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

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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 occurred 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.
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; Nwaigwu 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 plants 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. Glucose 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˚C. 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%.

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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 christopher, 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 9h of incubation. Thereafter a sharp increase in the activity was seen reaching 26 U/ml at the end of 16th 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 7th hour of incubation (8-12 x10^7 cfu/ml). By the end of 13th hour of fermentation the LAB count decreased to 2.5x10^7 cfu/ml (Fig. 3). Yeast cells were counted in the Neubar chamber. Each budding cell was counted as one. The cell count remained in the range of 3.4-4.4 x 10^7 till the 7th hour of fermentation. Thereafter, the proliferation of the yeast population was at a much faster rate reaching its peak at the 11th hour to a cell count of 8.75 x 10^7/ml. The cell count decreased 11th hour onwards reaching 5.8 x 10^7/ml. Budding yeast cells were predominant in the three sap samples collected from 5-9th hour of incubation. Invertase content which had increased at slow rate till 5h of incubation started increasing gradually till 9th 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 7th and 11th 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; Ouba 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 are indicated 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%.

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