Effect of time and temperature on the fermentation of Palm (*Borassus flabellifer*) sap by *Saccharomyces cerevisiae isolate* YN3

Krishna Prasad Nooralabettu* and Ronald Valder,

Abstract— Nutritionally rich palm sap tapped by chopping the inflorescence of palm tree (*Borassus flabelliffer*) is popular juice in several parts of India becomes heady with time due to fermentation. Effect of time and temperature on the fermentative characteristics of *Saccharomyces cerevisiae isolates* YN3 in fresh palm sap that was sterilized at 60°C for 25 minutes was studied to optimize the condition for intervention of fermentation. *Saccharomyces cerevisiae isolates* YN3 grown optimally at 60°C for 15 min with maximum alcohol production of 89.3g/L beyond which least degradative changes takes place in nutritionally rich palm sap.

Index Terms— Palmyra Palm, sterilization, *Saccharomyces cerevisiae*, *Borassus flabellifer*, disaccharides, Toddy, Palm sap. .

1 Introduction

Palm sap is the sweet and clear, which is tapped from the young inflorescence of the male or female trees(*Borassus flabellifer*). Fermentation of palm sap is an oxido-reductive process in which organic compounds such as sugars in an intermediate oxidation state act as electron acceptors [1]. Traditional method of fermentation is an uncontrolled spontaneous process occurs due to the wide varieties of lactic acid, alcohol and acetic acid producing yeast and lactic acid bacteria enters the stream through contact surface contamination of inflorescence, earthen pot, wind or insects [2]. Sucrose present in the sap initially hydrolyses to glucose and fructose by microbial enzyme 'invertase' located in the yeast cell wall. These end products are carried into the yeast cells and subjected to glycolysis via the "Embden-Meyerhof-Parna's" pathway leading to the formation of ethanol, carbon dioxide and energy. Nutritionally rich fresh palm sap is boiled to down to syrup or further boiled to produce palm sugar known in Hindi as jiggery[3]. Palm wine is also used in South India to produce fermented rice flour pan cakes such as 'Idli', 'Dosa', 'Appam' [4]. the plant also known to possess anti-laprotic, stimulant, antiphlogistic, diuretic, stomachic, anti-inflammatory, sedative, immunosuppressant properties and laxative properties[5], [6], [7], [8]. Palm sap is also a rich source of sugars, proteins, lipids, vitamin A, vitamin C, B-complex, and others minerals[9].

- *Corresponding author Krishna Prasad Nooralabettu is currently working as Professor and Head, Department of Biotechnology, P. A. College of Engineering, Mangalore, India, PH-+00919448529048. E-mail: hod_biotech@pace.edu.in*
- *Second author Ronald Valder is currently pursuing Doctoral degree program in Biotechnology Engineering in Visvesvarayya Technological University, Belgum, India, PH-+00919964277520. E-mail: ronald_bio@pace.edu.in*

2 MATERIALS AND METHODS

2.1 Sample Collection

Nine palm (*Borassus flabellifer* L.) trees were identified in Sajipa of Dakshina Kannada District (Karnataka, India). Freshly tapped palm sap was collected at around 6.00 AM over 14 tapping process in the month of December. Under sanitary condition, 50 mL of the samples are collected directly from the palm sap collection earthen pot into a sterile 50 mL sample collection vessels. Temperature of the environment varied from 18 to 24°C and within 30 min samples were transported to the laboratory of the Department of Biotechnology, P. A. College of Engineering, Mangalore, in an insulated container maintained at 4°C until analysis. Samples were immediately filtered using sterile muslin filter cloth and maintained at 4°C until analysis, and if any delay then preserved at –50°C in a deep freezer (Model C340, New Brunswick Scientific, England).

2.2 Chemicals

Analytical grade chemicals were procured from Merck Limited (Mumbai, India), and reagents were prepared as per the current American Chemical Society specifications [10]. Yeast Extract Potato Dextrose Agar (YEPDA), Yeast Extract Potato Dextrose Broth (YEPDB), were procured from Himedia, Mumbai and prepared as per manufacturer's instructions. Media was sterilized by autoclaving at 15 lbs pressure at 121°C for 15 min and after inoculation plates were incubated at 37 °C in incubator. The colonies are enumerated using digital colony counter (Systronics, Mumbai).

2.3 Sterilization of the palm sap

Sterilization of the palm sap was carried out at 60°C for 25 min. Sterilization was carried out in water bath (Rotek Instruments, Kerala) in 250 mL capacity flask. Properly plugged and wrapped flasks were arranged in the water bath with sufficient space to facilitate the moist heat penetration. Palm sap exposed to various temperatures for different intervals of time

was then analyzed for total protein content, total lipid content, Vitamin C, reducing sugar, non-reducing sugar, glucose, sucrose, ethanol, mould count and bacterial count to optimize the parameter.

2.4 Microorganisms and cultural preparations

A stock culture of *Saccharomyces cerevisiae isolate* YN3 was used for the present study as it was the predominant flora isolated from palm sap. *Saccharomyces cerevisiae isolate* YN3 was grown in YEPDB. The pH of the medium was adjusted by adding ammonium solution (1:1) or 2(N) HCl solution before autoclaving. YEPD was sterilized at 120°C in moist heat at 15 lbs pressure for 15 min. After sterilization 250 mL of YEPDB was inoculated with *Saccharomyces cerevisiae isolate* YN3 and incubated at a temperature of 30°C and pH 6.5 for 3 days under semi-aerobic condition, when the concentration of cells in optical density of the medium was reached 1.3 nm. At appropriate intervals of time, samples were drawn for analysis in quadruplicates. After attaining appropriate growth yeast cells were separated from the media by centrifugation at RCF of 3,500xg for 10 min (Remi C-24, Mumbai, India). Pellets were separated and stored at stored at -40°C in a cold store for further stud [11].

2.5 Kinetics for palm sap fermentation

Fermentative characteristics of *Saccharomyces cerevisiae isolate* YN3 and effect of different temperature and time interval of the fermentation were studied in the 1L laboratory fermenter [12]. Growth characteristics of the *Saccharomyces cerevisiae isolate* YN3 in sterile palm sap with associated changes in the components of the medium were carried at temperature of 10, 20, 30, 40, 50, and 60°C for time interval of 5 h up to 50 h **were carried out at pH 6.5.** Palm sap so treated was then analyzed for total protein content, lipid content, Vitamin C, reducing sugar, glucose, sucrose, ethanol, and total yeast count on YAPDA by drawing samples at different intervals of time to optimize the growth parameter.

2.6 Proximate analysis during incubation

At different intervals of experiments, palm sap samples were collected in quadruplicates. Cell free samples were prepared by centrifuging the samples at relative centrifugal force (RCF) of 1681.1 x *g* for 5 min at 4°C (C-24BL/CRP24 model centrifuge, Remi Laboratory Instruments, Mumbai, India). The pH was measured using Portable Glass electrode pH meter (Systronics, Mumbai). Incubation of the samples at 30°C was carried out in an incubator (Rotek Instruments, Kerala). Temperature of the samples was estimated using infra-red thermometer (Quicktemp 826 T4, Austria).

Absorbance of the cell free samples of *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 and 60°C (YEAST-AB-10, YEAST-AB-20, YEAST-AB-30, YEAST-AB-40, YEAST-AB-50, and YEAST-AB-60, respectively) was measured in 1 cm Quartz cell at 420 nm with a visible spectrophotometer (Systronics, Mumbai). Total protein content in the samples at 10, 20, 30, 40, 50, or 60°C (YEAST-TPC-10, YEAST-TPC-20, YEAST-TPC-30, YEAST-TPC-40, YEAST-TPC-50, and YEAST-TPC-60, respectively)for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50 h were estimated by Lowry's method and values were expressed in mg/mL[13]. Total lipid content (YEAST-TLC-10, YEAST-TLC-20, YEAST-LPC-30, YEAST-TLC-40, YEAST-TLC-50, and YEAST-TLC-60, respectively) in palm at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50 h were estimated after extraction with chloroform-methanol extraction method followed by reaction with sulfuric acid and vanillin phosphoric acid reagent and values were expressed in percentage (w/v) [14]. Vitamin C content in the samples at 10, 20, 30, 40, 50, or 60°C (YEAST-VC-10, YEAST-VC-20, YEAST-VC-30, YEAST-VC-40, YEAST-VC-50, and YEAST-CV-60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50 h were estimated by Redox Titration methods using 2, 4-dinitrophenyl hydrazine (DNPH) dye and standard ascorbic acid, and values were expressed as mg/mL [15].

Reducing sugar in palm sap at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50 h were estimated dinitrosalicylic acid reagent, and values were expressed in percentage (v/v) [16]. Glucose and Sucrose at 60, 70, 80, 90, 100, 110 or 120°C (YEAST-SU-10, YEAST-SU-20, YEAST-SU-30, YEAST-SU-40, YEAST-SU-50, and YEAST-SU-60, respectively, AND YEAST-GL-10, YEAST-GL-20, YEAST-GL-30, YEAST-GL-40, YEAST-GL-50, and YEAST-GL-60, respectively) for at 5, 10, 15, 20 or 25 min were estimated using High sensitive Glucose and Sucrose Assay kit provided by EMerck, India, and values were expressed in percentage (w/v).

Changes in ethanol content in the palm sap was estimated at different intervals of time at different temperature based on the colorimetric reaction of ethanol with sodium dichromate, and values were expressed in percentage (v/v) [17]. Ethanol content of the cell free samples of *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 and 60°C (YEAST-EOH-10, YEAST-EOH-20, YEAST-EOH-30, YEAST-EOH-40, YEAST-EOH-50, and YEAST-EOH-60, respectively) was measured.

Changes in mould count and bacterial count of the palm sap at 60, 70, 80, 90, 100, 110 or 120°C were performed as per APHA method and values were expressed in cfu/mL [18]. Microbial count of the palm sap samples of *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 and 60°C (YEAST-COUNT-10, YEAST-COUNT-20, YEAST-COUNT-30, YEAST-COUNT-40, YEAST-COUNT-50, and YEAST-COUNT-60, respectively) was measured.

2.7 Statistical analysis

The samples were collected and analyzed in quadruplicate and data was analyzed by One way-analysis of variance (ANOVA) using the Fisher's least significant difference (LSD) test to estimate the significant differences between each sample ($p \leq 0.05$) using Statgraphics Centurion XV software (Statpoint Technologies Inc., Warrenton, VA, USA).

3 RESULTS

3. Changes in temperature and time of incubation

3.1 Effect of Temperature and Time on absorbance of Palm Sap with yeast

Effect of temperature of incubation on the optical density of the palm sap incubated with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C of incubation for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 1.

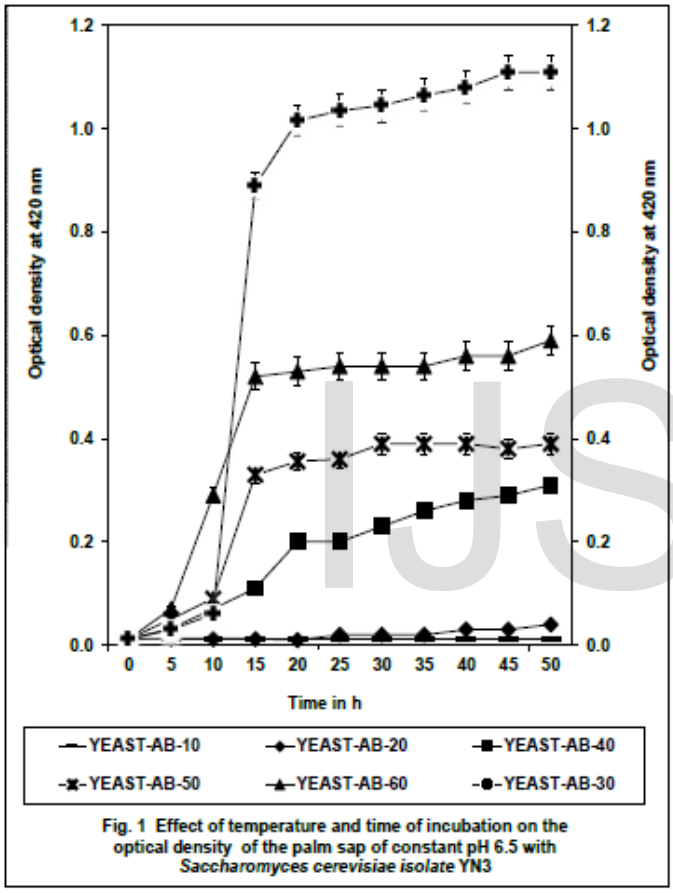

Fig. 1 Effect of temperature and time of incubation on the optical density of the palm sap of constant pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

Measuring optical density of the palm sap during fermentation by microbes provides valuable information on the number of cells in the media, the chemical components released into the media by living organisms and the physicochemical changes in the components of the media at various temperatures. Optical density of the fresh palm sap increased from 0.01±0.004 in initial sample to 1.068±0.5 in samples incubated for 55 h at 30°C. The palm sap incubated at 10, 20 and 60°C for 50 h did not show any significant(p>0.05) change in optical density in comparison to the optical density of its initial samples, as confirmed by One way ANOVA with *post hoc* Tukey's test. However, optical density of the palm sap inoculated with *Saccharomyces cerevisiae isolate* YN3 and incubated for 30, 40 and 60°C showed 93, 26, 33, and 49 folds increase in the optical density at 420 nm. Here, One way ANOVA with *post hoc* Tukey's test confirms that overall significant effect of temperature of incubation on the increase in optical density of the palm sap samples remained at 5% level of significance.

3.2 Effect of Temperature and Time on ethanol production in Palm Sap by yeast

Effect of temperature of incubation on the ethanol of the palm sap with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 2.

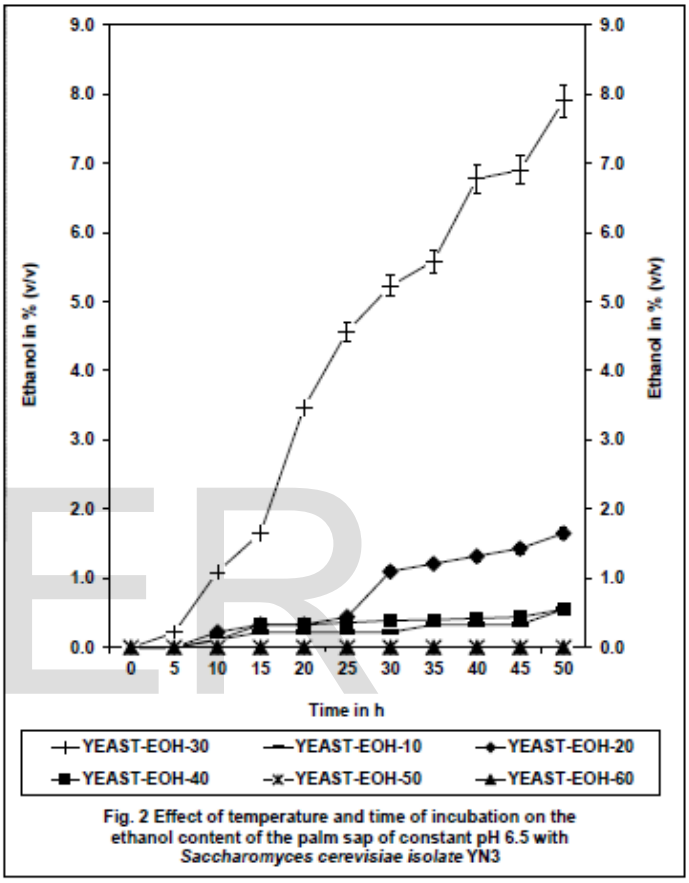

Fig. 2 Effect of temperature and time of incubation on the ethanol content of the palm sap of constant pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

Here, shows the interaction between the two variables of incubation of yeast isolates such as temperature and time but at constant pH of 6.5 on the absorbance and the ethanol content is shown. Ethanol content of the palm sap inoculated with *Saccharomyces cerevisiae isolate* YN3 increased from 0.011±0.001% in initial sample to 8.85±0.05% in samples incubated for 55 h at 30°C. One way ANOVA with *post hoc* Tukey's test was not able to establish any significant (p>0.05) change in ethanol content in the palm sap incubated at 10, 20, 50 and 60°C for 50 h in comparison to the ethanol content of its initial samples,. However, ethanol content of the palm sap inoculated with *Saccharomyces cerevisiae isolate* YN3 and incubated for 30 and 40°C showed 83, and 3 folds increase in the ethanol content. Here, the experiment confirms that overall significant effect of temperature of incubation on the increase in ethanol content of the palm sap samples remained at 5% level of significance, as indicated by One way ANOVA with *post hoc* Tukey's test.

3.3 Effect of Temperature and Time on viable

count of yeast in Palm Sap

Similarly, effect of temperature of incubation on the count of *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C of incubation for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 3.

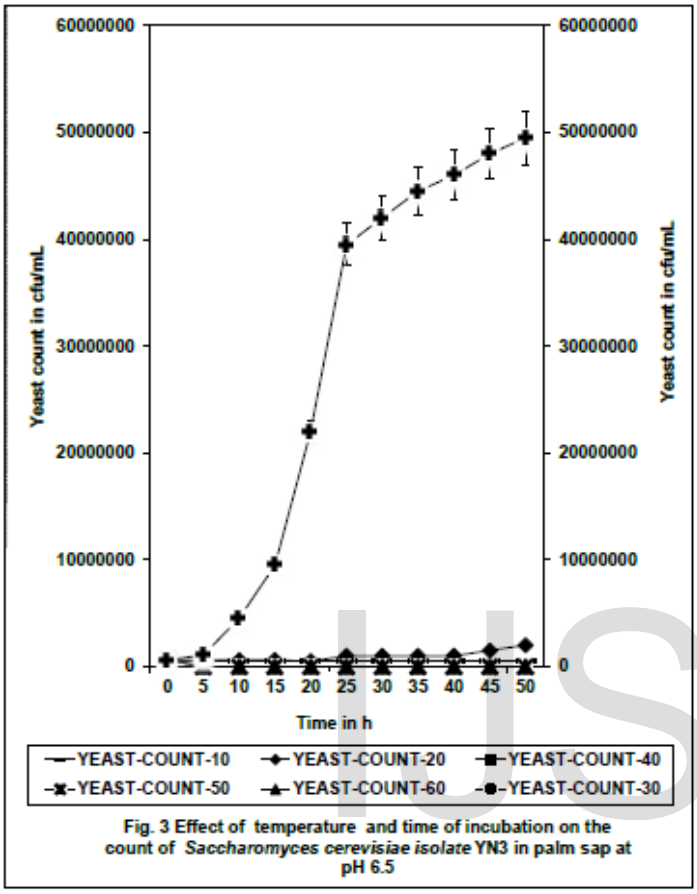

Fig. 3 Effect of temperature and time of incubation on the count of *Saccharomyces cerevisiae isolate* YN3 in palm sap at pH 6.5

During 50 h of incubation at 30°C, viable Count in palm sap that was sterilized and inoculated with *Saccharomyces cerevisiae isolate* YN3 was increased from 6.00×10^5 cfu/mL to 4.95×10^7 cfu/mL. One way ANOVA with *post hoc* Tukey's confirms that the palm sap incubated at 10, 40, 50 and 60°C for 50 h did not show any significant ($p>0.05$) change in viable count in comparison to the viable count of its initial samples. However, viable count of the palm sap inoculated with *Saccharomyces cerevisiae isolate* YN3 and incubated for 20 and 30°C showed 3 and 83 folds increase in the viable count. One way ANOVA with *post hoc* Tukey's test affirms that, overall significant effect of temperature of incubation on the increase in viable count of LAB of the palm sap in these samples remained at 5% level of significance.

3.4 Effect of Temperature and Time on sucrose utilization by yeast in Palm Sap

Similarly, effect of temperature of incubation on the sucrose level of palm sap with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 4.

Utilization of sucrose in palm sap that was sterilized and inoculated with *Saccharomyces cerevisiae isolate* YN3 was investigated. Sucrose level in the sample during 50 h of incubation at 30°C was reduced from 14.72 ±0.09% 1.60 ±0.05%. One way ANOVA with *post hoc* Tukey's test was able to establish significant ($p<0.05$) level of sucrose in samples incubated at 20, 30, and 40°C for 50 h in comparison to the sucrose levels of its initial samples. Sucrose in the palm sap inoculated with *Saccharomyces cerevisiae isolate* YN3 and incubated for 20, 30, and 40°C showed 2, 9 and 2 folds decrease in the sucrose level. Overall significant effect of temperature of incubation on the decrease in sugar by the yeast of the palm sap in these samples remained at 5% level of significance, as indicated by One way ANOVA with *post hoc* Tukey's test.

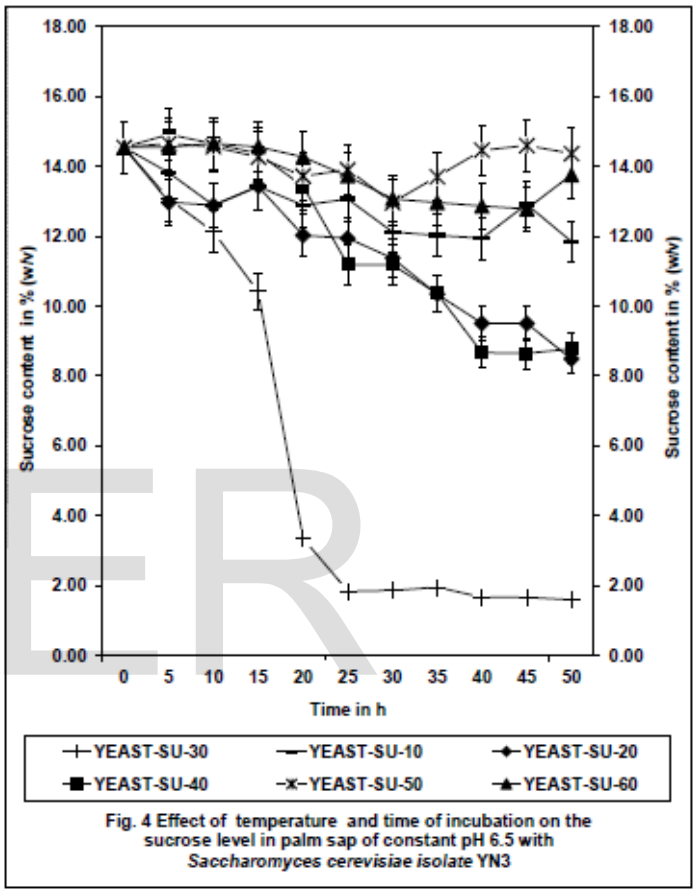

Fig. 4 Effect of temperature and time of incubation on the sucrose level in palm sap of constant pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

3.5 Effect of Temperature and Time on Glucose utilization by yeast in Palm Sap

Similarly, effect of temperature of incubation on the glucose level of palm sap with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 5.

Glucose is the immediate carbon source to microbes in the palm sap. Reduction in glucose level in the palm sap from 1.21±0.003% to 0.13 ±0.02% during 50 h of incubation of *Saccharomyces cerevisiae isolate* YN3 at 30°C indicates the microbial activity. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant ($p>0.05$) level of difference in glucose levels in between samples incubated at 10, 50, and 60°C for 50 h and the glucose levels in the initial samples. Glucose levels in the samples inoculated and incubated with *Saccharomyces cerevisiae isolate* YN3 for 20, 30, and 40°C showed 1.7, 9.1 and 1.6 folds reduction in the glucose level. One way ANOVA with *post hoc* Tukey's test confirms the overall significant effect of temperature of incubation on the decrease in glu-

cose of LAB of the palm sap in these samples remained at 5% level of significance.

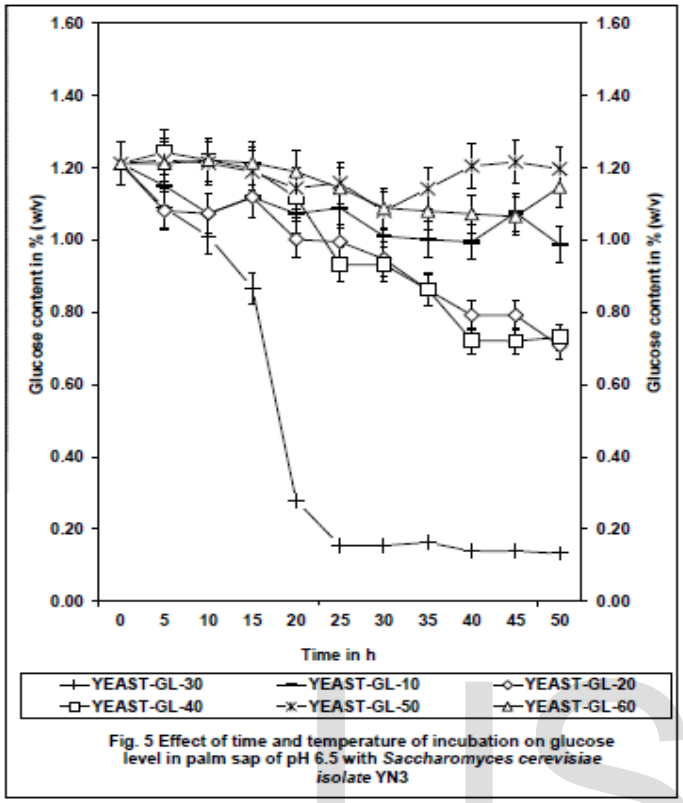

Fig. 5 Effect of time and temperature of incubation on glucose level in palm sap of pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

3.6 Effect of Temperature and Time on protein content in Palm Sap with yeast

Effect of temperature of incubation on the protein content of palm sap with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 6.

Protein content in the initial palm sap was 1.50±0.69 mg/mL, and the protein content during 50h of incubation with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 and 60°C was increased, respectively, to 1.33±0.45, 1.39±0.78, 1.37±0.65, 1.46±0.76, 1.33±0.81, and 1.17±0.83 mg/mL. One way ANOVA with *post hoc* Tukey's test was not able to establish any significant (p>0.05) difference in protein content in between samples incubated at 10, 20, 30, 40, 50 and 60°C for 50 h, and in comparison to protein content of the initial samples.

3.7 Effect of Temperature and Time on lipid content in Palm Sap with yeast

Effect of temperature of incubation on the lipid content of palm sap with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 7.

Lipid content in the initial palm sap was 0.05±0.008 mg/mL, and the Lipid content during 50h of incubation of *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 and 60°C was increased, respectively, to 0.04±0.009, 0.03 ±0.008, 0.03 ±0.007, 0.03 ±0.009, 0.05±0.008, and 0.05±0.009 mg/mL. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant (p>0.05) difference in Lipid content in between samples incubated at 10, 20, 30, 40, 50 and 60°C for 50 h, and in comparison to protein content of the initial samples.

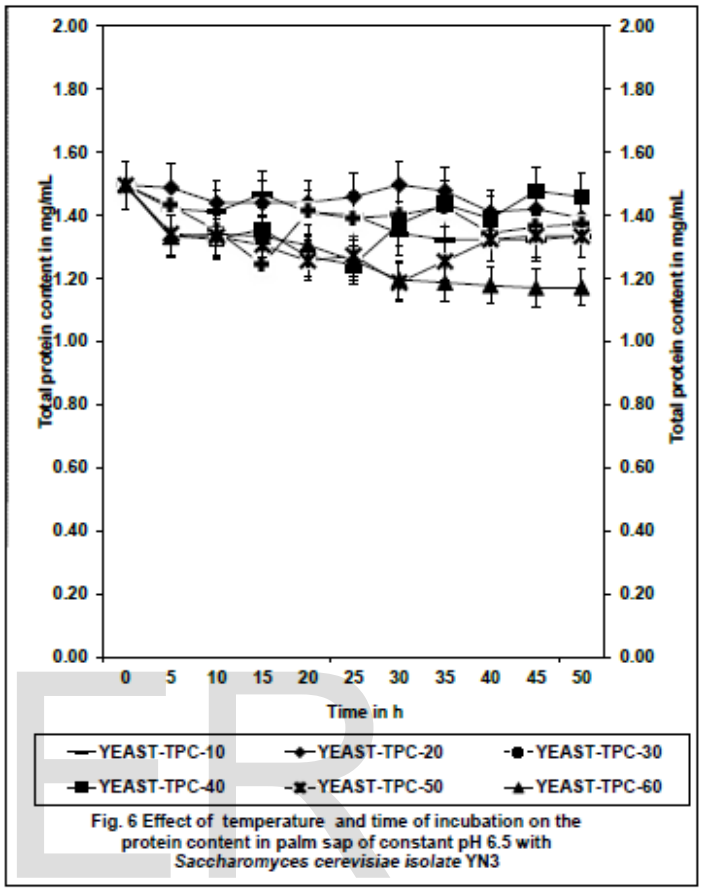

Fig. 6 Effect of temperature and time of incubation on the protein content in palm sap of constant pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

3.8 Effect of Temperature and Time on Vitamin C in Palm Sap with yeast

Similarly, effect of temperature of incubation on the lipid content of palm sap inoculated with *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C of incubation for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 8.

Vitamin C in the initial palm sap was 0.069±0.008 mg/mL, and the Vitamin C during 50h of incubation of incubation of *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 and 60°C was increased, respectively, to 0.056±0.006, 0.040 ±0.008, 0.040 ±0.007, 0.041 ±0.005, 0.068±0.006, and 0.065±0.007 mg/mL. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant (p>0.05) difference in Vitamin C in between samples incubated at 10, 20, 30, 40, 50 and 60°C for 50 h, and in comparison to protein content of the initial samples.

Here, effect of temperature on *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50, or 60°C of incubation for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50 h was studied. Here, interaction between the two variables of incubation of yeast isolates such as temperature and time but at constant pH of 6.5 on the absorbance and the ethanol content was analysed.

Changes in the physico-chemical properties of the Yeast inoculated in sterile palm sap was carried out at controlled physical factors such as temperature and pH that are important for the fermentation of ethanol. Freshly tapped palm

sap collected from inflorescence of *Borrasus flabellifer* was transparent without any colour and less viscous with an optical density of 0.01 at 420 nm.

The pH of the fresh palm sap was 7.8 and near to neutral. It was found that with the increase time at 30°C and pH 6.5 absorbance of the palm sap increased, but absorbance of the cell free palm sap did not vary much. During this period, it was found that with increase in time at 30°C and pH 6.5 ethanol production is increased. However, below 20°C or above 40°C no much change in absorbance or ethanol production was recorded. Maximum rate of alcohol proction was observed between 10-15 min of incubation at 30°C and pH 6.5. Longer period of incubation of both isolates in palm sap at higher temperatures reduced the production of ethanol. Even at optimum temperature but longer period of incubation also reduced the rate of alcohol production. It was registered that at 30°C for 50 h of incubation maximum production of ethanol was attained.

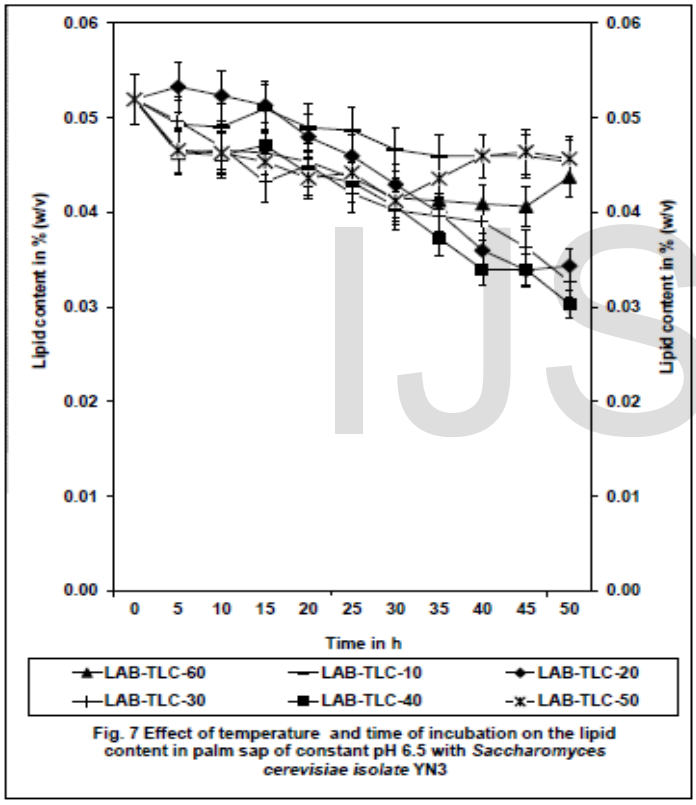

Fig. 7 Effect of temperature and time of incubation on the lipid content in palm sap of constant pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

Statistical significance of palm wine fermentation by *Saccharomyces cerevisiae isolate* YN3 is explained by analysis of variance (ANOVA). The probability of *p*-value for models of less than 0.05 indicates that the process were significant. Here, response of the variables temperature and time were significant with p-value of less than 0.05 at 30°C. For below 20°C or above 40°C, p-value was > 0.05 indicating that this value was insignificant.

Total sugar content in the fresh palm sap was 13.45±0.06% (w/v). Sucrose content, which is non-reducing sugar in the palm sap was 11.63±0.06% (w/v). Reducing sugar in the palm sap was varing between 1.81±0.09% (w/v). Glucose, which is reducing sugar in the palm sap was 0.90±0.01% (w/v). Fructose, which is non-glucose reducing sugar was 0.75±0.05% (w/v). Protein content of palm sap was 1.54±0.76 mg/mL. Similarly, Vitamin C in palm sap was 0.071 ±0.003 mg/mL.

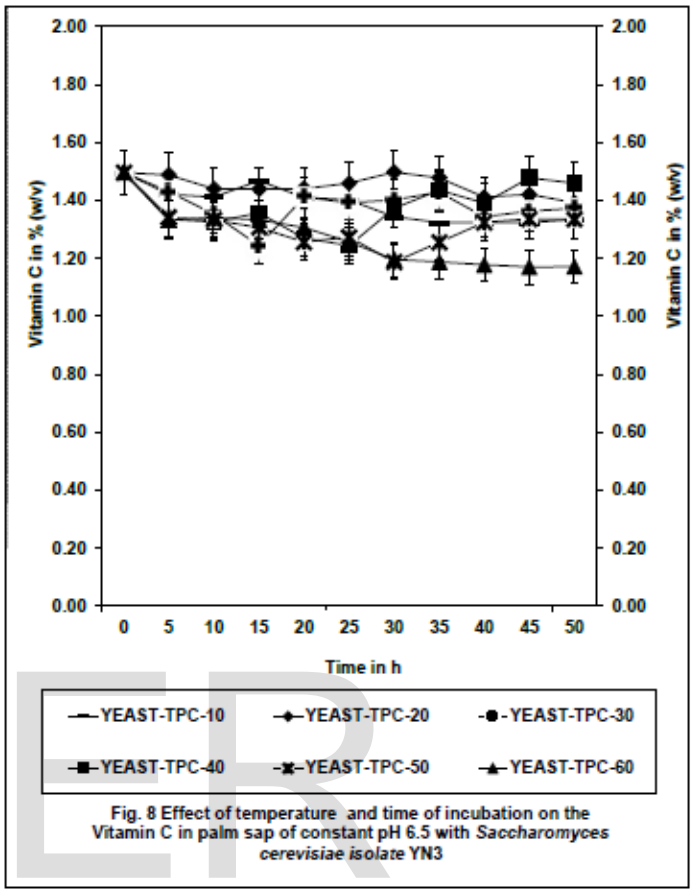

Fig. 8 Effect of temperature and time of incubation on the Vitamin C in palm sap of constant pH 6.5 with *Saccharomyces cerevisiae isolate* YN3

Changes in the Count of *Saccharomyces cerevisiae isolate* YN3 on YEPD, in palm sap at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h was studied. Count of *Saccharomyces cerevisiae isolate* YN3 on YEPDA was 1.8×10^4 cfu/mL.

In one hand, optimum growth of *Saccharomyces cerevisiae isolate* YN3 on YEPD at 30°C was recorded in palm sap between 15 and 30 h. On the other hand sharp reduction in the total sugar content was recorded in palm sap at 30°C and pH 6.5 between 15 and 30 h. Increase time at 30°C and pH 6.5 incresed the consumption of sugar in the palm sap. But, no much change in microbial load or sugar consumption was recorded below 20°C or above 40°C.

Maximum rate of sugar consumption was recorded between 15-30 h of incubation at 30°C and pH 6.5. Even at 30°C and pH 6.5, longer period of incubation of both isolates in palm sap at higher temperatures reduced the consumption of alcohol. Longer period of incubation also reduced the rate of alcohol production even at optimum temperature but. It is interesting to note that at 32°C optimum temperature after 50 h of incubation maximum utilization of sugar was recorded.

Analysis of variance by ANOVA was able to establish statistical significance of consumption of sugar of palm sap by *Saccharomyces cerevisiae isolate* YN3 as indicated by the probability of p-value for process of less than 0.05. In this study, response of the variables temperature and time on microbial

growth and carbohydrate utilization were significant with p-value of less than 0.05 at 30°C. For at and below at 20°C or at and above at 40°C, *p*-value was > 0.05 indicating that this value was insignificant.

Changes in the sucrose and glucose of palm sap by *Saccharomyces cerevisiae isolate* YN3 at 10, 20, 30, 40, 50 or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50h is illustrated in the figure 4 and 5. Sucrose content in palm sap reduced drastically at 30°C and pH 6.5 between 15 and 20 h. However, temperature above 40 °C and below 20°C not much changes in sucrose lavel in palm sap with *Saccharomyces cerevisiae isolate* YN3.

Maximum rate of sucrose consumption was recorded between 15-20 h of incubation at 30°C and pH 6.5. Even at 30°C and pH 6.5, increase in incubation period in both the isolates in palm sap at higher temperatures not much sucrose change was recorded. Here, One way ANOVA with *post hoc* Tukey's test was able to establish a significant(p<0.05) difference in the sucrose content amongst the samples collected at 10, 20, 30, 40, 50 or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 45, or 50h of incubation in both of the strains. However, in samples collected above 40 °C and below 20°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50h did not significantly (p>0.05) exhibit any changes in sucrose level, as indicated by One way ANOVA with *post hoc* Tukey's test .

Glucose consumption was recorded at highest level between 15-20 h of incubation at 30°C and pH 6.5. Increase in incubation period in both the isolates in palm sap at higher temperatures did not record much glucose change even at 30°C and pH 6.5. Here, significant(p<0.05) difference in the glucose content was recorded amongst the samples collected at 10, 20, 30, 40, 50 or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50h of incubation in both of the strains, as established by One way ANOVA with *post hoc* Tukey's test.

However, One way ANOVA with *post hoc* Tukey's test was not able to establish as significant (p>0.05) difference in samples collected above 40 °C and below 20°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50. Here interesting note here that One way ANOVA with *post hoc* Tukey's test was not able to establish as significant (p>0.05) difference protein content, lipid content and Vitamin C content in samples incubated at 10, 20, 30, 40, 50 or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50H.

4 DISCUSSION

Values obtained from the proximate analysis of the palm sap more or less similar to the values published in previous works [19], [20]. In this work, first lot of the sterilized fresh palm sap was divided into six sub-lots and inoculated with *Saccharomyces cerevisiae isolate* YN3, and temperature of incubation of the inoculate was optimized by incubating each sub-lots at different temperature, but at constant pH of 6.5. Second lot of the sterilized fresh palm sap was divided into six sub-lots and inoculated with *Saccharomyces cerevisiae isolate* YN3, and pH of the palm sap was optimized by incubating each sub-lot at different pH levels, but at optimised temperature condition. Total sugar content in the fresh palm sap was ranging between 09.88±0.08% (w/v) and 17.32±0.04% (w/v), which means that the sugar concentration in the palm sap was ranging between 98.80 g/L to 173.32 g/L, the value confirms with the previously reported values[21]. Temperature is an important parameter that influences the fermentation in batch mode. However, temperature tolerance in yeast during fermentation was previously reported by research work [22]. Production of ethanol was very high during the initial 24 hours of incubation at 40°C; however, thereafter the ethanol production was only 76.7 g/L at 35°C. Decline in ethanol production after 24 hours of incubation at 40°C may be due to the inactivation of yeast cells due to high temperature for longer time period. Previous reports suggest that ethanol producing yeast grows rapidly at temperature between 25 °C and 33°C, and rate of ethanol production increases between 30 °C and 37°C[23]. Ethanol production was least at and below 20 and at and above 40°C and therefore not favourable temperature for the strain *Saccharomyces cerevisiae isolates* YN3, as there extreme temperature conditions were stressful. Temperature of 30°C was moderator as the ethanol production increased even up to 50 h of incubation. Hence temperature of incubation plays a major role in the production of ethanol as it controls the viability of the cells, growth rate of the isolate, exponential phase of the ethanol production strain, enzyme activity of the alcohol producer, and function of the semipermeable membrane of the cells [24]. Change of colour from transparent to whitish colour is due to the production of a gum probably dextrans by lactic acid bacteria, and in addition to this heavy suspension of yeast and bacteria gives milky-white colour to the palm sap[25], [26] . Volatile profile of the palm sap is due to the ethanol and acetic acid produced by the microbes [26]. Decrease in the sugar content in palm sap during initial stage of fermentation is due to the microbial metabolic activity [27]. Reduction in pH during the initial fermentation is due the production of lactic acid by Lactic acid producing bacteria [28].Accumulation of ethanol in palm sap during fermentation depends on several factors such as type of the microorganism, composition of the palm sap, species of palm tree, and environmental factors such as temperature [28].

Growth optimum of between 14 to 25, decreased at 30°C and 10°C, indicate the mesophylic characteristics of the isolate [29]

5 CONCLUSION

Freshly tapped palm sap is sweet and clear, but microbial activity changes it to milky white and sour. *Saccharomyces cerevisiae isolates* YN3 grown optimally at 60°C for 15 min with maximum alcohol production of 89.3g/L beyond which least degradative changes takes place in nutritionally rich palm sap. Degradative activity of yeast can be intervened by adjusting the temperature of the medium and period of incubation to a level beyond the optimum levels where least degradative changes takes place. Present study gives valuable information for the intervention of the palm sap from fermentation.

REFERENCES

[1] R. K. Chrystopher and K. Theivendirajah, "Palmyrah palm wine 1: microbial and biochemical changes", *J. Notes. Sci. Conn, Sri Lanka,* vol. 16, no. 1, pp. 131-141, 1988

[2] J.F. Morton, "Notes on Distribution, Propagation, and Products of *Borassus* Palms (Arecaceae)," *Economic Botany,* vol. 42, no. 3, pp. 420-441, 1988

[3] P.V.K.J. Rao, M.Das, and S.K. Das, "Changes in physical and thermophysical properties of sugarcane, palmyra-palm and date-palm juices at different concentration of sugar," *Journal of Food Engineering, no.* 90, vol. 1, pp. 559–566, 2009

[4] T.A. Davis and D.V. Johnson, "Current utilization and further development of the Palmyra Palm (*Borassus flabellifer* L., Areeaceae) in Tamil Nadu State, India," *Economic Botany*, vol. vol. 41, no.2, pp. 247-266, 1987
[5] P. S. Vaidyaratnam, "Variers Arya Vaidya Sala - Indian Medicinal Plants A Compendium of 500 species," *Orient Longman: Chennai, India.* vol. 4, pp. 293-296, 2002
[6] L.D. Kapoor, "Handbook of Ayurvedic medicinal plants: Herbal reference library", *CRC Press, USA, Florida,* p 82, 2000.
[7] L. Revesz, P. Hiestand, L. La Vecchia, R. Naef, H.U. Naegeli and L. Oberer, "Isolation and synthesis of a novel immunosuppressive 17 alpha-substituted dammarane from the flour of the Palmyrah palm (*Borassus flabellifer*)," *Bioorganic and Medicinal Chemistry Letters,* vol. 9, no. 1 pp. 1521-1526, 1999
[8]. M.S. Paschapur, M.B. Patil, R. Kumar, and S.R. Patil, "Evaluation of anti-inflammatory activity of ethanolic extract of *Borassus flabellifer* L. male flowers (inflorescences) in experimental animals," *Journal of Medicinal Plants Research*, vol. 3, no. 2, pp. 49-54, 2009
[9]. 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," *Research Journal of Medicine and Science,* vol. 3, no. 2, pp. 173-176, 2008
[10] American Chemical Society (ACS), Lab Guide, Washington D.C., American Chemical Society, pp. 98-99, 1999
[11] D. Richwood, T. Ford and J. Steensgaard, Centrifugation-Essential Data, John Wiley & Son Ltd., pp. 1-128, 1994.
[12] P. Naknean and Meenune, "Impact of Clarification of Palm Sap and Processing Method on the Quality of Palm Sugar Syrup (*Borassus Flabellifer Linn.*)," *M. Sugar Tech*, vol. 17, no. 1, pp 195, 2015
[13] 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
[14] E.V. Handel, "Rapid Determination of Total Lipids in Mosquitoe," *J Aru Mosq. Conrnol Assoc,* vol.1, no.3, pp. 302-304, 1985
[15] 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
[16] G.L.Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Anal. Chem.,* vol. 31, no. pp. 426, 1959
[17] S.V Sumbhate, S. Nayak, D. Goupale, A. Tiwari, R.S. Jadon, Colorimetric methods for the Estimation of Ethanol from alcoholic drinks, Journal of Analytical Techniques, Vol. 1, pp.1-6, 2012
[18] APHA, Recommended Procedure for the Examination of Seawater and Shellfish, 2nd ed., American Public Health Association, Washington, pp. 12-13, 1982
[19] P.V.K.J. Rao, M. Das and S.K. Das, "Changes in physical and thermophysical properties of sugarcane, palmyra-palm and date-palm juices at different concentration of sugar," *Journal of Food Engineering*, vol. 90, no. 1, pp. 559–566, 2009.
[20] A. S. Aldiguier S. Alfenore X. Cameleyre G. Goma, J. L. Uribelarrea S. E. Guillouet C. Molina-Jouve, "Synergistic temperature and ethanol effect on *Saccharomyces cerevisiae* dynamic behaviour in ethanol bio-fuel production", Bioprocess Biosyst. Eng., Vol. 26, 217–222, 2004
[21]. P. Naknean, M. Meenune, and G. Roudaut, "Characterization of palm sap harvested in Songkhla province, Southern Thailand," *International Food Research Journal*, vol. 17, no.1, pp. 977-986, 2010.
[22]. S. Rousseau, D. Rouleau, L.Yerushalmi and R.C. Mayer, "Effect of temperature on fermentation kinetics of waste sulfite liquor by *Saccharomyces cerevisiae," Journal of Chemical Technology and Biotechnology*, vol. 53, no.1, pp. 285-291, 1992
[23]. F. Ozcelik, and Y. Denli. "Sarap mayalarinin teknolojik ozellikleri," *Gida,* vol. 24, no. 6, pp. 385-389, 1996
[24]. M.J.Torija, G.Beltran, M. Novo, M. Poblet, J. Manuel, G.A. Mas and N. Roze, "Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile ompounds in wine," *International Journal of Food Microbiology*, vol. 8, no, 1, pp. 127– 136, 2003.
[25]. 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
[26]. 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
[27] J.A. Santiago-Urbina, A.G. Verdugo-Valdez, F. Ruíz-Terán, "Physicochemical and microbiological changes during taping 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
[28] 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
[29] F.Krier, A. Revol-Junelles and P. Germain, "Infuence of temperature and pH on production of two bacteriocins by *Leuconostoc mesenteroides* subsp. mesenteroides FR52 during batch fermentation," *Appl Microbiol Biotechnol* , vol. 50, no. 1, p 359, 1998.