Sains Malaysiana 46(9)(2017): 1505–1512
http://dx.doi.org/10.17576/jsm-2017-4609-20

Effects of Fermentation Time and pH on Soursop (*Annona muricata*) Vinegar Production towards its Chemical Compositions

(Kesan Masa Fermentasi dan pH terhadap Penghasilan Cuka Durian Belanda (*Annona muricata*) dan Komposisi Kimianya)

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ABSTRACT

*Vinegar is a liquid product that undergoes both alcoholic and acetous fermentation of sugar (carbohydrate) sources. Soursop (*Annona muricata*) is easily available in Malaysia throughout the year. However, it is also highly perishable and has a short shelf-life. Therefore, in this research, soursop was used in the production of vinegar, to increase its utilisation and reduce wastage. The objectives of this research were to determine the effects of fermentation time and pH on soursop vinegar using a 3 × 5 factorial design and to determine its chemical compositions. It was found that pH and fermentation time showed significant (*$p<0.05$*) effects on the reduction of sugar content and the production of acetic acid, while only fermentation time showed a significant effect on the production of ethanol. The interaction between factors did not exhibit any statistical significance (*$p>0.05$*). It was evident that the sugar concentration reduces over time and it was inversely proportional to the ethanol and acetic acid concentrations, due to the conversion of sugar to ethanol and subsequently acetic acid. It was found that higher pH (pH5.5) gave significantly (*$p<0.05$*) higher acetic acid production in the vinegar, while pH has no significant (*$p>0.05$*) effect on ethanol production. There were no significant differences (*$p>0.05$*) in vitamin C content in all vinegar samples. Thus, it can be established that at fermentation time of 120 h and pH5.5, more sugar was used and more ethanol and acetic acid were produced.*

Keywords: Acetous fermentation; alcoholic fermentation; soursop; vinegar; yeast

ABSTRAK

*Cuka merupakan produk cecair yang telah melalui proses fermentasi alkohol dan aselom pada sumber gula (karbohidrat). Durian belanda (*Annona muricata*) merupakan buah-buahan yang amat popular dan senang diperoleh di Malaysia sepanjang tahun. Walau bagaimanapun, durian belanda merupakan buah-buahan yang sangat mudah rosak dan mempunyai jangka hayat yang pendek. Oleh itu, dalam kajian ini, durian belanda telah digunakan untuk menghasilkan cuka untuk mengurangkan pembaziran serta meningkatkan penggunaannya. Objektif kajian ini adalah untuk mengenal pasti kesan masa fermentasi dan pH terhadap penghasilan cuka durian belanda dengan menggunakan reka bentuk eksperimen berfaktor 3 × 5 dan mengenal pasti komposisi kimianya. Hasil daripada kajian ini menunjukkan bahawa masa fermentasi dan pH memberi kesan yang bererti (*$p<0.05$*) ke atas kandungan gula dan penghasilan asid asetik, manakala hanya masa fermentasi memberi kesan yang bererti (*$p<0.05$*) ke atas penghasilan etanol. Interaksi antara faktor tidak menunjukkan kesan yang bererti (*$p>0.05$*). Dapat diperhatikan bahawa kepekatan gula menurun dengan peningkatan masa fermentasi dan ia adalah berkadar songsang dengan kepekatan etanol dan asid asetik. Ini disebabkan oleh penukaran gula kepada etanol dan kemudiannya asid asetik. Didapati juga pada pH yang lebih tinggi (pH5.5), penghasilan asid asetik adalah lebih tinggi secara bererti (*$p<0.05$*), tetapi nilai pH tidak memberi kesan bererti (*$p>0.05$*) pada penghasilan etanol. Kandungan vitamin C tidak menunjukkan perbezaan bererti (*$p<0.05$*) dalam semua sampel. Secara keseluruhannya, pada masa fermentasi 120 jam dan pH5.5, didapati gula paling banyak digunakan manakala etanol dan asid asetik paling banyak dihasilkan.*

Kata kunci: Cuka; durian belanda; fermentasi alkohol; fermentasi asetous; yis

INTRODUCTION

Vinegar is usually used as food preserving agent, food condiment or food enhancer or as a drink since many years ago (Tesfaye et al. 2002) and it has a long history of more than 3,000 years in China (Chen et al. 2013). Recently, there are a variety of popular vinegars that have been produced and different fermentation techniques have been developed for vinegar production in Europe and Asia (Chen et al. 2013). According to the Malaysian Food Act and Regulations (1985), vinegar is defined as a liquid product prepared from the alcoholic fermentation and subsequently acetous fermentation of any suitable food, and it shall contain not less than 4 percent (w/v) of acetic acid, and it may contain permitted preservatives, caramel as a colouring substance and spices as permitted flavouring substances.

Vinegar is claimed to have high antioxidant activity, antimicrobial and antidiabetic effects and therapeutic properties and could promote a healthier body to consumers (Budak et al. 2014). Besides that, vinegar is widely used as dressing on foods to bring beneficial effects to health, such as improved digestive system, stimulating appetite, lowering lipid levels and regulating blood pressure (Fushimi et al. 2001; Qui et al. 2010). Juices are normally inoculated with *Saccharomyces cerevisiae* to allow alcoholic fermentation occurs under anaerobic condition (Pooja & Soumitra 2013), where the conversion of table sugars to ethanol would happen (Tesfaye et al. 2002). The addition of *Acetobacter* species is the second stage of fermentation, acetous fermentation where it allows the oxidation of ethanol occur under aerobic condition (Pooja & Soumitra 2013) and therefore ethanol would be convert into acetic acid, thus, vinegar (Tesfaye et al. 2002). The quality and characteristics of vinegar are mainly influenced by the microbial diversity and its dynamic changes (Chen et al. 2013).

Soursop fruit (*Annona muricata*), also known as *durian belanda* in Malaysia is easily available in Malaysia. It is becoming more popular due to its highly aromatic juicy and distinctive flavour (Quek et al. 2013) and also reported to be a major source of antioxidants (Umme et al. 1996). Since soursop is a highly perishable fruit and easily damaged, soursop fruit is now manufactured into other form of products to prolong its shelf life (Quek et al. 2013). Undesirable pH will influence the production vinegar as the optimum pH for *S. cerevisiae* to grow is in the range of pH4.0 to pH5.5 (Narendranath & Power 2005). Besides that, fermentation time could affect the quality of vinegar or wine produced as the longer the fermentation time, the higher the ethanol production (Dung et al. 2014). Therefore, the objective of this study was to determine the effects of pH and fermentation time on soursop vinegar, and to determine its chemical composition.

Materials and Methods

Soursop Juice, Vinegar and Yeast Samples

Soursop juice was prepared in the ready-to-drink form of product by Malaysian Agricultural Research and Development Institute (MARDI). Yeast (*Saccharomyces cerevisiae*) (brand: Mauri-Pan) was purchased on February 2015 in a local market at Bandar Baru Bangi, Selangor, Malaysia. The mother of vinegar, where it contains *Acetobacter* sp. was directly obtained from Bragg's apple cider vinegar with an expiry date of 7th of May 2019, where it was purchased from a shopping complex located at Bandar Baru Bangi, Selangor, Malaysia. This mother of vinegar was directly used in this study.

Production of Vinegar

A 3×5 factorial experimental design was used in this study and two factors were chosen, pH and fermentation time, as shown in Table 1. The levels of pH were set at pH4.5, 5.0 and 5.5; whereas the levels of fermentation time were set at 24, 48, 72, 96 and 120 h. The fermentations were performed in triplicated, where soursop juice samples were prepared at 100 mL each in 45 conical flasks (500 mL) and 1 gram of yeast in powder form was added into the soursop juice for alcoholic fermentation. According to Narendranath and Power (2005), it showed that these are the optimum level factors for yeast to grow and sufficient time for the production of alcohol and acetic acid. The pH of samples was adjusted using 1 M potassium carbonate. The temperature was fixed at 30°C, as it is the optimum for yeast to grow. All the samples were placed into incubator shaker (*Ecotron*, Switzerland) at 150 rpm. After the alcoholic fermentation, samples were added with 2 mL of mother of vinegar for acetous fermentation to occur. The fermentation treatments were similar to that of alcoholic fermentation stated above.

Chemical Composition Analysis

Determination of Sugar Content Using Phenol-Sulphuric Acid Method

The basic principle of this method is the dehydration of carbohydrates by reacting with concentrated sulfuric acid and produce furfural derivatives, which reacts with phenol, giving a detectible colour compound. Samples (2 mL) were mixed with 1 mL of 5% aqueous solution of phenol in a test tube, and followed by adding 5 mL of concentrated sulfuric acid. Then, the mixture was allowed to stand for 10 min, vortexed for 30 s and placed in water bath at room temperature for another 20 min. Vinegar sample was replaced with deionised water as sample blank. The absorbance was read at 490 nm using UV-visible spectrophotometer (BioTek Epoch, Vermont). A standard curve was prepared using glucose at concentrations of 0.002%-0.02% (Ammar et al. 2013). The glucose concentration was calculated using the equation below.

$$\text{Glucose equivalence (\%)} = R \times DF,$$

where R is reading from the standard curve; and DF is dilution factor.

Determination of Ethanol Content Using Dichromate Method

Samples (1 mL) were added with 5 mL of sodium dichromate solution (2.5% w/v), 5 mL of 0.1 M acetate buffer (pH4.3) and 25 mL of 1 M sulfuric acid. The mixture was shaken gently for 1 min and allowed to stand for 2 h at room temperature. Vinegar sample was replaced with deionised water as sample blank. The absorbance was read at 578 nm using UV-visible spectrophotometer (BioTek Epoch, Vermont). A standard curve was prepared using ethanol at concentrations of 5 to 40% (Betiku & Taiwo 2015). The results were expressed in ethanol equivalent calculated using the equation below.

$$\text{Ethanol equivalent (\%)} = R \times DF,$$

where R is reading from the standard curve; and DF is dilution factor.

DETERMINATION OF ACETIC ACID CONTENT USING TOTAL TITRATABLE ACIDITY (AOAC METHOD)

The samples (2 mL) were added with 20 mL of deionised water and 5 drops of phenolphthalein. A 0.1 M sodium hydroxide was prepared and used to titrate the sample until it shows a faintest discernible pink colour persisting for 30 s. Vinegar sample was replaced with deionised water as sample blank (Cairns et al. 2002). A standard curve was prepared using acetic acid at concentrations of 0.25%-1.5%. Acetic acid content in samples was calculated using the following equation:

$$\text{\% Acetic acid in vinegar} = ((M_{NaOH} \times V_{NaOH} \times MW_{\text{acetic acid}})/SV) \times 100,$$

where M_{NaOH} is molarity of NaOH; V_{NaOH} is the volume of NaOH used in titration; $MW_{\text{acetic acid}}$ is the molecular weight of acetic acid (60); and SV is the sample volume.

DETERMINE VITAMIN C CONTENT BY USING INDOPHENOL METHOD

Dye factor of ascorbic acid in indophenol method was determined by pipetting 2 mL of 1 mg/mL ascorbic acid standard solution into conical flasks containing 5 mL metaphophoric acid-acetic acid solution. Indophenol dye solution was titrated into the conical flasks, until rose pink colour was formed as the end point. Ascorbic acid standard solution was replaced with deionised water as blank. The dye factor was calculated as follows:

$$F = m_{\text{ascorbic acid}}\ (2.0\ \text{mg})/V_{\text{indophenol}},$$

where F is the dye factor (mg ascorbic acid/ mL dye); $m_{\text{ascorbic acid}}$ is the mass of ascorbic acid in the conical flask; and $V_{\text{indophenol}}$ is the indophenol titration volume.

The dye factor was used to calculate the ascorbic acid content in the vinegar samples. The vinegar samples (5 mL) were added with 5 mL of 3% metaphosphoric acid-acetic acid-sulfuric acid. Indophenol dye solution was titrated into the sample solution until a rose pink colour was formed as the end point (Hernandez et al. 2006). Vitamin C contents were calculated using the following equation:

$$\text{Ascorbic acid (AAE mg/ 100 mL juice)} = F \times (V - V_0) \times (TV/SV) \times 100,$$

where F is the dye factor (mg ascorbic acid/ mL dye); V is the average titration volume for sample; V_0 is the verage titration volume for blank; TV is total volume of sample prepared; and SV is the volume of sample used for the assay.

STATISTICAL ANALYSIS

Analyses were performed in triplicate (n=3). Data were obtained as the mean and standard deviation and analysed using Minitab Statistical Software (Release 15). The difference in mean values was considered significant when $p<0.05$.

Results and Discussion

The results were analysed using the main effects and the interaction between factors. Fifteen experiments were carried out and each of them was replicated three times. All possible combinations of factors were used and a matrix was established according to the levels of factors that have been fixed, respectively. Overall, pH5.5 would give a better result in fermentation as it consumes more sugar compounds to produce more ethanol and subsequently the highest production of acetic acid in this study according to Table 1.

Table 1 shows that as the fermentation time increases, the sugar content was reduced in all the pH been set (pH4.5, 5.0 and 5.5). Factors of pH and fermentation were significantly ($p<0.05$) reduced the sugar concentration in all samples as shown in Table 2(a). However, it was found that there were no significant ($p>0.05$) effect of interaction between the factors. The regression model for sugar content in vinegar production is: Sugar = 5.134 - 0.196*pH - 1.263*Time. In Table 2(b), the sum of squares used to estimate the effects of factors and F ratios are shown. It shows that R^2 and $R^2_{(adj)}$ values which were important due to the test obtained mathematical model were close to each other at approximately 1.0 (R^2 = 97.08%, $R^2_{(adj)}$ = 96.28%) that of expected result statistically. R^2 is a statistical measure of how close the data are to the fitted regression line and it is also known as the coefficient of determination or the coefficient of multiple determinations for multiple regressions. When R^2 is approaching to 100%, it indicates that the model explains most of the data variability of the remove bold is approaching to 0%, it indicates that the model explains none of the variability of response data around its mean. In general, the higher the R^2, the better the model fits the data. However, $R^2_{(adj)}$ is a modified version of R^2 that has been adjusted for the number of predictors in the model, and it is always lower than R^2 (Cameron & Windmeijer 1996; Heinzl & Mittlbock 2003). Therefore, the regression model for sugar content fits the data well.

Apart from that, as for the ethanol production in samples, it shows increases in ethanol content when fermentation time increases according to Table 1. However, only fermentation time shows significant influence ($p<0.05$) on ethanol content. However, it was found that pH as well as interaction between factors do not have significant ($p>0.05$) effect on ethanol production. Therefore, the regression model for ethanol production is: Ethanol = 14.4811 + 10.738*Time. Besides that, Table 3(b), shows that R^2 and $R^2_{(adj)}$ values were close to each

TABLE 1. Experimental factorial design and its response of sugar, ethanol and acetic acid content

Factor levels		Response			
pH	Time (hrs)	Sugar content (%)	Ethanol content (%)	Acetic acid content (%)	Ascorcic acid content (AAE mg/100 nmL)
4.5	24	6.33 ± 0.23	4.00 ± 0.92	0.57 ± 0.04	13.11
4.5	48	5.93 ± 0.10	8.71 ± 1.02	0.68 ± 0.03	13.11
4.5	72	5.44 ± 0.24	14.67 ± 0.97	0.86 ± 0.04	13.11
4.5	96	4.76 ± 0.19	22.13 ± 0.75	1.02 ± 0.06	13.11
4.5	120	4.01 ± 0.20	24.15 ± 0.90	1.13 ± 0.05	13.11
5.0	24	6.38 ± 0.17	3.77 ± 0.21	0.63 ± 0.03	13.11
5.0	48	5.58 ± 0.14	8.22 ± 0.96	0.83 ± 0.04	13.11
5.0	72	5.48 ± 0.18	14.17 ± 1.07	1.07 ± 0.02	13.11
5.0	96	4.53 ± 0.29	21.78 ± 0.77	1.15 ± 0.04	13.11
5.0	120	4.03 ± 0.18	23.76 ± 1.08	1.25 ± 0.06	13.11
5.5	24	6.52 ± 0.30	3.77 ± 0.20	0.68 ± 0.03	13.11
5.5	48	5.63 ± 0.16	8.23 ±0.20	0.88 ± 0.03	13.11
5.5	72	4.69 ± 0.21	14.10 ± 1.05	1.07 ± 0.04	13.11
5.5	96	4.06 ± 0.16	21.92 ± 0.24	1.19 ± 0.09	13.11
5.5	120	3.62 ± 0.07	23.83 ± 1.25	1.26 ± 0.05	13.11

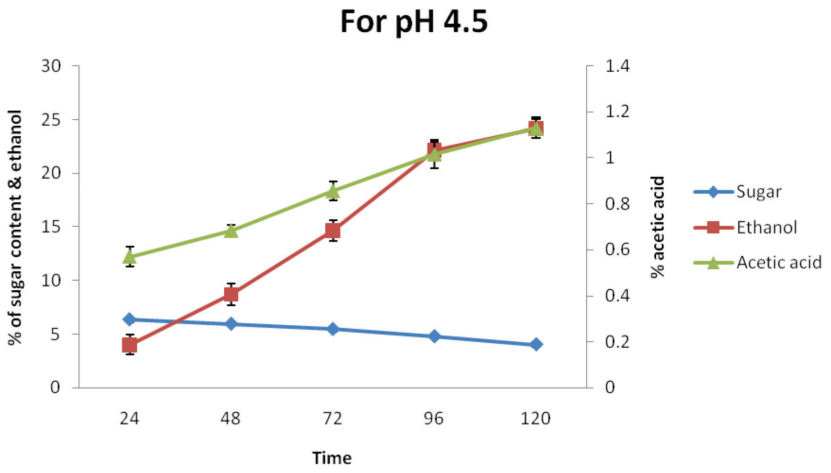
other at approximately 1.0 ($R^2 = 97.72\%, R^2_{(adj)} = 97.10\%$), suggesting that the regression model fits the data well.

Glucose is transported via facilitated diffusion, glucose uptake requires concentration gradient across the plasma membrane. After this reaction, glucose will dissimilate convert glucose to two pyruvates (Maris et al. 2006). This will cause the formation of two ATP per glucose. Sugar in the juice was converted into alcohol by alcohol dehydrogenase of the yeast (Iersel et al. 1999). The presence of alcohol dehydrogenase is dependent on the coenzyme NAD under the anaerobic conditions. Sugar was reduced to different alcohol compounds, such as ethanol, butanol, propanol, isobutanol, isoamyl alcohol (Iersel et al. 2000). This indicates that the longer the period of alcoholic fermentation, the more ethanol/alcoholic compounds were produced. This is due to the fact that the yeast has sufficient time to produce more alcohol dehydrogenase in the sample which in turn converts the sugar into alcohol during the alcoholic fermentation (Okamura et al. 2001; Rajko & Janez 1999). This is evident in the results showed in Figure 1, in which it indicates that as the fermentation time increases, the sugar content reduces while the ethanol content increases.

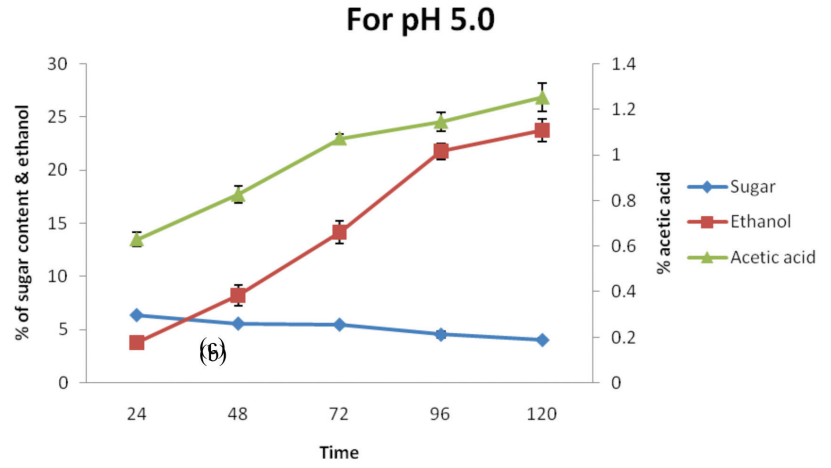
As for the acetic acid production, Table 1 shows that the fermentation time has directly proportional to the production of acetic acid, where when fermentation time increases, it would increase the acetic acid content as well. Table 4(a) shows positive effect on both pH and fermentation time, where the production of acetic acid increases as the factors was changed from low to high levels. As reported in Table 4(a) and 4(b), the effects of interaction between factors showed p-value of more than 0.05, thus making the effects of interaction between factors not significant. Therefore, the regression model for effects of pH and fermentation time on acetic acid production is: Acetic acid = 0.9509 + 0.082*pH + 0.298889*Time. The R^2 and $R^2_{(adj)}$ values from Table 4(b) are close to each other approximately 1.0 ($R^2 = 95.87\%$, $R^2_{(adj)} = 94.75\%$). However, the fermentation is incomplete as vinegar needs to contain at least 4% acetic acid. This might be due to insufficient oxygen present during the acetous fermentation, as acetic acid bacteria needs aerobic conditions to produce acetic acid. The low oxygen concentration could influence the production of acetic acid and the speed of the fermentation process (Dabija & Hatnean 2014). It was also reported by Buyuksirit and Kuleasan (2014) that low production of acetic acid during the acetous fermentation under aerobic conditions could occur due to the presence of toxic-secreting strains of *Saccharomyces cerevisiae* (yeast), which might inhibit the growth of bacteria (*Acetobacter* species). Therefore, in order to increase the acetic acid concentration, it is suggested that the oxygen concentration to be increased by aeration during the acetous fermentation to increase the production of acetic acid in the soursop vinegar.

The ethanol substrate was first oxidised to acetaldehyde in the presence of alcohol dehydrogenase and subsequently oxidised to acetic acid. These dehydrogenase enzymes consist of quinoproteins and flavoproteins which have pyrroloquinoline quinine and will form covalent bond with flavin adenine dinucleotide as prosthetic groups. The alcohol dehydrogenase consists of two or three subunits, including the dehydrogenase and cytochrome which is essential in the enzymatic reaction (Raspor & Goranovic 2008). According to Ubeda et al. (2011), the insufficient oxygen could lead to accumulation of acetaldehyde during acetous fermentation and a lower production of acetic acid.

Fermentative microorganisms, such as *Acetobacter* species would be adapting the sudden change on the medium conditions, which was transferred from mother



(a)



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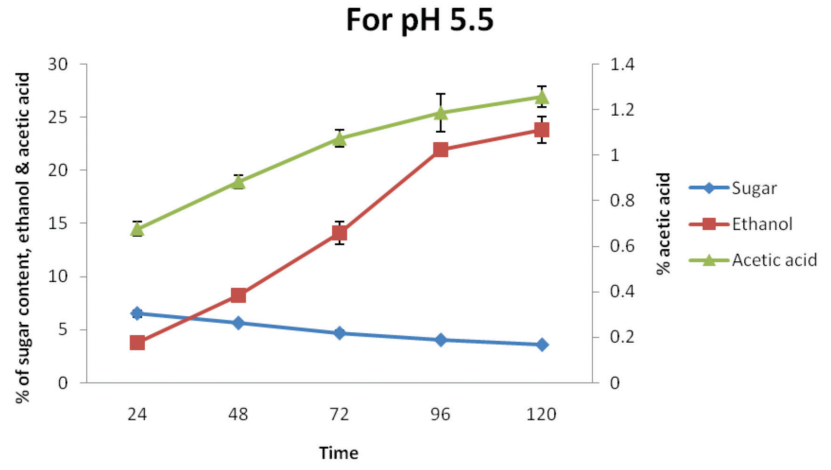

(c)

FIGURE 1. The reduction of sugar and production of ethanol and acetic acid over fermentation time; where (a) is at pH4.5, (b) is at pH5.0 and (c) is at pH5.5

TABLE 2(a). Statistical parameters for factorial design on sugar

Term	Effect	Coefficient	Standard Error	t-statistic	p
Constant		5.134	0.04765	107.75	0.000
pH	-0.392	-0.196	0.05836	-3.36	0.006
Time	-2.525	-1.263	0.06739	-18.73	0.000
pH * Time	-0.312	-0.156	0.08253	-1.89	0.085

TABLE 2(b). Analysis of variance for sugar

Source	Degrees of freedom	Sum of Square	Mean square	F	p
Main Effects	2	12.3378	6.16890	181.13	0.000
2-Way Interaction	1	0.1221	0.12207	3.58	0.085
Residual Error	11	0.3746	0.03406		
Total	14	12.8345			

$R^2 = SS_{MODEL}/SS_{TOTAL}$; $R^2_{adj} = 1 - [(SS_{ERROR}/DF_{ERROR})/(SS_{TOTAL}/DF_{TOTAL})]$; $R^2 = 97.08\%$, $R^2_{(adj)} = 96.28\%$

TABLE 3(a). Statistical parameters for factorial design on ethanol

Term	Effect	Coefficient	Standard Error	t-statistic	p
Constant		14.4811	0.3499	41.39	0.000
pH	-0.3613	-0.1807	0.4285	-0.42	0.681
Time	21.4760	10.7380	0.4948	21.70	0.000
pH * Time	0.0153	0.0077	0.6060	0.01	0.990

TABLE 3(b). Analysis of variance for ethanol

Source	Degrees of freedom	Sum of Square	Mean square	F	p
Main Effects	2	865.111	432.556	235.55	0.000
2-Way Interaction	1	0.000	0.000	0.00	0.990
Residual Error	11	20.200	1.836		
Total	14	885.311			

$R^2 = SS_{MODEL}/SS_{TOTAL}$; $R^2_{adj} = 1 - [(SS_{ERROR}/DF_{ERROR})/(SS_{TOTAL}/DF_{TOTAL})]$; $R^2 = 97.72\%$, $R^2_{(adj)} = 97.10\%$

TABLE 4(a). Statistical parameters for factorial design on acetic acid

Term	Effect	Coefficient	Standard Error	t-statistic	p
Constant	0.164000	0.950889	0.01387	68.57	0.000
pH	0.597778	0.082000	0.01698	4.83	0.001
Time	0.002000	0.298889	0.01961	15.24	0.000
pH * Time		0.001000	0.02402	0.04	0.968

TABLE 4(b). Analysis of variance acetic acid

Source	Degrees of freedom	Sum of Square	Mean square	F	p
Main Effects	2	0.737249	0.368625	127.78	0.000
2-Way Interaction	1	0.000005	0.000005	0.00	0.968
Residual Error	11	0.031734	0.002885		
Total	14	0.768988			

$R^2 = SS_{MODEL}/SS_{TOTAL}$; $R^2_{adj} = 1 - [(SS_{ERROR}/DF_{ERROR})/(SS_{TOTAL}/DF_{TOTAL})]$; $R^2 = 95.87\%$, $R^2_{(adj)} = 94.75\%$

of vinegar to the alcohol samples. During the lag phase, acetic acid bacteria use the main proportion of their energy resources in this synthesis (Bazirake et al. 2014), and therefore the production of acetic acid was produced in a small amount.

Besides that, vitamin C content has been determined using indophenols method. It was found that there were no changes in vitamin C content of all the vinegar samples, where all samples contain 13.11 mg of ascorbic acid equivalence in 100 mL juice. This shows that no ascorbic acid were metabolised or produced during the fermentation process. There has been contradicting statements in published work, where according to Adetuyi and Ibrahim (2014), fermented product will have increased vitamin C content, but Okigbo and Obire (2009) reported that fruit wine which has undergone alcoholic fermentation has reduced vitamin C content by 70% from the original value. Adetuyi and Ibrahim (2014) also reported that as the fermentation period increased the loss in ascorbic acid may occur due to the increased ascorbate oxidase enzyme activity, which might be produced by fermentation microorganism in the favourable fermentation conditions.

CONCLUSION

This study proved the concept of soursop vinegar production and it could increase the economical and food values as well as innovate a way of utilising and preserving soursop fruit in Malaysia. It was found the main effect of pH and fermentation time had significant effect on the reduction of sugar and production of acetic acid, while only fermentation time shows significant effect on the production of ethanol. Interaction between factors did not exhibit any statistical significance. However, the acetous fermentation was not complete as it did not achieve minimum 4% acetic acid concentration. It is suggested that samples should be properly aerated during acetous fermentation to create an aerobic condition which is favourable for acetous fermentation. Vitamin C contents in the vinegar samples were found to be similar in all vinegar samples.

ACKNOWLEDGEMENTS

This study was supported by the Dana Top Down-Geran Penyelidikan Universiti-Industri, Universiti Kebangsaan Malaysia (INDUSTRI-2014-005) Geran Ganjaran Penerbitan (GP-K020181). The authors would like to acknowledge Malaysian Agricultural Research and Development Institute (MARDI) which provided the soursop juice and the School of Chemical Sciences and Food Technology, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, which provided the facilities necessary for this research.

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Received: 31 August 2016
Accepted: 28 April 2017