugar, which is the best substrate for yeast cells. The activity of yeast cells results in the production of alcohol (Fig. 5). The results also revealed that alcohol production started only after cell density reached its maximum. As the fermentation phase follows the growth phase of yeast, it may be suggested that a higher inoculum density should be used in alcohol fermentation in order to reduce the sugar utilisation by yeast for their growth and multiplication. Under such circumstances total amount of sugar in the medium will be available for production of alcohol and will make the process more efficient. It took almost five days to obtain maximum ethanol concentration.

Results of chromatographic analysis revealed, that unfermented *Caryota urens* sap contained only traces of glucose and fructose in addition to the major sugar sucrose, while fermenting sap contained more reducing sugars, glucose and fructose. After sixth day of fermentation, fructose was the only remaining sugar in the sap. These results are similar to changes observed in coconut palm sap(3). The remaining sugar was fermentable fructose, but a decrease in rate of alcohol production observed may be due to the low alcohol tolerance of yeast cells involved in fermentation (Fig.6). Thus, it is revealed that alcohol tolerant yeast strains should be used to bring about maximum utilization of sugar, which in turn would give higher alcohol yields.

Increase in acidity at the initial stages of fermentation is due to the activity of lactic acid bacteria. The acetic acid bacteria bring about the conversion of alcohol to acid and increased the pH at the final stages. This process allows manufacture of vinegar from all kinds of palm wines. At present only coconut toddy has been utilized in the production of vinegar. If the technology were provided, production of palmyrah vinegar would be a good industrial venture in areas where tree is found in abundance.

5. Acknwledgement

The authors are grateful to the University of Sri Jayewardenepeura for granting financial assistance for this research. The assistance of the National Collection of Yeast Cultures, Institute of Food Research, Norwich Laboratory, in the identification of yeast cultures is gratefully acknowledged.

6. References:

1. Liyanage, A.W. and Shanmuganathanm, S.(1970) Comparative studies of yeasts isolated from fermented saps of palms of Ceylon, Proc. Cey. Assoc. Advance. Sci. **26**,97

2. Jayatissa, P.M., Pathirana, R.A. Sivayogasundaram, K. and Jeyaraj, E. E.(1978), Yeasts of the coconut palm wine of Sri Lanka. J.Sci. Food Agri. **29**,975-978.

3. Athputhrajah, J.D., Widanapathirana, S. and Samarajeewa, U.(1986) Microbiology and Biochemistry of natural fermentaion of coconut palm sap. Food Microbiol. 3, 273-280

4. Chrystoper, R. K. , Thevendrarajah. K. (1988), The Microbial and Biochemical changes of Palmyrah palm wine. J. Nat.. Sci. Coun., Sri Lanka 16, 131-141

5. Hayashida, S.,Feng, D. D. and Hongo, M. (1974). Function of the high alcohol producing factor. *Agricultural Biological Chemistry 38: 2001 - 2006*

6. Lodder , J. A.(1984)*"The Yeast" A taxonomic study* 3rd. ed. pp.1385, North Holland Publishing Company. Amsterdam.

7. Kreger-van Rij, N. W. J. (1984) Ed., "The Yeasts, a Taxonomic Study" 3rd . revised edition, Elsevier, Amsterdam.

8. Bergey's Manual of Determinative Bacteriology, 8th. Ed., Editors, R.E Buchanan and N.E. Gibbons, Pub., The Williams & Wilkins Comp., Baltimor.

9. Official Methods of Analysis. Association of Analytical Chemist, 4th. Edition,(1984), Published by Association. of Official Analytical Chemists Inc., Virginia U.S.A.

10. Block, R. J., Durrum, E. L. and Zwig G. (1958) A manual of paper chromatography and paper electrophoresis. 2nd . Ed. p. 107-203. New York, Academic Press Inc.

11. Okafora, N. (1990). Traditional Alcoholic Beverages of Tropical Africa : strategies for scale up. Process Biochem. p. 213-220