Optimization of Fermentation for Bioethanol Production from sour *Citrus aurantifolia* Fruit Juice using Natural Palmyrah Toddy Yeast

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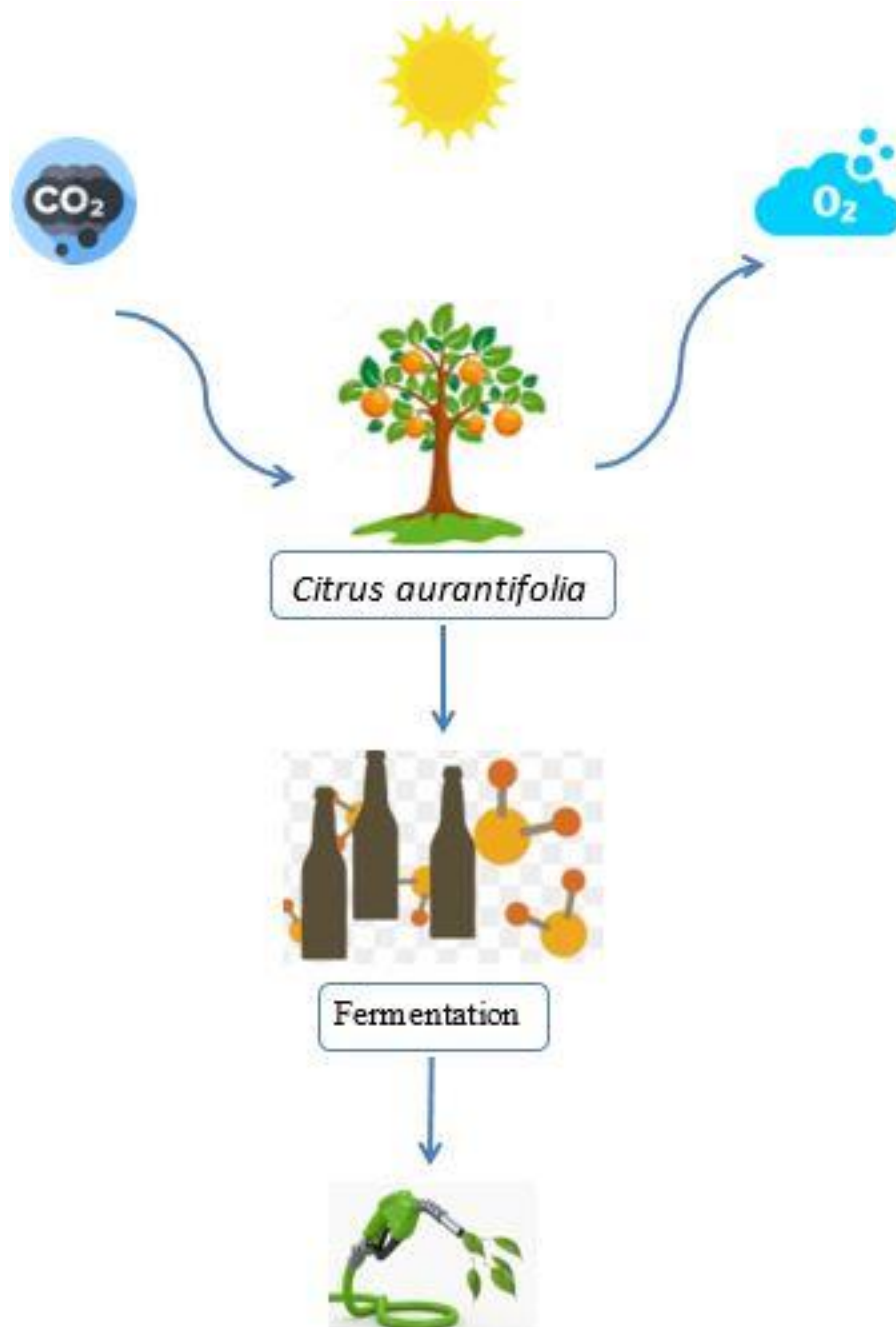
Graphical Abstract



Abstract

Large scale consumption of fossil fuel to meet the increasing demand causes adverse effects on the environment due to the emission of harmful greenhouse gases. The production of bioethanol from diverse fruit juices that are underutilized because of poor taste quality could be one of the alternative fuels to overcome the issues. The objective of the study was to produce bioethanol from sour *Citrus aurantifolia* fruit juice using palmyrah toddy yeast and to optimize the conditions to increase the bioethanol yield. The sour *Citrus* fruit juice was inoculated with palmyrah toddy yeast (*Saccharomyces cerevisiae* - 2g/L) in the fermentation media (100ml, sour *Citrus* fruit juice: distilled water = 1:3) composed of 10 g/L yeast extract, 10 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 2 g/L peptone and 0.5 g/L $MgSO_4 \cdot 7H_2O$ and allowed for fermentation for 24 hours at room temperature. The amount of ethanol produced from the *Citrus* juice was 0.8% (V/V) after 24 hours of fermentation. In order to optimize the fermentation process for *Citrus aurantifolia*, a variety of experimental parameters were studied, including the type of nitrogen source (ammonium sulphate, ammonium nitrate, ammonium chloride, and urea), inoculum size (0.4 to 1.0 g/100 mL), temperature (20 to 40 °C), rotation speed (50 to 250 rpm), concentration of raw fruit juice (5 to 100%), amount of urea (0.1 to 2.0 g/100 mL), carbon source (glucose, sucrose, maltose, and dextrose), amount of sucrose (1 to 20 g), pH of the medium (3.0 to 8.0), and incubation period (24 to 96 h). After conducting the experiments, it was found that using *Citrus aurantifolia* at 100% concentration resulted in significantly higher ethanol yields of 11.50%, which was 14.37 times higher than the non-optimized conditions. The highest yield was achieved when the fermentation was carried out at 35 °C for 24 h with an inoculum concentration of 0.5 g/100 mL, a rotation speed of 150 rpm, a pH of 4.0, 0.1 g/100 mL urea as the nitrogen source, and 10 g/100 mL sucrose as the carbon source. Large scale fermentation study using bioreactor should be done to determine whether this finding could be commercialized.

Key words: *Bioethanol, Fermentation, Palmyrah toddy yeast, Citrus fruit juice, Urea*

1. Introduction

Bioenergy can be explicated as energy obtained from biomass, which is the biodegradable fraction of products, waste and residues from agriculture like vegetables and animal origin, forestry and related industries (Nigam and Singh, 2011). Biofuels produced from biomasses are the best alternatives for fossil fuels and nowadays, they are commonly used for transport purposes in many countries all over the world (Hill et al., 2006). Different forms of bioenergy can be produced by using a wide range of biomass sources, such as agricultural residues, underutilized fruits and vegetables and food wastes (Hossain et al., 2011). Bioethanol is frequently used as motor fuel or as an additive in gasoline and is an option for more "renewable" energy. This is a clear colourless liquid, it is biodegradable, low in toxicity and causes little environmental pollution if released. By blending ethanol with gasoline fuel

mixture is oxygenated. Therefore, the quantity of the polluting emissions will be highly reduced (Thenmozhi and Victoria, 2013).

Bioethanol is generally produced from the 1st and 2nd generations of biomass (Scaife et al., 2015). The first generation of bioethanol is generated from edible biomass that contains higher levels of starch and sugar materials (Ho et al., 2014) including wheat, sugar beet, corn, barley, sugar cane, and molasses (Sarkar et al., 2012). Fruits are widely recognized as one of the most abundant sources of fermentable sugars, and thus are commonly used as the first generation feedstock for bioethanol production (Sarkar et al., 2019). One of the major advantages of using fruits for ethanol production is their high sugar content, which makes them highly desirable for the process. Additionally, compared to other biomasses, fruits do not require any pre-treatment processes, making them a more efficient and cost-effective source of ethanol (Sarkar et al., 2012). Second-generation bioethanol is obtained from lignocellulosic materials such as energy crops, forestry products, and solid wastes like sugarcane bagasse. Researchers focused on lignocellulosic materials due to their abundance and immense potential for conversion into sugars and fuel. When comparing first and second generations of bioethanol feedstock, sugary and starchy materials can easily convert to bioethanol compared with lignocellulosic materials, which require additional physical, chemical, and enzymatic pre-treatment techniques (Ruiz et al., 2012).

Liquid stage fermentation by yeast from palmyrah toddy is a very popular, traditional, well-established natural metabolic process for conversion of lignocellulosic biomass to bioethanol where a micro-organism transforms complex carbohydrate into simple sugar and sugars transformed into an alcohol (Thenmozhi and Victoria, 2013). This fermentation process can be facilitated by lot of different micro-organisms that are naturally available in the environment. In fermentation process, *Saccharomyces cerevisieae* will ferment the sugar to ethanol and carbon dioxide (Powell and Hill, 2013). Naturally grown yeast cultivated from the palmyrah toddy sap is capable of tolerating a wide range of pH, which makes the fermentation less susceptible to microbial contamination (Sarathadevi et al., 2018, Kapilan, 2015, Kapilan et al., 2015). It also tolerates higher concentrations of ethanol better than the other ethanol producing microorganisms. Using the bioethanol as an alternative fuel, the greenhouse gas emissions will be reduced as the fuel crops absorb the CO_2 they emit through growing (Balat, 2011). Bioethanol is carbon neutral and very less toxic than fossil fuels. For bioethanol to become more sustainable to replace petrol the production process has to be more efficient e.g:- the cost of conversion to be reduced, to increase yields and to increase the diversity of crop used (Irfan et al., 2011).

Citrus aurantifolia is one of the lemon types that are commonly found in the tropical region. Since the fruit of this *Citrus* is sour in taste, it is not in the prime list of a food / drink of the public (Srikantha et al., 2017; Srikantha et al., 2016). Since the usage of the fruits of *Citrus aurantifolia* is very limited due

to its poor taste and low economic value for human and animals, the fruits are left on the tree until they fall off naturally. Though bioethanol production from food crops is banned in Sri Lanka, it was planned to use this underutilized, but abundantly found *Citrus* variety as source for bioethanol production. Therefore, the objective of the present study was to produce bioethanol from under-utilized sour *Citrus aurantifolia* fruit juice using natural yeast obtained from palmyrah toddy and to optimize the culture and fermentation conditions to increase the yield.

2. Methodology

2.1 Source of strain and fruit

Yeast (*Saccharomyces cerevisiae*) was obtained from palmyrah toddy by centrifugation at 3000 rpm for 5 minutes at 30 ^{o}C and grown on Potato Dextrose Agar medium (PDA). Sour *Citrus* fruits were grabbed from the Botanical Garden of the Department of Botany, University of Jaffna and the juice was prepared.

2.2 Chemicals and Media

All the chemicals used were obtained from standard sources. Basal medium containing 10 g/L yeast extract, 10 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$,2 g/L peptone and 0.5 g/L $MgSO_4 \cdot 7H_2O$ was prepared. After the autoclaving of the conical flask containing 100ml media, it was inoculated with 0.2 g of yeast (*Saccharomyces cerevisiae*) collected from palmyrah toddy.

2.3 Production of alcohol and its measurement

To the fermentation medium (100 mL), (2 g/L) inoculum (*Saccharomyces cerevisiae*) was added and incubated at room temperature (30 ^{o}C) in a rotatory shaker (100 rpm) and allowed for fermentation. Each flask was cultured at room temperature under oxygen limited condition up to 24 hr. The oxygen limited condition was prepared by sealing the flask tightly with parafilm and kept in an anaerobic chamber. Medium was mixed with distilled water and the suspension was mixed and the extract was centrifuged using a TOMY CAX-571 centrifuge. Reducing sugar was measured by di nitro salicylic acid method (Miller, 1959) and the concentration of alcohol (v/v %) in the substrate was determined using an ebulliometer (Christy et al., 2021).

2.4 Optimization of culture conditions for bioethanol production

2.4.1 Production of bioethanol in sour Citrus medium

Fermentation medium (100 mL) was inoculated with 0.2 g of palmyrah toddy yeast (*Saccharomyces cerevisiae*) and incubated at room temperature and bioethanol yield was monitored.

2.4.2 Optimization of nitrogen source

Fermentation media were prepared by taking different nitrogen sources (Ammonium sulphate, ammonium nitrate, ammonium chloride, and urea) with 0.2 g/100 mL (Rasmey et al., 2018).

2.4.3 Optimization of the size of inoculum

Fermentation media were prepared by mixing the optimized nitrogen source (urea) with liquid fermentation media. Different amount of yeast inoculum (0.4, 0.5, 0.6, 0.8 and 1.0 g/100 mL) was added in the media and incubated at room temperature (30 ^{o}C). Bioethanol production was determined after 24 hours.

2.4.4 Optimization of temperature

Media were prepared by mixing the optimized nitrogen source (urea) with liquid fermentation media. Optimized amount of yeast inoculum was added in the media and incubated at different temperatures (20 – 40 ^{o}C). Bioethanol production was determined after 24 hours.

2.4.5 Optimization of rotation speed

Media were prepared by mixing the optimized nitrogen source (urea) with liquid fermentation media. Optimized amount of yeast inoculum was added in the media and incubated at the optimized temperature (35 ^{o}C) at different rotation speed (50, 100, 150, 200, 250 rpm). Bioethanol production was determined after 24 hours (Liu and Shen, 2008).

2.4.6 Optimization of raw fruit juice concentration

Fermentation media were prepared by mixing all the substances with different concentration of substrate (5, 10, 25, 50 and 100%) of liquid fermentation media. The fermentation medium was inoculated with yeast inoculum (0.5 g/100 mL) and incubated at 35 ^{o}C at 150 rpm and bioethanol production was monitored for 24 hours.

2.4.7 Optimization of amount of Urea

Media were prepared by mixing all the substances with different amount of urea (0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 g/100 mL) with 100% of sour *Citrus* juice concentration of liquid fermentation media. The medium was inoculated with 0.5 grams/100 mL and incubated at 35 ^{o}C at 150 rpm. Bioethanol production was determined after 24 hours (Rasmey et al., 2018).

2.4.8 Optimization of carbon source

Fermentation media were prepared by mixing all the substances with 100% of sour *Citrus* juice concentration and 0.1 g/100 mL of urea of liquid fermentation media. Different carbon sources such as glucose, sucrose, maltose and dextrose (2 g/100 mL) were added in the media and inoculated with yeast inoculum and incubated at 35 ^{o}C at 150 rpm. Bioethanol production was determined after 24 hours.

2.4.9 Optimization of amount of sucrose

Media were prepared by mixing already optimized substances at the appropriate level in the liquid fermentation media. Different amount of carbon source (Sucrose - 1, 2, 4, 6, 8, 10, 15 and 20 g) was added to the media and inoculated with yeast inoculum and incubated at 35°C at 150 rpm. Bioethanol production was determined after 24 hours.

2.4.10 Optimization of pH of the medium

Media were prepared by mixing already optimized substances at the appropriate level in the liquid fermentation media. The medium was set at different pH values such as 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 and inoculated with yeast inoculum and incubated at 35 °C at 150 rpm. Bioethanol production was determined after 24 hours.

2.4.11 Optimization of incubation period

Media were prepared by mixing already optimized substances at the appropriate level in the liquid fermentation media. The medium was set at pH 4.0 and inoculated with yeast inoculum and incubated at 35 °C at 150 rpm. The set ups were incubated at different incubation periods (24, 48, 72 and 96 h). Bioethanol production was determined after 24 hours.

2.5. Statistical analysis

The experiments conducted in this study were performed in triplicate to ensure the accuracy of the results, and the mean values were used to generate the graphs. Minitab 17.0 software was employed to perform the statistical analysis. A one-way ANOVA was employed to analyze the data, and significant differences were determined using Tukey's multiple comparison tests with a significance level of $p < 0.05$.

3. Results

3.1 Production of bioethanol in sour Citrus medium

The amount of ethanol produced from the *Citrus* juice was 0.8 % initially at room temperature after 24 hours of fermentation. Sugar concentrations before the commencement of the experiment and after the end of optimization of fermentation process, were measured by using refractometer method and tabulated in Table 1.

Table 1: Sugar concentration measurements of the fermentation media, before & after the fermentation by using di nitro salicylic acid method (Miller,1959) & refractometer method.

	Sugar concentration	
	Before the fermentation	ter the optimization of ermentation conditions
nitro salicylic acid meth 540nm)	0.57 moldm^{-3}	0.09 moldm^{-3}
fractometer method	11° Brix	3.6° Brix

3.2 Optimization of nitrogen source

Fermentation media were prepared by taking different nitrogen sources (Ammonium sulphate, ammonium nitrate, ammonium chloride, and urea) with 0.2 g/100 mL. When different nitrogen sources

such as urea, ammonium sulphate, ammonium carbonate and ammonium nitrate were used in the fermentation media, highest ethanol production (1.25 times) was obtained in the medium containing urea. Among these tested nitrogen sources, urea was the best for bioethanol production.

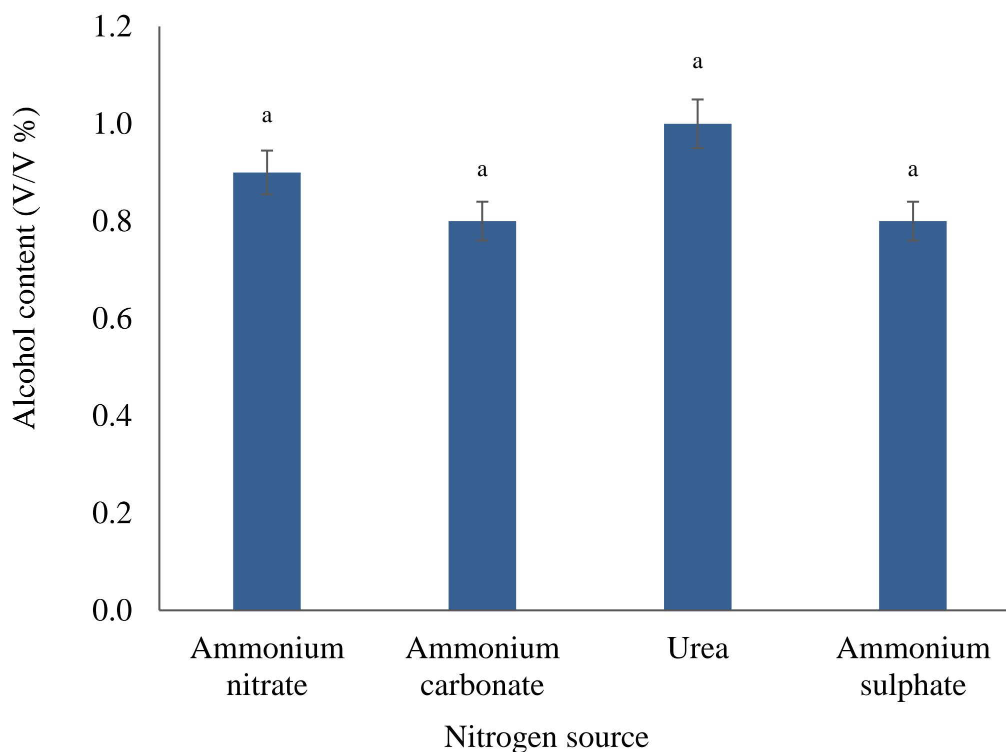


Figure 1: Effect of different nitrogen sources on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast *Saccharomyces cerevisiae*. Different letters showed the significant differences between the mean values at $p<0.05$.

3.3 Optimization of the size of inoculum

Media were prepared by mixing the optimized nitrogen source with liquid fermentation media. Different amount of yeast inoculum (0.4, 0.5, 0.6, 0.8, 1.0 g/ 100 mL) was added in the media and incubated at room temperature (30 °C). When the amount of initial yeast inoculum was optimized as 0.5 g/100 mL, ethanol yield was increased by 1.10 times (Alcohol yield 1.10 %) than the control which contained the amount of initial yeast inoculum 0.2 g/100 mL.

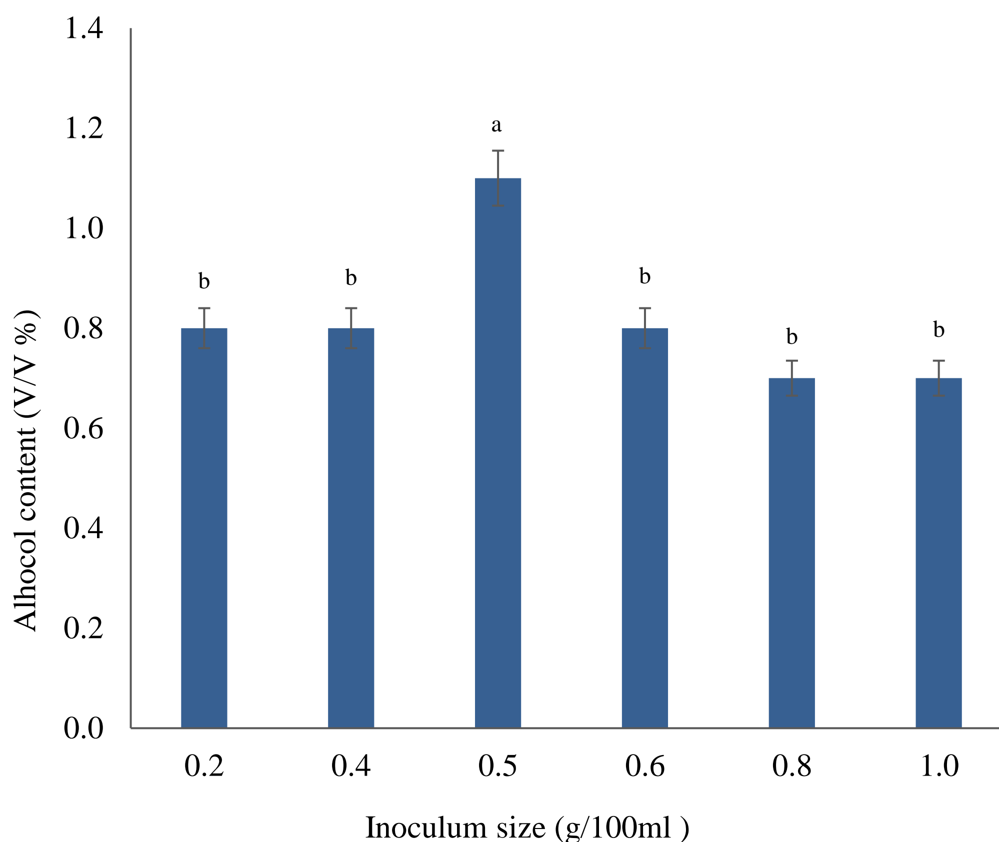


Figure 2: Effect of different inoculum sizes on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast *Saccharomyces cerevisiae*. Different letters showed the significant differences between the mean values at p<0.05.

3.4 Optimization of temperature

Media were prepared by mixing the optimized nitrogen source with liquid fermentation media. Optimized amount of yeast inoculum was added in the media and incubated at different temperatures (20 – 40 °C). The bioethanol produced after 24 hours at 20, 25, 30, 35 and 40 °C were 0.9%, 0.9%, 1%, 1.2% and 0.8% respectively.

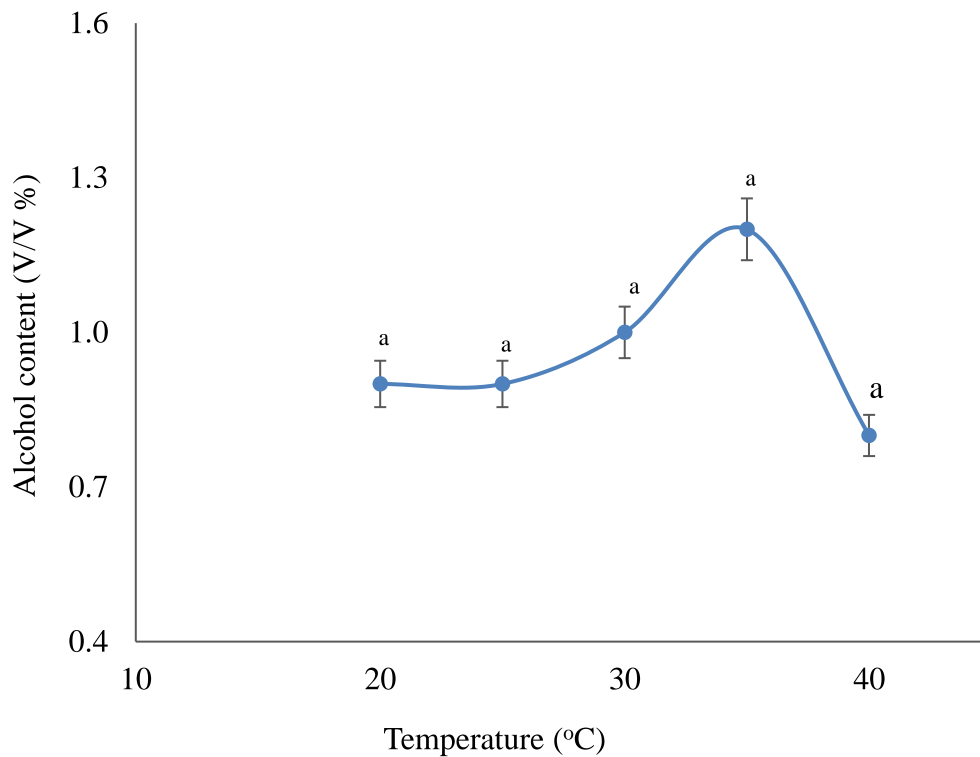


Figure 3: Effect of different temperatures on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast (*Saccharomyces cerevisiae*). Different letters showed the significant differences between the mean values at $p<0.05$.

3.5 Optimization of rotation speed

When different rotation speeds (50, 100, 150, 200, 250 rpm) were chosen, highest ethanol production was obtained when 150rpm was used. When the rotation speed of the media was optimized as 150 rpm, ethanol yield was increased by 1.08 times (Alcohol yield 1.30 %) than the control speed of 100 rpm.

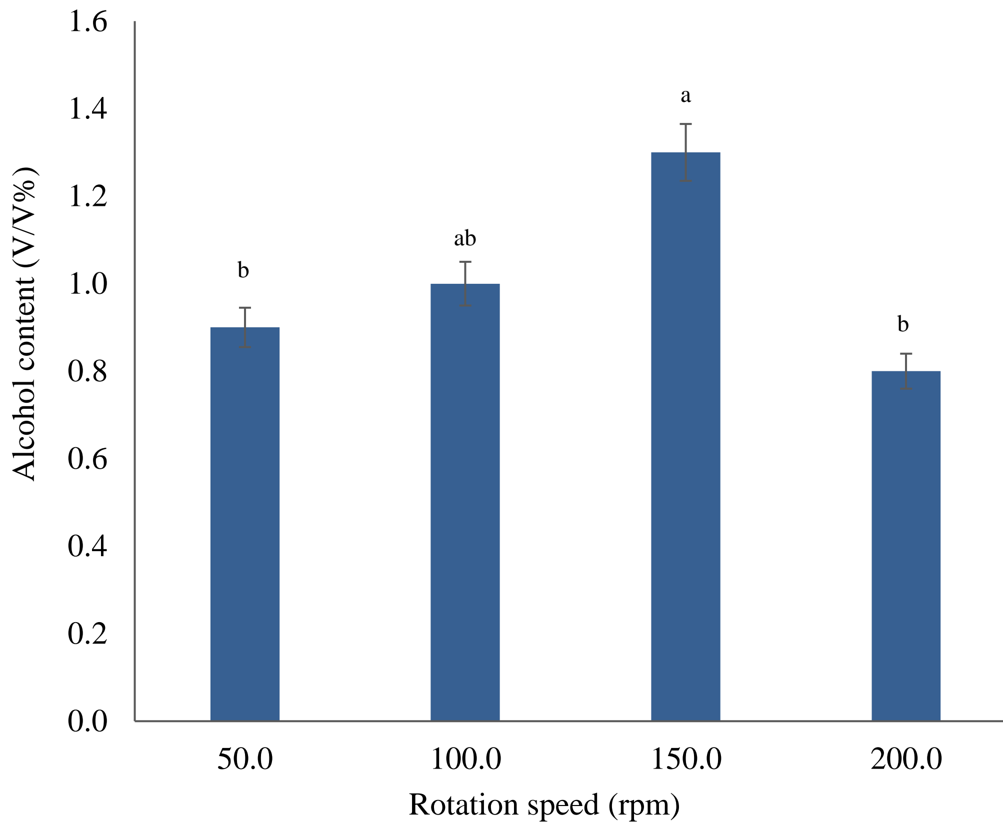


Figure 4: Effect of different rotation speeds on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast (*Saccharomyces cerevisiae*). Different letters showed the significant differences between the mean values at p<0.05.

3.6 Optimization of substrate concentration

When different concentration of substrates (5,10, 25, 50 and 100%) were chosen the highest ethanol production was obtained at 100% of substrate concentration (4.50%). When the substrate concentration of the media was optimized as 100%, ethanol yield was significantly increased by 3.75 times than the non-optimized substrate concentration (25%).

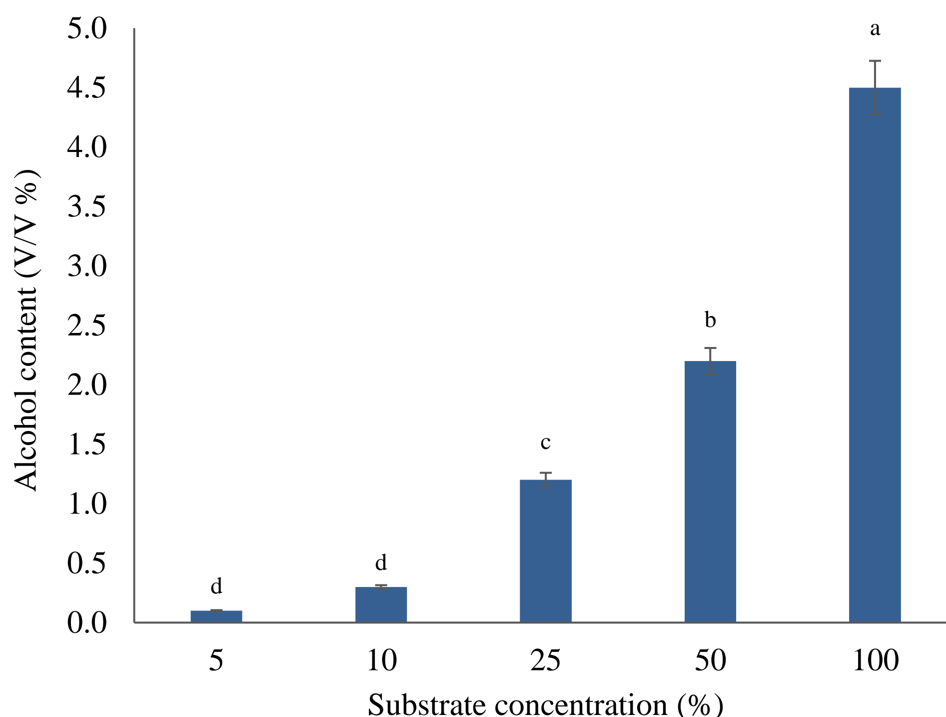


Figure 5: Effect of different concentrations of raw fruit juice on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast (*Saccharomyces cerevisiae*). Different letters showed the significant differences between the mean values at $p<0.05$.

3.7 Optimization of amount of nitrogen source

When the amount of urea was optimized as 0.1 g/100 mL, there was an increase in the ethanol yield than the non-optimized amount of urea (0.2 g/100 mL). Fermentation medium containing 0.1 g of urea yielded significantly higher ethanol production than the other concentrations except 0.2 g/100 mL.

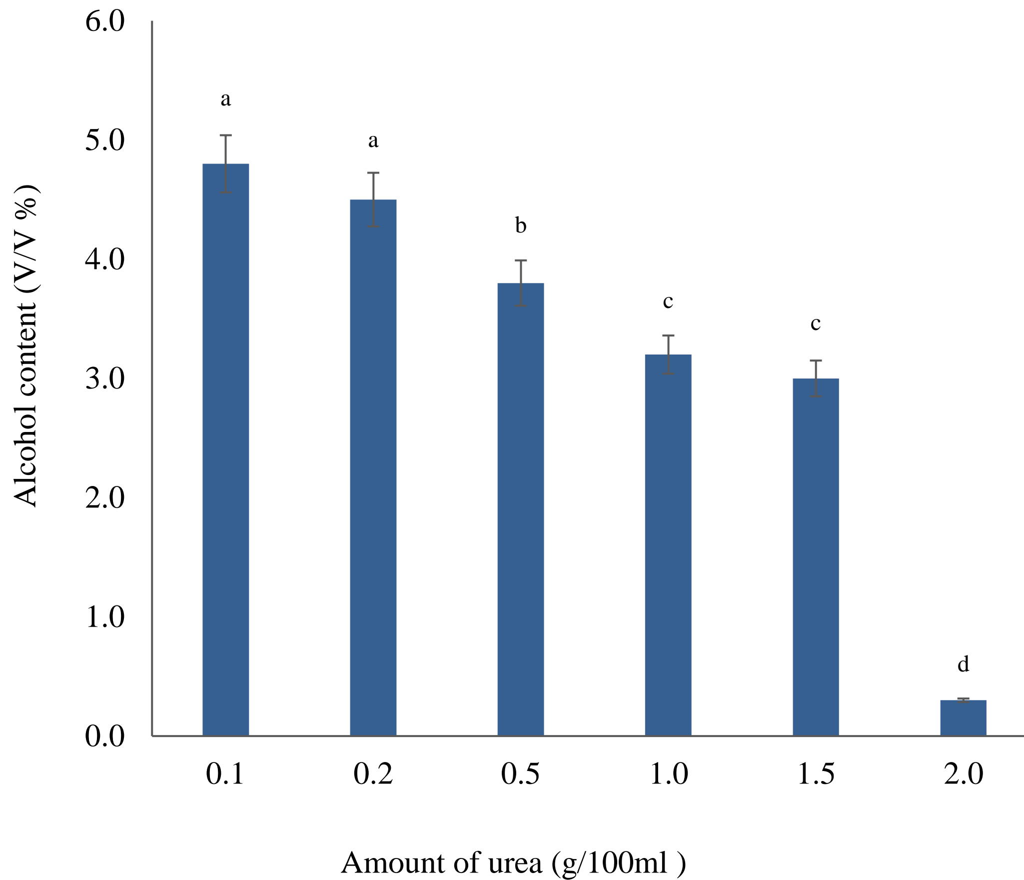


Figure 6: Effect of different amount of urea on bioethanol production from *Citrus* fruit juice using *Saccharomyces cerevisiae*. Different letters showed the significant differences between the mean values at $p<0.05$.

3.8 Optimization of carbon source

When different carbon sources such as glucose, sucrose, maltose and dextrose (2 g/100 mL) were separately added in the media set ups, highest ethanol production (1.16 times, 5.60 %) was obtained in the medium containing sucrose.

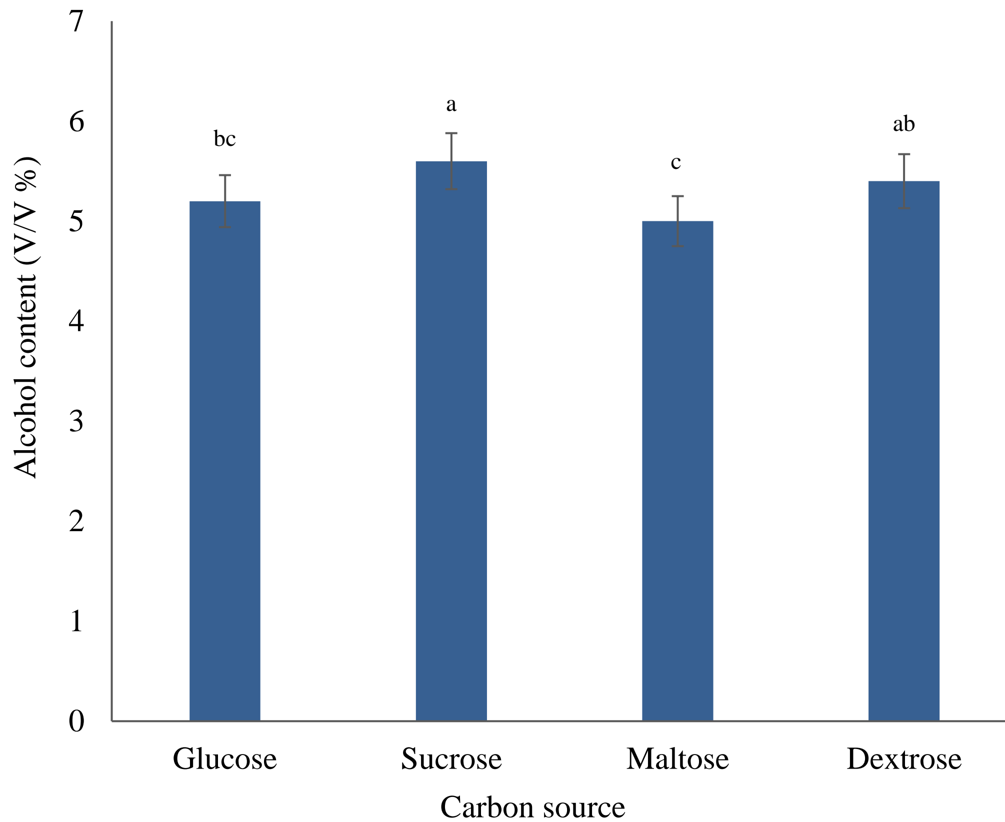


Figure 7: Effect of different carbon sources on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast (*Saccharomyces cerevisiae*). Different letters showed the significant differences between the mean values at $p<0.05$.

3.9 Optimization of amount of carbon source

When the amount of sucrose in the media was optimized as 10 g/100 mL, the ethanol yield was significantly increased by 1.89 times (10.60%) than the non-optimized amount (2 g/100 mL).

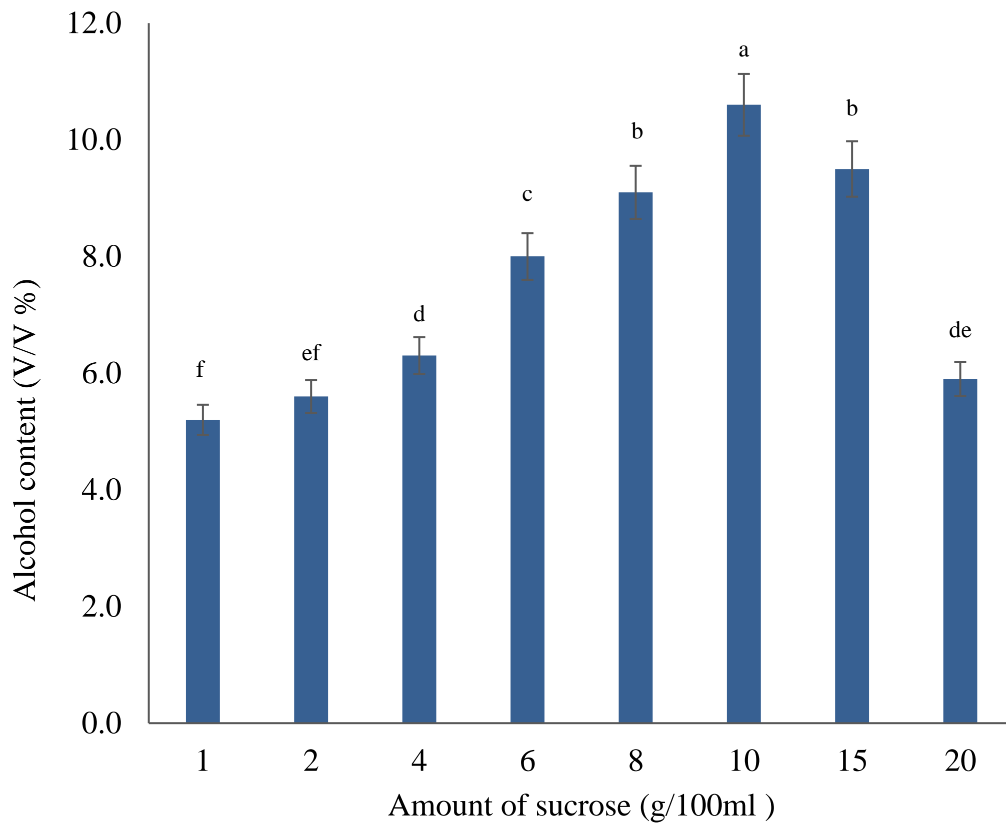


Figure 8: Effect of amount of Sucrose used in the fermentation media on bioethanol production from *Citrus* fruit juice using palmyrah toddy yeast (*Saccharomyces cerevisiae*). Different letters showed the significant differences between the mean values at $p<0.05$.

3.10 Optimization of pH of the medium

When the pH of the media was kept at 4.0, ethanol yield was significantly increased by 1.08 times than the control pH 7.0.

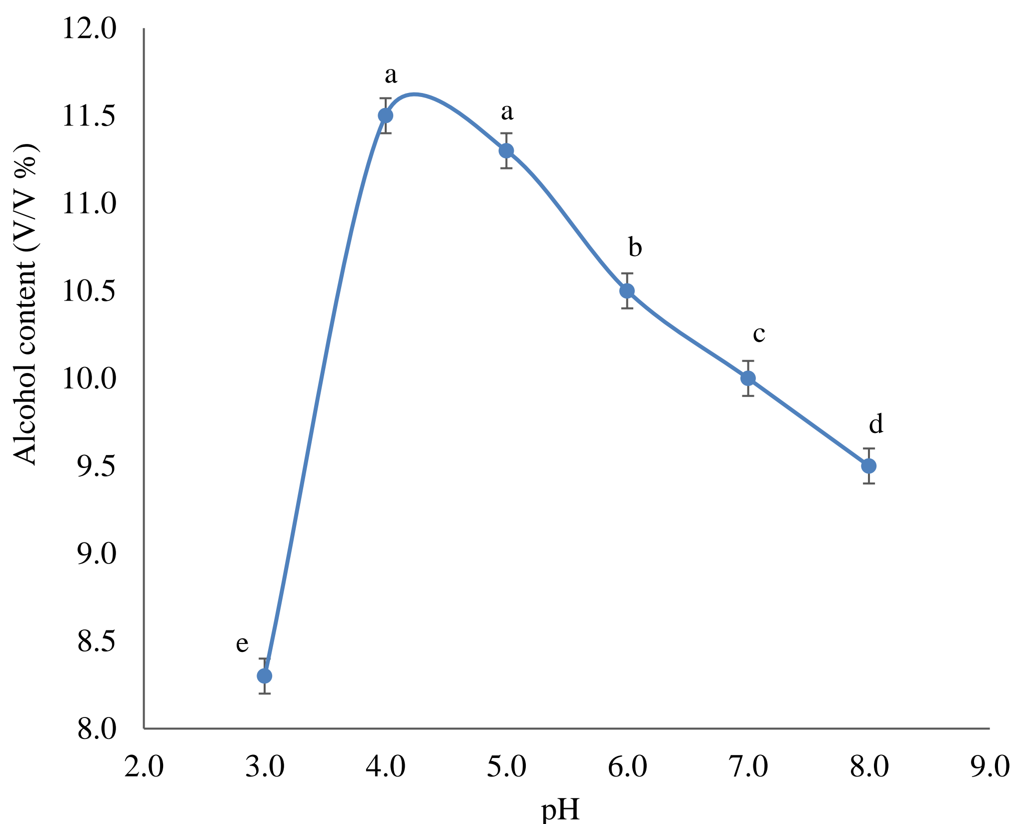


Figure 9: Effect of different pH on bioethanol production from *Citrus* fruit juice using natural yeast (*Saccharomyces cerevisiae*) from palmyrah toddy. Different letters showed the significant differences between the mean values at $p<0.05$.

3.11 Optimization of incubation period

The quantity of bioethanol produced after 24, 48, 72 and 96 h were 11.5, 11.2, 6.5 and 6.4% respectively. Since there was no significant difference in the alcohol yield between the different incubation periods of the media (24, 48, 72 and 96 h) it was decided to use 24 h as the incubation period for the future experiments in order to save energy and time.

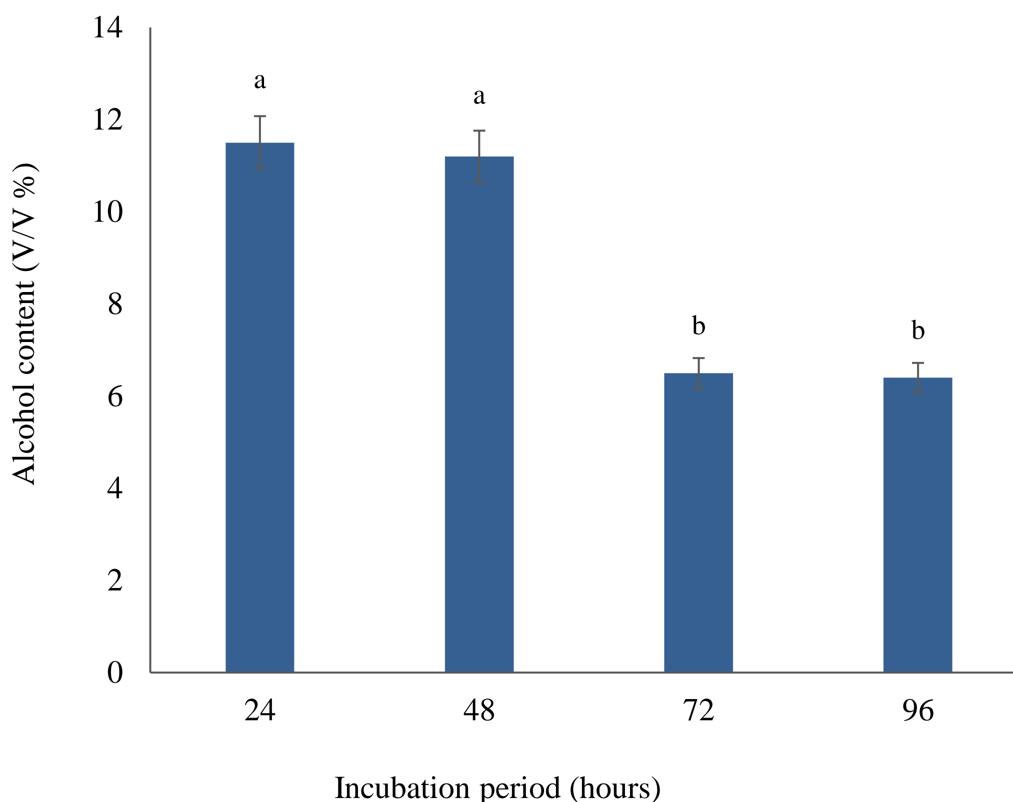


Figure 10: Effect of different incubation periods on bioethanol production from *Citrus* fruit juice using natural yeast (*Saccharomyces cerevisiae*) from palmyrah toddy. Different letters showed the significant differences between the mean values at $p<0.05$.

4. Discussion

The bioethanol production process from *Citrus aurantifolia* fruit juice using yeast is significantly influenced by various culture conditions, including the type of nitrogen source, inoculum size, temperature, rotation speed, concentration of raw fruit juice, amount of urea, carbon source, amount of sucrose, pH of the medium, and incubation period. These parameters are crucial in optimizing the bioethanol production yield and overall efficiency of the process. After conducting experiments to optimize the nitrogen sources for the bioethanol production process, it was determined that urea was the most effective nitrogen source, yielding a significantly higher amount of alcohol compared to the other nitrogen sources tested. Ammonia is known to be more soluble in water and also more toxic compared to urea, its accumulation during the bioethanol production process can result in adverse effects, such as elevated pH levels and cellular damage. Therefore, in order to minimize the risk of such complications and ensure a successful production outcome, urea was selected as the nitrogen source for subsequent studies (Chai and Adnan, 2018; El-Refai et al., 1992). When urea was used as a nitrogen source, ethanol production of 9.82% was obtained with sugarcane molasses by *Saccharomyces cerevisiae* Y17 (Rasmey et al., 2018).

Inoculum concentration does not have significant influence on final ethanol concentration but significantly affects sugar consumption rate and ethanol productivity (Laopaiboon et al., 2007). However, it was reported that ethanol production was increased with the increase in the initial cell numbers from 1×10^4 to 1×10^7 cells/mL and no significant difference in ethanol production was found between 10^7 and 10^8 cells/mL. Increased cell concentration within a certain range also reduces fermentation time considerably due to the rapid growth of cells in the fermentation media utilizing the fed sugars (Hosney et al., 2016). Hence 0.5 g/100 mL of yeast inoculum was chosen for further studies. Temperature is crucial element that is carefully regulated during fermentation as it has a vital impact on the fermentation and enzymatic action. The growth rate of the microorganisms is directly affected by the temperature (Charoenchai et al., 1998). The organisms which grow well at temperatures between 30 to 50 ^{o}C are called as thermophiles (Kapilan and Arasaratnam, 2010). When the culturing temperature was optimized as 35 ^{o}C, ethanol production was increased by 1.09 times, than the non-optimized condition, that is the room temperature (30 ^{o}C). Microorganisms produce heat-shock proteins in response to the high temperature and inactivate their ribosomes (Zabed et al., 2014). In addition, microbial activity and fermentation process are regulated by different enzymes which are also sensitive to high temperature since it denatures their tertiary structure eventually inactivating them (McMeekin et al., 2002; Kapilan and Arasaratnam, 2010). Moreover, Higher the temperature used lower the bioethanol production. The rate of enzyme reaction increases with increasing temperature up to a certain temperature and then the enzymes started to denature. Higher temperatures, normally inhibits the growth of the yeast and bacterial cells and significantly decreases the quantity or rate of fermentation (Zabed et al., 2014). The higher bioethanol yield of 60 ml/l was achieved from sewage sludge broth at an incubation period of 10 days at 30 °C by yeast (Manyuchi et al., 2018). Hence 35 ^{o}C, a moderate temperature was chosen for further culture growing studies.

Agitation plays major role in the production of higher ethanol yield after fermentation by increasing the permeability of nutrients from the fermentation broth to inside the cells and in the same way removing ethanol from the cell interior to the fermentation broth. Agitation also increases the sugar consumption and reduces the inhibition of ethanol on cells. Agitation rate commonly used is 150–200 rpm for yeast cells in fermentation (Zabed et al., 2014). If the agitation rate is high, it would affect the ethanol production by the restricted metabolic activities of cells. Hence 150 rpm rotation speed was chosen for further studies. Agitation enhances the production of higher ethanol by fermentation. This is possible by increasing the permeability of nutrients from the fermentation media to inside the cells and in the same method removing the bio ethanol from the inside of the cell to the fermentation media. Agitation also facilitates the sugar consumption and makes sure the rate of inhibition of the produced bio ethanol is low inside the cells (Liu and Shen, 2008). Since Initial sugar concentration has a direct

effect on fermentation rate and microbial cells, it directly influences the bioethanol yield (Zabed et al., 2014). Generally, fermentation rate will be increased with the increase in substrate concentration up to a certain level. But when the sugar concentration become excessive, the uptake capacity of the yeast cells leading to a steady rate of fermentation. Increased ethanol productivity and yield can be obtained at higher initial sugar concentration (Laopaiboon et al., 2007). Hence 100% substrate concentration was chosen for further studies. Higher concentration of nitrogen sources may inhibit the growth of yeast by their toxic nature in the fermentation medium & this might be the reason for the reduction of the ethanol production. Hence 0.1 g/100 mL of nitrogen source (urea) was chosen for further studies. When the different carbon sources were optimized a significantly higher alcohol yield was observed with sucrose was used and it may be due to its ability to make the yeast cells develop a foam surface for efficient fermentation than the other carbon sources. Therefore, sucrose was chosen as carbon source for the media in the future studies. The rate of anaerobic respiration in the yeast cells increases with the concentration of sucrose. If the availability of the substrate increases then there will be more cells involved in to use the substrate up for respiration, and this would lead to the increase in the amount of released CO_2. Higher concentration of ethanol may cause toxic effects to the yeast cells and it can retard the rate of cell respiration, or even lead to cell death (Phisalaphong et al., 2006). Higher concentrations of sucrose in the fermentation media might lead to decrease in the bioethanol production due to its inhibitory reaction. Hence a moderate 10 g /100 mL of sucrose concentration was chosen for further studies. Enhanced ethanol production through fermentation can be obtained by controlling pH of the broth as it is one of the key factors for ethanol production having direct influence on organisms as well as on their cellular processes (Kasemets et al., 2007, Piršelová et al., 1993). In general, H^+ concentrations in fermentation broth can change the total charge of plasma membrane affecting the permeability of some essential nutrients into the cells through the plasma membrane. When the fermentation medium is highly acidic, the fermentation rate also increases. This may be due to the enzymes produced by yeast to ferment glucose and these enzymes are highly active and adapted in acidic conditions. Yeast cells form resistant to acidic conditions than basic conditions. The organic and inorganic chemicals used in the media either directly or indirectly change the pH values of the media due to the different types of ions released. Hence pH 4.0 was chosen for further optimization studies. If the fermentation time is very short, it would reduce the growth of microorganisms eventually causing inefficient fermentation. On the other hand, longer fermentation time might lead to accumulation of toxic substances in the media especially in batch mode due to the presence of higher concentration of ethanol in the fermented broth (Nadir et al., 2009; Hossain et al., 2011; Asmamaw and Fassil, 2014).

5. Conclusion

Citrus juice has been identified as a valuable raw material for bioethanol production using palmyrah toddy yeast and has demonstrated efficient yields in this study. A higher ethanol yield of 11.50% was achieved under the following conditions: 100% of *Citrus aurantifolia* concentration, 35 °C of temperature, 24 h of incubation period, and 0.5 g/mL of inoculum concentration, 150 rpm rotation speed, 0.1 g/mL of urea, 10 g/mL of sucrose and pH of 4.0. Large scale fermentation study needs to be done with the bioreactor to determine whether the yield could be magnified and the process be commercialized.

6. Acknowledgement

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