Optimization of Submerged Batch Fermented Coconut Water Vinegar Production Process

H. K. Vidulani Shanika[1], M.A. Jagath Wansapala[2]

[1, 2] Department of Food Science and Technology, University of Sri Jayewardenepura, Sri Lanka

Abstract: *Vinegar is a condiment made from various sugary and starchy materials by alcoholic and subsequent acetic fermentation. The present study was aimed to improve the efficiency of coconut water vinegar production process by reducing the fermentation time, along with identifying the product conformity to SLS 168:1999-Specification for Coconut Vinegar. Optimum conditions for ethanol fermentation was investigated by Taguchi's factorial design with L_8 orthogonal array. The optimum inoculum type, pH, Total soluble solids, and concentration of nutrient respectively was brewer's yeast, 4.97 (natural pH of coconut water), 16° brix and 1% w/w yeast nutrient for the highest yield of ethanol (8.28±0.029 % m/V). This optimum treatment combination was proceeding for next levels of acetic acid fermentation 20% w/w 1:1 ratio of coconut water mother (solid scum) and liquid culture (unpasteurized coconut water vinegar) was most efficient in producing acetic acid (within 10 days 4.02±0.21 % m/V total acidity as acetic acid) in submerged batch fermented condition compare to other two inoculums (20% w/w liquid culture and 20% w/w coconut water vinegar mother). Descriptive sensory evaluation of products shows that there is a significant (at 95% CI) acceptance in terms of aroma, taste, astringency and overall acceptability to the coconut water vinegar made with 20% w/w 1:1 ratio of coconut water mother (solid scum) and liquid culture (unpasteurized coconut water vinegar) compare to other two.*

Keywords: coconut water vinegar, ethanol and acetic acid fermentation, Taguchi's factorial design, sensory evaluation

1. Introduction

Coconut water vinegar is defined as 'coconut vinegar produced by the alcoholic and acetous fermentation of the coconut water (enriched with sucrose) from mature nut of the coconut palm (*Cocos nucifera*) [1].

Sri Lanka is the fourth largest coconut producing country in the world and average annual production is around 2870 million nuts and 30% of country's production is for the industries [2]. Since Sri Lanka produce of desiccated coconut is around 50000 MT per year, over 60 million liters coconut water discarded factories annually.

Even though production of coconut water vinegar is beneficial to utilize wasting coconut water, the production of coconut water vinegar is unfavorable among the manufacturers due to long fermentation time (12-15 weeks).

This study was carried out in order to identify most efficient coconut water vinegar production process having shorter fermentation time (cycle time) as well as yielding a high concentration of acetic acid and to conform it to SLS 168: 1999-Specification for Coconut Vinegar.

Taguchi's design of experiment (factorial design) was used to optimize the parameters of first phase of fermentation (Ethanol fermentation). Then the best treatment combination was proceeding to second phase of fermentation (acetic acid fermentation). Acetic acid fermentation was conducted to elucidate the effectiveness of 20% m/m of *Acetobacter* liquid culture (unpasteurized coconut water vinegar having 3.75% m/V acetic acid, 1.20×10^7 CFU/ml), Coconut water vinegar mother (solid scum, 4.08 % m/V acetic acid, 2.94×10^7 CFU/ml) and 1:1 of liquid culture and coconut water mother scum. Fresh coconut water (from mature coconut) was collected from selected local suppliers.

2. Literature Review

Vinegar
Vinegar is defined as "a liquid fit for human consumption, produced from a suitable raw material of agricultural origin, containing starch, sugars, or starch and sugars by the process of double fermentation, and acetous, and contains a specified amount of acetic acid" [3].

Its color and aroma are greatly dependent on the material from which it is made [4]. Natural vinegar is a better food additive than synthetic vinegar as it carries essential amino acids from its fruit source and is reported to act as medicine for many illnesses.

Production of vinegar
Grape, apple, and other fruit juices are the primary starting materials used for vinegar production although rice vinegar, malt vinegar, and beer vinegar are also produced in some countries [5].

The transformation of wine or fruit juice to vinegar is a chemical process that the production of vinegar involves two types of biochemical reactions: alcoholic fermentation and oxidation of alcohol in to acid. Alcoholic fermentation of carbohydrate is the first critical step in the production of vinegar and takes place under anaerobic condition. In this step sugar is fermented to alcohol by the action of yeast species as follows.

$$C_6H_{12}O_6 \xrightarrow{\text{Yeast}} 2C_2H_5OH + 2CO_2 + \text{Energy}$$
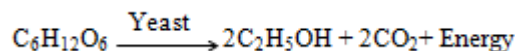
Oxidation of alcohol to acid is the second major step in the production of vinegar and is aerobic process. In this step alcohol is oxidized to acetic acid by the action of acetic bacteria; the species of *Acetobacter* [6].

The major raw materials for the production of vinegar are alcohol containing liquid, *Acetobacter*, a genus of aerobic

bacteria, oxygen, and sometimes herbs and fruits as a flavoring agent.

Alcohol containing liquid

Vinegar can be made from a variety of diluted alcohol products, the most common being wine and beer. Alternatively, an alcohol product can be prepared through fermenting carbohydrate in rice, sugar cane, Coconut water, coconut toddy or malt anaerobically by yeast.

Bacterial cultures

Acetobacter aceti cultures, perfectly work at a temperature of 28°C (82°F) with full air injection are used for vinegar production. The lowest temperature, bacteria can tolerate is 20°C (68°F) and the maximum temperature is 33°C (91°F). The starting alcohol should be lower than 7.5 % (v/v) and there should be no free Sulfites. In the natural processes, *Acetobacters* are allowed to grow over time. However, mother of vinegar is added as a source of *Acetobacter* for commercial production. Mother of vinegar is the gooey film that appears on the surface of the alcohol product as it is converted to vinegar. Mother of vinegar is skimmed off the top and added to subsequent batches of alcohol to speed the formation of vinegar. It consists natural carbohydrate called cellulose and this film holds the highest concentration of *Acetobacter*. Sometimes in the vinegar factory acetozym nutrients are added in to the alcohol liquid as a bacterial culture. Acetozym nutrients are manmade powdered form of mother of vinegar [7].

Flavoring agent

Herbs and fruits are often used to flavor vinegar. Commonly used herbs include tarragon, garlic, and basil. Popular fruits include raspberries, cherries, pine apple, lime, grapes and lemons

Processing methods of vinegar

Several methods of vinegar production exist but primarily two methods are used commercially. The first is a traditional method classified as a "surface method" in which the culture of AAB grows on the surface of wood shavings and provides oxygen at the surface. The second method, classified as a "submerged culture" is a method in which oxygen is supplied in fermentation to accelerate industrial production.

Commercial vinegar is produced either by fast or slow fermentation processes. In the slow, or natural, process, vats of cider are allowed to sit open at room temperature. During a period of several months, the fruit juices ferment into alcohol and then oxidize into acetic acid. The longer fermentation period allows for the accumulation of a nontoxic slime composed of acetic acid bacteria and soluble cellulose, known as the mother of vinegar.

Fast methods add mother of vinegar (i.e., bacterial culture) to the source liquid before adding air using a venturi pump system or a turbine to promote oxygenation to obtain the fastest fermentation. In fast production processes, vinegar may be produced in a period ranging from 20 hours to three days.

Coconut water vinegar

Coconut water is technically liquid endosperm, forming small quantities in the third month of coconut maturation and reaching a maximum in eight months, declining as the nut ripens. It is a faintly turbid to clear liquid, colorless, sweet, naturally flavored and slightly acidic with reported pH ranging from 4.0 to 6.0 [8].

Coconut water is a perishable product, after nut opening on contact with the atmospheric oxygen, coconut water leads to reduction in enzymes and external microbial contamination [9].

Coconut vinegar is made from fermented coconut water or coconut toddy. Coconut vinegar is also made from the sap of the coconut tree and is similar to the fresh coconut water. Naturally fermented coconut vinegar is rich in minerals and vitamins such as Beta carotene, calcium, iron, magnesium, phosphorous, potassium and sodium. Raw, unfiltered organic coconut vinegar is similar to the one that is fermented naturally. Coconut vinegar helps in digestion and improves the quality of cooked meat and fish [10].

3. Materials and methods

3.1 Application of Taguchi's Design of Experiment factorial design- L8 orthogonal array) to optimize ethanol fermentation condition

Materials
Coconut water
Fermenting yeast (Saccharomyces cerevisiae)
- Thermo tolerant alcoholic yeast (Angel yeast Co.Ltd, China Single culture of **Saccharomyces cerevisiae** with emulsifying agent (E491), TPC$<2.3\times10^5$ CFU/g)
- Bravo instant yeast (China, TPC$< 1\times10^5$ CFU/g, wild yeast$< 1\times10^4$ CFU/g)

Refined sugar (148IU, Thailand cane refined sugar, Thailand)

Yeast nutrient (Wyeast Yeast Nutrient from Homebrew.org, USA. A blend of vitamins, minerals, Inorganic nitrogen, organic nitrogen, zinc, phosphates and other trace elements) pH regulator

Method
The fractional factorial design a standard L_8 orthogonal array was employed. Each raw of the matrix represents one treatment combination. The factors and their levels were assigned in table 1 and Table 2 shows the standard L_8 orthogonal array.

Table 1: Factors and their levels for design of experiment

Notation	Factors	Low level	High level
A	Type of the yeast	A_1=Baker's yeast	A_2= Brewer's yeast
B	Concentration of yeast nutrient	B_1=0%	B_2=1%
C	pH of the medium	C_1=4.97	C_2=3.2
D	Brix value of the medium	D_1=12^0	D_2=16^0

Interactions of AB, and BC also were considered. Eight treatment combinations were prepared according to above orthogonal array and repeated three times. Static anaerobic condition was maintained throughout the incubation period of 48hrs.

Analysis of output array

Mean Yield of ethanol v/v percentage after 2 days of fermentation is considered as output array determined using Dujardin-salleron ebulliometer (LDS model 360, France).

Table 2: L_8 Orthogonal array (using Minitab version 17 statistical software)

Treatment	Factors			
	1 A	2 B	4 C	7 D
1	1	1	1	1
2	1	1	2	2
3	1	2	1	2
4	1	2	2	1
5	2	1	1	2
6	2	1	2	1
7	2	2	1	1
8	2	2	2	2

The MINITAB version 17 (Minitab Inc., PA, United States) was used to perform one-way analysis of variation (ANOVA) to evaluate statistical significance (level of $p < 0.05$) of differences between the treatments and to compare the means among the samples and S/N ratio analysis. Also, response curves were drawn for individual factors and interactions and the Y (Dependent Variable-Ethanol v/v percentage) optimum was calculated.

3.2 Optimizationofaceticacidfermentation

Then the best treatment of ethanol fermentation was employed to prepare alcoholic seed broth for Acetic acid fermentation and study on the inoculum type for acetic fermentation was performed to identify the most efficient inoculum for the acetic acid production in coconut water medium.

Materials

Fermented alcoholic broth
Acetic acid bacteria culture (*Acetobacter aceti*)
- *Acetobacter* liquid culture (Unpasteurized coconut water vinegar with 3.75% m/V acetic acid, 1.89×10^7 CFU/ml)
- Coconut water vinegar mother (solid scum, 4.08 % m/m acetic acid, 2.86×10^7 CFU/ml)
- 1:1 of liquid culture and coconut water mother scum

Method

The acetic acid fermentation was proceeded by inoculating 20% m/m of *Acetobacter* liquid culture, Coconut water vinegar mother and 1:1 of liquid culture and coconut water mother scum in to separate ethanol fermented coconut water seed broths (four samples for each) and incubated at the ambient temperature under aerobic condition for a month where the vessels were connected to an aeration system with pressure of 0.1 MPa for 5 min.

This aeration was designed to aerate with normal atmospheric air in to the vessels with 8 min intervals. During the fermentation period samples were withdrawn at 2 days interval to quantify total acidity in terms of acetic acid through titration assay, brix value (total soluble solids), and pH value of the medium, and Total & viable cell count of Acetic Acid Bacteria was monitored within 3 days of intervals.

Total soluble solid content was determined usingpotable refractometer (Atago® M, 0.00-32.0, USA) and expressed as Brix degree indicating the mass in gram of dry matter 100 g juice. The measurements were made in triplicate for each sample.

The pH of the fermenting medium was measured with a digital display pH meter. (HANNA, HI-8314, France)

Determination of ethanol concentration of fermented broth was carried using Dujardin-salleron ebulliometer (LDS model 360, France).

Determination of Acetic acid degree was carried out by titration assay using standardized sodium hydroxides as indicator where phenolphthalein was used as acid base indicator and expressed as Total acidity, as acetic acid g per 100 ml = $0.6 \times X \times V$. Where, V is the volume in ml, of sodium hydroxide required for the titration. X is the concentration in dm^{-3}, of standardized sodium hydroxide solution.

Determination of Viable and Total Cell Count (Total viable count total plate count) which gives a quantitative idea about the presence of microorganisms such as bacteria, yeast and mold in the sample. Results were expressed as colony forming units (CFU) per ml of the medium (30-300 numbers of colonies were considered as countable range).
Finally Counting of microorganisms was done using digital colony counter.

3.3 Sensory evaluation of finished products

Coconut water prepared using different *Acetobacter* bacteria cultures was subjected to descriptive sensory analysis to evaluate more comprehensive profile of vinegars.

Mixed pickle was used to test the basic taste and the astringency (mouth feel after tasting) of the products.

As total acidity of coconut water vinegar prepared using different *Acetobacter* bacteria cultures was identified through titration assay, ranking test was carried out to verify whether the sour taste of the product increases with increment of total acidity of the product.

ISO 8587:2006 (sensory analysis methodology -ranking) standard was referred to develop ranking test. 30 untrained Panelists were asked to consume same amount of vinegar (full of a tea spoon) and to allow the product to be retained in the mouth for few second before swallow.

3.4 Finished product quality testing

Finished product from each Acetic acid fermentation was tested to ensure its conformity to SLS specification for coconut vinegar (second revision) SLS 168: 1999 by Sri Lanka Standards Institution. Minimum Total acidity as acetic acid, Minimum Total solids g per 100 ml, Minimum permanganate oxidation value, iodine value, maximum residual ethyl alcohol percent by volume was tested according to the methods specified in particular standard.

4. Results and Discussion

Used coconut water was with 4^0 Brix, pH 4.97, 95.967± 0.0569% moisture and 4.0333± 0.0569 % of total dry matter. According to the literature mature coconut water is with 94.45% of moisture and 5.55 % of total dry matter which is most similar to observed results.

4.1 Application of Taguchi's Design of Experiment (factorial design- L_8 orthogonal array) to optimize ethanol fermentation condition

Eight experiments were carried out according to the L_8-OA and each experiment was repeated three times in order to reduce experimental errors

The observed results (Taguchi array response) were tabulated in table 3.

Table 3: L_8-OA response values and S/N ratio for yield of ethanol[a]

Treatment (Run)	Factors				Results (ethanol concentration %)			Average Ethanol %	S/N ratio
	1	*2*	*4*	*7*	*1*	*2*	*3*		
	A	*B*	*C*	*D*					
1	1	1	1	1	5.45	5.35	5.45	5.42±0.058	39.4459
2	1	1	2	2	3.45	3.45	3.35	3.42±0.058	35.4433
3	**1**	**2**	**1**	**2**	**8.30**	**8.30**	**8.25**	**8.28±0.029**	**49.1559**
4	1	2	2	1	5.15	5.15	5.10	5.13±0.029	44.9998
5	2	1	1	2	8.05	8.00	8.00	8.02±0.029	48.8717
6	2	1	2	1	3.05	3.10	3.15	3.10±0.050	35.8478
7	2	2	1	1	5.70	5.65	5.70	5.68±0.029	45.8839
8	2	2	2	2	5.70	5.65	5.75	5.70±0.050	41.1381

[a]Experimental results were the average of three replicates
Mean± Standard Deviation

Analysis of output array

Analysis of the S/N ratio
In the Taguchi method, the term 'signal'(S) represents the desirable value (mean) for the output characteristics and the term 'noise' (N) represents the undesirable value for the output characteristics. Taguchi uses the S/N ratio to measure the quality characteristic deviating from the desired value. When consider about this experimental analysis higher is the better (HB) because higher yield of alcohol is desirable.

A quality characteristic of the higher is better was calculated according to the following equation:

$$\frac{S}{N} = -10 \, log_{10} \left[\frac{1}{n} \sum \frac{1}{y^2} \right]$$

Where *n* is the number of measurements in a trial/row and *y* is the measured value in a run/row. The S/N ratio values were listed in Table 3 for parameters of yield of ethanol in percentage.

Table 4 shows the response table for S/N ratio of yield of ethanol in percentage for "higher is better" obtained for different parameter levels.

Table 4: Response Table for Signal to Noise Ratios Larger is better

Level	A	B	C	D
1	14.48	13.31	**16. 5**	13.45
2	**14.53**	**15.70**	12.45	**15.56**
Delta	0.05	2.38	4.10	2.11
Rank	4	2	1	3

The analysis of S/N ratio of yield of ethanol in v/v % revealed, that the first factor that causes parameter ethanol in percentageto be great is the type of the yeast, Brix of the medium, Concentration of yeast nutrients and pH of the medium respectively.

Regardless of the category of the performance characteristics, a greater S/N value corresponds to a better performance.

Therefore, the optimal level of the ethanol fermenting process parameters is the level with the greatest S/N value. Based on the analysis of the S/N ratio, the optimal process conditions for alcoholic fermentation was obtained with brewer's yeast type (Level 2) at 1% yeast nutrients (Level 2), natural pH of the coconut water medium (level 1) and Brix of the medium was 16^0 (Level 2).

Main effect plot

Main effect plot for the main effect terms viz. factors A, B, C and D are shown in figure 4.2. From the main effect plots, it has been observed that yield of ethanol in percentage increases with changing of yeast type from baker's yeast to brewer's yeast due to their activity on substrates.

Also yield of ethanol increases due to addition of yeast nutrient rich with vitamins, minerals, Inorganic nitrogen, organic nitrogen, zinc, phosphates and other trace elements which are essential for the growth of *Saccharomyces cerevisiae*. When the pH of the medium decreases to 3.2 using mineral acid (hydrochloric acid) yeast activity

retarded resulting low yield of ethanol. Yield of ethanol increased with increment of Brix value (total soluble solids) of the medium due to increment of the amount of substrate available for fermentation.
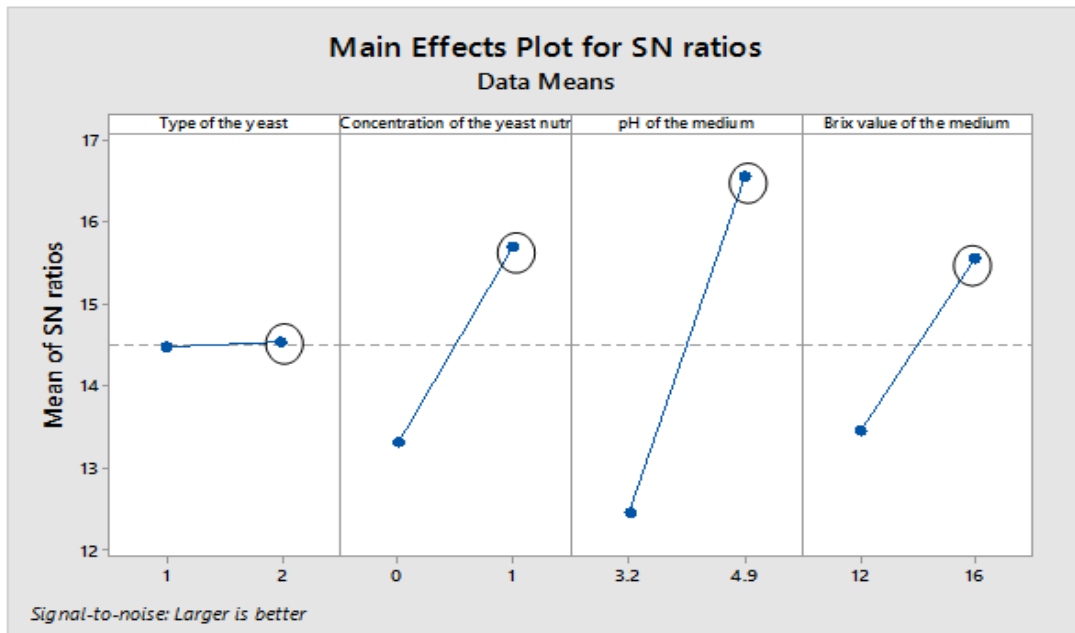

Figure 1: Main effect plot

Interaction plot

Whether interactions between factors exist or not can be shown by plotting a matrix of interaction plot Parallel lines in an interaction plot indicate no interaction. However, the interaction plot doesn't tell if the interaction is statistically significant. Interaction plot visualized the interactions during the design of experiment. Matrix of interaction plot for yield of ethanol in percentage shown in figure 4.3.

It can be visualized that there are no parallel lines between Type of the yeast (A) and Concentration of yeast nutrient (B), type of the yeast(A) & pH of the medium (C) and B & C. It means all three interactions show significant interaction for yield of ethanol in percentage.
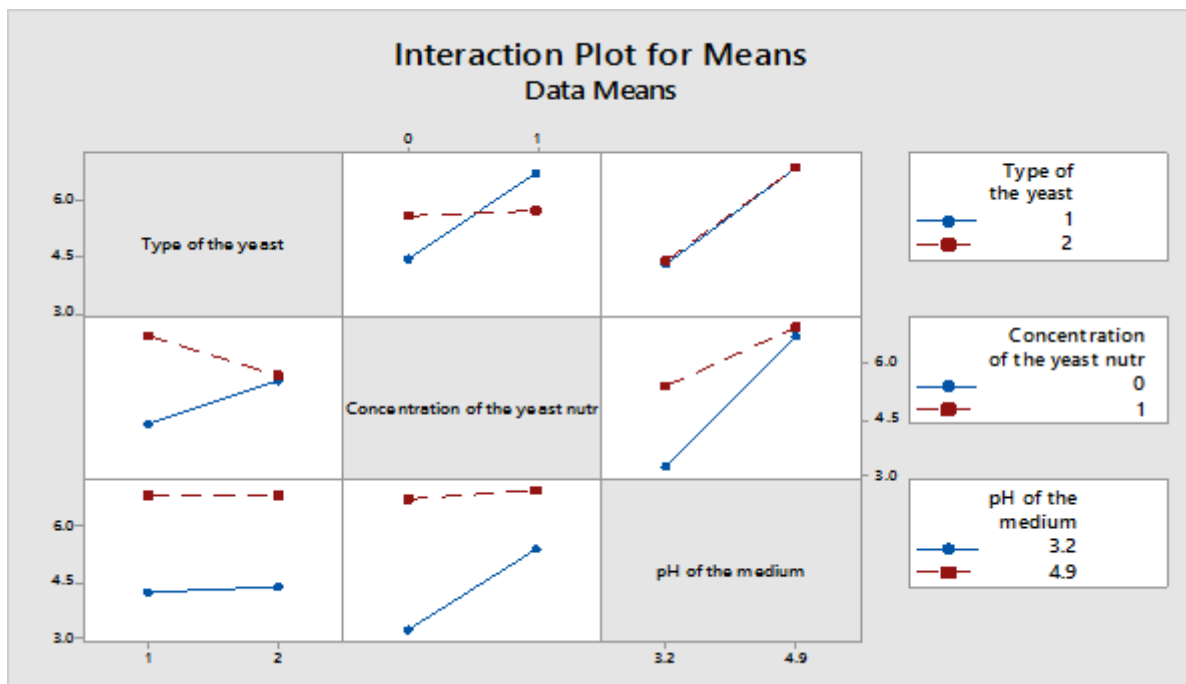

Figure 2: Interaction plot

Analysis of Variance for Means

ANOVA is a statistically based objective decision-making tool for detecting any differences in the average performance of groups of items tested. ANOVA helps in testing the significance of all main factors and their interactions by comparing the mean square against an estimate of the experimental errors at specific confidence levels.

Table 4: ANOVA output

Source	DF	Seq SS	Adj SS	Adj MS	F	P
A	1	0.0078	0.0078	0.0078	1.00	0.500
B	1	2.9403	2.9403	2.9403	376.36	0.033
C	1	12.6253	12.6253	12.6253	1616.04	0.016
D	1	4.6259	4.6259	4.6259	592.11	0.026
A*B	1	2.3292	2.3292	2.3292	298.14	0.037
B*C	1	1.7892	1.7892	1.7892	229.02	0.042
Residual Error	1	0.0078	0.0078	0.0078		

The P-value reports the significance level (suitable and unsuitable) in Table 10. The Percent (%) is defined as the significance rate of the process parameters on the yield of alcohol in percentage. The percent numbers depict that the Concentration of yeast nutrient (B), pH of the fermenting medium(C), and soluble sugar content of the medium (D), have significant effects on the yield of alcohol (%) at 5% significant level. Also, the interactions between A*B and B*C has significant effect on the output array at 5% significant level.

Factor A (type of the yeast) was not show significant effect on ethanol fermentation at 5% significant level.

A confirmation of the experimental design was necessary in order to verify the optimum alcoholic fermentation conditions.

Theoretical conformation of results

The confirmation test was the final step in verifying the results drawn based on Taguchi's design approach.

Theoretical confirmation by calculating,

Y $_{optimum}$ for main effects (without considering interactions)

$$\bar{T} = \frac{Total\ of\ output\ array}{Number\ of\ experiments}$$

According to the experiment results the best combination of four factors are $A_2B_2C_1D_2$, which gives maximum out puts.

$Y_{opt} = \bar{T} + (\overline{A_2} - \bar{T}) + (\overline{B_2} - \bar{T}) + (\overline{C_1} - \bar{T}) + (\overline{D_2} - \bar{T})$
$Y_{opt} = (\overline{A_2} + \overline{B_2} + \overline{C_1} + \overline{D_2} - 3\bar{T})$
$Y_{opt} = (5.6250 + 6.1999 + 6.8500 + 6.3542 - (3*5.5938))$
$Y_{opt} = 8.2480$

Y$_{optimum\ calculated}$ without considering interaction is 8.2480, but according to interaction plot for mean values of main effect and according to the ANOVA A*B and B*C interactions are significant and as factor A (Type of the yeast) is insignificant it was kept in economical level A_1 (Bravo Yeast).

Y with considering interactions

$Y_{opt} = \bar{T} + (\overline{A_1B_2} - \bar{T}) + (\overline{B_2C_1} - \bar{T}) + (\overline{D_2} - \bar{T})$
$Y_{opt} = (\overline{A_1B_2} + \overline{B_2C_1} + \overline{D_2} - 2\bar{T})$
$Y_{opt} = (6.70833 + 6.9833 + 6.8500 - (2*5.5938))$
$Y_{opt} = 20.0458 - 11.1875$
$Y_{opt} = 8.8583$

According to the findings treatment number three gives maximum ethanol yield (in %) and with treatment combination $A_1B_2C_1D_2$. This combination was employed to improve the ethanol fermenting phase for further analysis.

4.2 Optimization of acetic acid fermentation

Study on the inoculum type for acetic fermentation was performed to elucidate its effect on the acetic acid production in coconut water medium. Inoculation was done as below mentioned three types also for easy presenting notations were introduced as below.

Type of the inoculums (20% m/m)	Notation
Acetobacter liquid culture (3.75% m/V acetic acid, 1.2×10^7 CFU/ml)	A
Coconut water vinegar mother (solid scum, 4.08 % m/V acetic acid, 2.94×10^7 CFU/ml)	B
1:1 of liquid culture and coconut water mother scum	C

The initial concentrations of ethanol prior acetic acid fermentation in all samples were 8.63% v/v with the pH of 3.98

During acetic acid fermentation, ethanol will be oxidized to acetic acid by two step reactions, the oxidation of ethanol to acetaldehyde, followed by the oxidation of acetaldehyde to acetate.
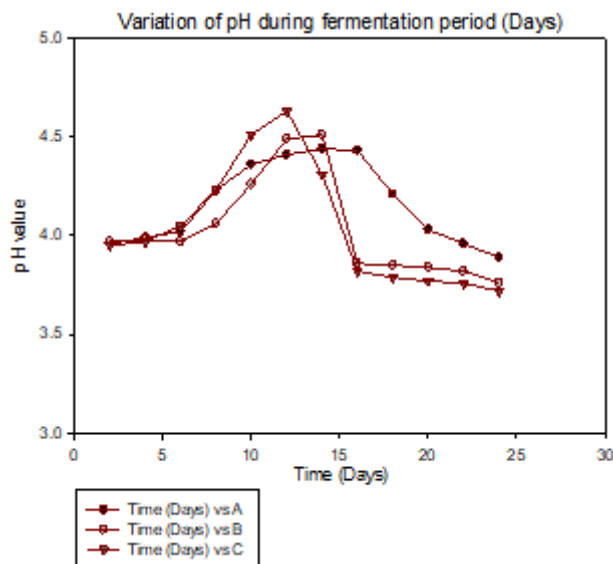

Figure 3: Variation of pH during fermentation period (Days)[b]
[b]Average values were plotted

4.2.1 Determination of pH of acetic acid fermenting broth

Figure 3 clearly illustrates the pH variations of the acetic acid fermenting broths with the time (days). pH of all three samples has found to be decreased to a desired range during

the fermentation process. The decrease in pH value during alcoholic fermentation may be due to the presence of carbon dioxide and hydrogen ions **(Ning, 2008).** Yeast transformed sugar present in coconut water into alcohol and carbon dioxide gas during alcoholic fermentation. First phase of fermentation reduces the pH from 4.97 to 3.99. after introducing of acetic acid inoculums pH of the three mediums was dropped down to 3.96±0.02, 3.97±0.09, 3.95±0.078 respectively in A, B, and C.

The acidity of coconut water vinegar in the second stage of fermentation process was most probably due to the presence of organic acids. The increasing concentration of acetic acid caused the lower pH in vinegar.

During the second phase of fermentation the pH variations in fermenting broths lie in between 3.72 to 4.63. The maximum range was observed in liquid: scum 1:1 culture medium. The pH changes become stable at the end of fermentation was due to the production of acetic acid was reducing.

Fermenting broth which inoculated with *Acetobacter* liquid culture (unpasteurized coconut vinegar) is with the lowest pH change from 3.96±0.02to 3.89±0.07.

pH of finished products was 3.89±0.07, 3.76±0.17 and 3.72±0.04 respectively in A, B, and C.

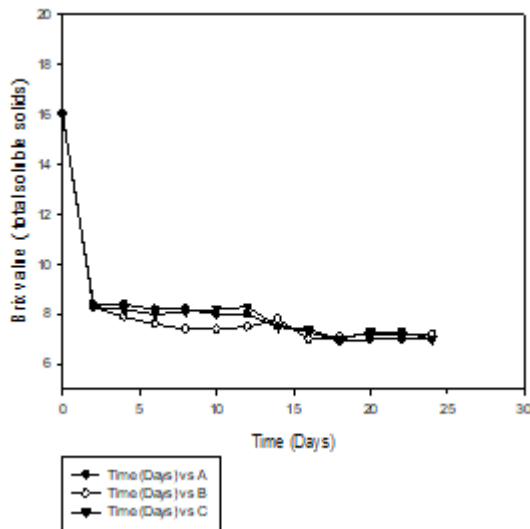### 4.2.2 Determination of total soluble solids of acetic acid fermenting broth

Figure 4: Brix value variation during fermentation period (days)[c]
[c]Average values were plotted

Brix values donate the total soluble solids in the medium. As fermentation progress Brix value decreases due to utilization of carbohydrate and other soluble nutrients by yeast and *Acetobacter aceti* bacteria. Initial 16^0 brix of the medium rapidly decreases down to 8.3 within 2 days of alcoholic fermentation Producing 8.25% v/v ethanol. During this period *Saccharomyces cerevisiae* yeast strain will convert sugars prominently sucrose in to ethanol by anaerobic fermentation.

Brix value variation during the acetic acid fermentation was with decreasing trend; it was decreasing slightly and eventually got constant value in all three types of inoculums. During acetic acid fermentation *Acetobacter* bacteria oxidize the ethanol into acetic acid. This conversion is not affect to the total soluble matter in the broth. However, yeast which survives in the medium has the ability to utilize carbohydrate sources which responsible for total soluble solids.

The final brix values of the vinegars are 7.0, 7.2, and 7.5 respectively in A, B and C.

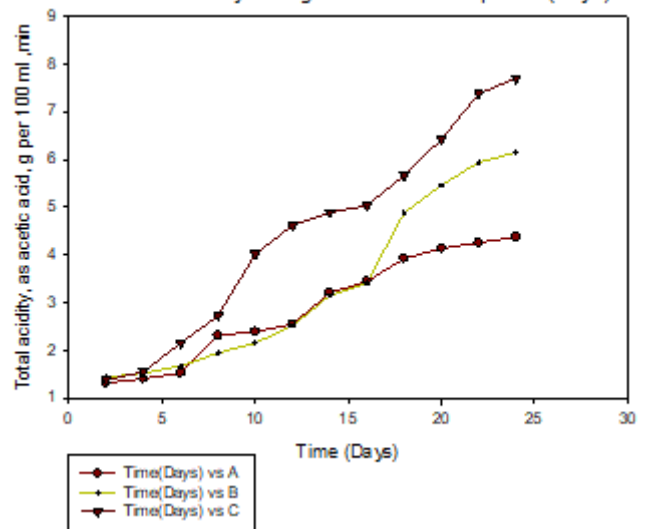### 4.2.3 Determination of total acidity of acetic acid fermenting broth

Figure 5: Variation of Total acidity during fermentation period (days)

According to the SLSI specifications for coconut vinegar, coconut vinegar must have minimum total acidity as acetic acid % m/V 4%. Total acidity of the samples showed an increasing trend and sample with liquid: scum 1:1 reach to this expected level (>4%) within 10 days of acetic acid fermentation while sample with liquid culture took longest time of

So, liquid: scum 1:1 acetic acid culture was more efficient in producing acetic acid compare to two others. At the end of 24 days of acetic acid fermentation A, B, and C fermenting broths reach to 4.3875± 0.0512, 6.0975±0.0709 and 7.7025± 0.037 of total acidity as acetic acid % m/V respectively.

4.2.4 Determination of total and viable cell count of acetic acid fermenting broth
Total and viable cell count gave a quantitative idea about presence of microorganisms in the vinegar samples.

The growth of microorganisms is a highly complex and coordinated process, ultimately expressed by increase in cell number or cell mass. The process of growth depends on the availability of requisite nutrients and their transport into the cells, and the environmental factors such as aeration, O_2 supply, temperature and pH. Specific culture media *Acetobacter* agar (glucose) M238-Himeida which facilitates the growth of acetic acid bacteria was used. *Acetobacter*

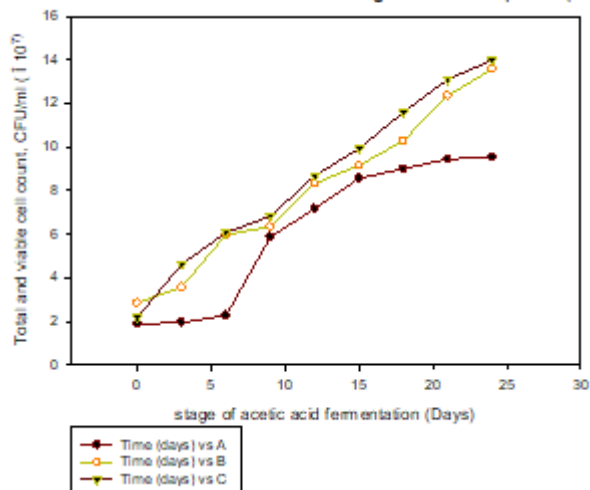agar is with yeast extract, calcium carbonate, glucose and agar.

Figure 6: Variation in total & viable cell count during fermentation period (Days)

Acetobacter species are aerobic, gram negative organisms. Acetic acid bacteria are found in vinegar oxidizes ethanol to acetic acid. Various synthetic and maintenance media for *Acetobacter* cultures have been cited. A typical maintenance medium, *Acetobacter* Agar is formulated as per Manual of Microbiological Methods and used for the maintenance of *Acetobacter* species utilizing glucose. Yeast extract in the medium provides nitrogen, vitamins and minerals necessary to support bacterial growth. Glucose acts as energy source. Calcium carbonate acts as a buffer.

Average colony count in CFU/ml was presented in above figure 4.7. Total viable cell count in all three samples shows an increasing trend. Highest cell count was observed in sample with 1:1 scum: liquid culture, which is with highest total acidity as acetic acid g per 100ml. final colony count was reported as 9.525×10^7, 13.58×10^7, 9.925×10^7 in liquid culture, Scum culture and 1:1 scum: liquid culture respectively. Lowest colony count was observed with liquid cultured sample and it showed an exponential increment of microorganisms during 6 to 9 days. Also, colony count reach to constant after 21 days of fermentation under batch fermenting condition.

Microbial Growth in Batch fermenter is associated with,
- Lag Phase
- Exponential Phase
- Stationary Phase and
- Death Phase

Liquid culture and scum culture was in lined with this microbial growth in batch fermenter but liquid: scum 1:1 culture showed a deviation from it. During this fermentation period liquid culture has reached to the stationary phase after its 20 days of fermentation.

4.3 Characteristics of finished products according to SLSI standards

Finished products were tested in accordance with SLSI Specification for coconut vinegar (third revision) SLS 168:1999

Table 5: Finish product quality characteristics according to SLS 168: 1999

Characteristic	SLS requirement	Finished product results		
		A	B	C
Total acidity as acetic acid g per 100 ml, minimum	04	4.3875± 0.0512	6.0975±0.0709	7.7025± 0.037
Total solids, g per 100 ml, minimum	01	3.4133 ±0.1007	3.4644±0.0752	3.4153±0.021
Permanganate oxidation value, minimum	750	2560.0±4.00	2690.7±2.31	2700.0±4.00
Alkaline oxidation value, minimum	80	169.87±0.462	184.00± 0.800	152.53± 0.462
Iodine value, minimum	160	170.67 ±2.31	293.33 ±4.62	285.33± 2.31
Residual ethyl alcohol, percent by volume, maximum	01	0.87	0.80	0.66

[d]Experimental results were the average of three replicates
Mean value± Standard Deviation

All three coconut vinegar products were complied with SLS specification for coconut water vinegar SLS 168: 1999. But total acidity of Pineapple flavored coconut water vinegar was lower than specified level of 4%.

4.4 Sensory evaluation

Descriptive analysis
Three coconut water vinegar samples were subjected to descriptive analysis on Appearance, basic taste (sour), aroma, astringency (mouth feel)and overall acceptability.

Statistical analysis
Selected data were analyzed using non-parametric analysis (kruskalwallis test method) as untrained sensory panel was used. Total number of panelist was 30.

392- Vinegar prepared with liquid culture

927- Vinegar prepared with solid coconut water vinegar mother (scum)
458- Vinegar prepared with liquid: scum 1:1 ratio

M_1 = median of sample 392
M_2 = median of sample 927
M_3 = median of sample 458

H₀ (null hypothesis)= There is no significant difference between samples ($M_1=M_2=M_3$)

H₁ (alternative hypothesis) = At least one sample median is significant compare to others corresponding to particular sensory attribute (appearance, aroma, taste, astringency, and overall acceptability) ($M_i \neq M_j$ where $i \neq j$)

At 95 % confidence level p values for appearance, aroma, taste, astringency and overall acceptability were 0.005,

0.003, 0.000, 0.000, 0.000 (α = 0.05) respectively. As all P value < α value H_o rejected and H_1 accepted, means at least one sample median is significant compare to others corresponding to particular sensory attribute (appearance, aroma, taste, astringency, and overall acceptability)

To find out which sample is odd or where there is a significant difference between all three samples non-parametric paired comparison for independent samples were carried out according to Mann-Whitney U test.
Results were summarized in below table 6.

Table 6: Mann Whitney U test results

Sensory attribute	P values for Sample combination			Conclusion (at 95% CI)
	392:927	392:458	927:458	
Appearance	0.0492	0.0023	**0.0863**	(M1<M2=M$_3$)
Aroma	**0.4077**	0.0014	0.0103	(M$_1$=M$_2$<M$_3$)
Taste	0.0392	0.0000	0.0006	(M$_1$<M2<M3)
Astringency	**0.0736**	0.0000	0.0042	(M$_1$<M$_2$=M$_3$)
Overall acceptability	0.0034	0.0000	0.0000	(M$_1$<M$_2$<M$_3$)

According to the statistical analysis it is proven that three samples prepared using different inoculums were with different consumer acceptances. In term of appearance samples made with scum: liquid 1:1 and scum has similar acceptance which is higher than sample made with liquid culture.

When consider about aroma attribute sample with scum: liquid 1:1 has superior acceptance than other two. Astringency of sample with liquid culture is lower than other samples.

Sample with Liquid: scum 1:1 has superior taste and overall acceptability compare to other two. Also, taste is the most significant attribute as p value is smaller than other attributes.

Lowest consumer overall acceptance is with product made with liquid culture while highest acceptance is with product made with liquid: scum 1:1 culture.

Sensory evaluation also shows that product made with liquid: scum 1:1 culture is superior same as total acidity.
- **Graphically representation**

Table 7: Average values of sensory attributes

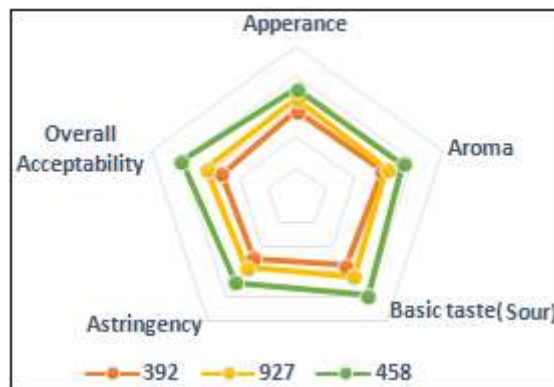| Sample No:	Sensory attribute				
	Appearance	Aroma	Basic taste (Sour)	Astrin-gency	Overall Acceptability
392	2.87	2.94	2.74	2.42	2.61
927	3.26	3.10	3.16	2.81	3.13
458	3.61	3.68	3.97	3.42	4.00

Figure 7: Web diagram

Web diagram illustrates that sample made with liquid: scum 1:1 is with more superior sensory characteristics compare to other two. However, any sample is not attained extreme likeness as the average maximum response were 4.00 for overall acceptance of sample made with liquid: scum 1:1 ratio.

Ranking test results
To determine if a difference exists between three samples with regard to sour taste as they are having different amount of total acidity as acetic acid per 100ml of the sample.

Sour taste of the product mainly depends on the acids present in the samples.
α = 0.05 S = 25.40 DF = 2 P = 0.000

Sum of Treatment	N	Est. Median	Ranks
392	30	1.3333	41.0
458	30	2.6667	80.0
927	30	2.0000	59.0

Grand median = 2.0000

The Friedman test, which evaluated differences in medians among the three vinegars, was significant X^2 (2, *N* = 30) = 25.40, *p* < 0.000.

Next, follow-up tests were conducted to evaluate comparisons between pairs of medians and used the Wilcoxon signed test at 95 % confidence level.

P value (0.000) is lower than α value (0.05) so H_o was rejected and H_1 was accepted. At least median of one sample is different from others.

Table 8: Wilcoxon signed rank test results

Sample pairs	P value	Conclusion (95% CI)
392-927	0.014	H_o rejected & H_1 accepted
392-458	0.000	H_o rejected & H_1 accepted
927-458	0.005	H_o rejected & H_1 accepted

The Least Significant Difference (LSD) procedure controls adequately for Type I error across pair-wise comparisons if there are three levels and the overall test is significant. In this ranking test for vinegar all three comparisons (392-458, 392-927, and 927-458) were significant at the 0.05 alpha level.

Based on the results produced from the statistical analysis, the median sour taste of vinegar sample made out with liquid: scum 1:1 ratio culture was significantly greater than the median for vinegar sample made out with liquid culture, $p < 0.000$, and the median for vinegar sample made out with solid scum, $p < 0.005$, also the median of vinegar sample made out with scum culture was significantly greater from the vinegar sample made out with liquid culture $p = 0.014$.

Amount of acid present in the samples made significant impact on the taste of vinegar. And sour taste of the samples can be line up from highest to lowest like below, 458> 927> 392 same as acetic acid present in the samples also decreases respectively.

5. Conclusion

Best inoculum size (baker's yeast) for alcoholic fermentation in 16° Brix (total soluble solids) coconut water medium was 1% w/w. and the maximum output ethanol concentration was 6.1375 ± 0.0479 %v/v.

According to the Taguchi's factorial design (L_8 orthogonal array) the best combination for ethanol fermentation was baker's yeast, at pH of natural coconut water, and 16^obrix, with 1% w/w Wyeast Yeast Nutrient from Homebrew.org, USA (a blend of vitamins, minerals, Inorganic nitrogen, organic nitrogen, zinc, phosphates and other trace element) under that conditions maximum theoretical ethanol yield was 8.8583 and maximum practical ethanol yield was **8.28±0.029 % v/v.**

Best inoculum for acetic acid fermentation was 1:1 ratio of acetic acid mother (Solid scum): liquid culture which gave >4% acidity within 10 days of fermentation.

6. Acknowledgement

References

[1] SLS specifications for coconut vinegar, SLS 168:1999, Sri Lanka standards institute, Sri Lanka.

[2] Department of Census and Statistics Sri Lanka, 2014.

[3] FAO/ WHO Food Standards Programme, 1987.

[4] C.L. Kehrer, "The chemistry of vinegar", Journal of Food Product and The American Vinegar Industry,1, pp.5-20, 1921. (journal style)

[5] M.R. Adam, "Vinegar in microbiology of fermented foods",New York Elsevier Applied Science Publishers,(1), pp.140-147, 1985. (journal style)

[6] I. De Ory,L.E.Romero, D.Cantero, "Maximum yield acetic acid fermenter. Comparative fed-batch andcontinuous operation studies at pilot plant scales", Bioprocess Engineering, 2(1), pp.187-190, 1999.(journal style)

[7] Y.S.Park, H.Otade, M.Fukaya, H. Okumura, Y. Kawamura, K.Toda,"Effect of dissolved oxygen and acetic acid concentrations on acetic acid Production in continuous culture of Acetobacter aceti" Journal of Fermentation Bioengineering, 68, pp.96-101, 1989. (journal style)

[8] K.K.D.P. Gunathilake, J.A.K.M. Fernando, and D.S.Jayawicrama, "Development of low cost coconut water vinegar production process and evaluation of generator process to accelerate the acetification", Coconut development board (CDB), 27 (1), 2011. (conference style)

[9] I. De Ory, L.E. Romero and D.Cantero, "Optimum starting-up protocol of pilot plant scale acetifier for vinegar production", Journal of Food Engineering, 5, pp.31-37, 2002.(journal style)

[10] D. Divina, "Processing manual of virgin coconut oil, its and by products for products for pacific island countries & territories",Journal of Food Engineering, pp.142-145, 2011. (journal style)

Author Profile

H.K.Vidulani. S Nimalsiri is a final year (2016) undergraduate student in Department of Food Science and Technology at University of Sri Jayewardenepura who is following the degree of B.Sc. (Special) in Food Science and Technology. She has successfully completed this research project under the expert guidance of M.A. JagathWansapala, Senior lecturer in Food Science and Technology who holds B.Sc. in Chemistry, M.Sc. in Food Science and Technology andPhD in Food Science.