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Effect of inoculum size and agitation speed on bioethanol production by *Kluyveromyces marxianus* using sugarcane molasse

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KEYWORDS

Agitation speed
Inoculum concentration
Kluyveromyces marxianus
Molasses

ABSTRACT

In this study, bioethanol production was carried out at a high temperature (40 ^{0}C) using *Kluyveromyces marxianus* in sugar cane molasses media with a study of combinations in fermentation conditions in the form of inoculum concentration and agitation speed. This study investigated the effect of inoculum concentration and agitation speed on bioethanol production. This study used a factorial randomized block design with two factors: inoculum concentration (with levels of 5%, 10%, and 15% (v/v)) and agitation speed (with levels of 100, 150, and 200 rpm). The results showed that the interaction of inoculum concentration and agitation speed significantly decreased pH, total sugar consumption, total reducing sugar (TRS) consumption, and total dissolved solids (TDS). However, an increase in cell optical density (OD), ethanol concentration, and ethanol yields was evident from all treatments. The best treatment was from a combination of 10% inoculum with an agitation speed of 150 rpm, giving the highest ethanol concentration of 3.44% and ethanol yields of 89.91%.

Introduction

The current world energy supply is still very dependent on the availability of various fossil fuels such as oil, coal, and natural gas. The most widely used is oil, with a light content of 27%. The scarcity of fossil energy sources, coupled with the enormous environmental impact caused by burning fossil fuels, encourages the search for alternative sources of renewable and safe energy for the environment (Sivarathnakumar et al., 2019). This aligns with the environmental pillar as one of the concepts of sustainable development in the sustainable development goals (SDGs). Bioethanol is today's most common and widely used alternative renewable energy source (Tsigie et al., 2013).

The efficiency of bioethanol production is strongly influenced by the accuracy of the selection of raw materials, types of microorganisms, and control of the fermentation process (Wardani et al., 2013). Molasses is a second-generation bioethanol raw material with high prospects in Indonesia. A previous study stated that bioethanol production using *S. cerevisiae* on molasses media had an optimum growth rate at 30 ºC and would be depressed when it reached 37 ºC (Wardani et al., 2013). Another study stated that bioethanol production at high temperatures could be carried out using a strain of *K. marxianus*. Based on research by Limtong et al. (2007), bioethanol fermentation using *K. marxianus* DMKU 3-1042 on sugarcane juice media can be carried out in a temperature range of 37-45 ºC, with ethanol concentration reaching 7.56% (w/v) at 37 ºC.

The control of factors influencing fermentation is essential to optimize bioethanol production. A previous study reported that optimal conditions for bioethanol production should consider the inoculum concentration and the agitation speed during fermentation (Rastegari, 2019). A previous study also stated that bioethanol fermentation in sugarcane juice media using *K. marxianus* strain was optimal at 5% (v/v) inoculum concentration at 37 ^{o}C with an agitation speed of 300 rpm for 72 hours (Limtong et al., 2007). Another study stated that bioethanol production from blackstrap molasses media using *K. marxianus* strain was optimal at an agitation speed

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of 150 rpm with an inoculum concentration of 5% (v/v) at 33 ºC for 72 hours (Eiadpum et al., 2012).

Based on the description above, molasses has the potential as a carbon source for bioethanol production using the yeast *K. marxianus*. The many variations of inoculum concentration factors and agitation speed in previous studies also encouraged researchers to find the best conditions for molasses fermentation using *K. marxianus*. This study aimed to investigate the effect of inoculum concentration and agitation speed on bioethanol production.

Material and Methods

Materials

Molasses were freshly collected from PG Krebet Baru Malang. Other materials were strain *Kluyveromyces marxianus* UB-5, aquadest, H_2SO_4, NaOH, Na-EDTA, growth media (Yeast extract 1%, Glucose 2%, Peptone 2% , Agar 1.5%), phenol, DNS reagent, cotton, plastic wrap, aluminum foil, brown paper, rubber, petromax plastic, spirit, and 70% alcohol.

Yeast K. marxianus UB-5 used was isolated from soil in the Wonokromo River, Surabaya (Putri, 2019), which was stored in the Bioindustry Laboratory, Department of Agricultural Industrial Technology, Faculty of Agricultural Technology, Universitas Brawijaya.

Experimental set-up

This study used a factorial Randomized Block Design (RBD) with two factors. The first factor was inoculum concentration of 5% (v/v), 10% (v/v), and 15% (v/v). The second factor was agitation speed, which consisted of 100 rpm, 150 rpm, and 200 rpm. In total, there were 9 treatment combinations. Sampling for parameter analysis was done once every 12 hours and all were repeated in triplicate.

Fermentation procedures

As much as 100 mL of pretreated molasses was inoculated with inoculum with concentration variations of 5%, 10%, and 15% (v/v) in vials clear glass size 100 mL. The glass bottle was closed and placed on a water bath shaker at a temperature of 400ºC and treatment settings were carried out at agitation speed with variations of 100 rpm, 150 rpm, and 200 rpm. Sampling for analysis of optical density parameters was carried out at 8, 24, 33, and 48 hours, while taking samples for parameter analysis. Ethanol content, reducing sugar, and yield were measured at 0 and 48 hours. The fermentation treatment was repeated in triplicate.

Parameters analysis

Reducing sugar test was carried out on molasses before pre-treatment, after pre-treatment, and at 0 and 48 hours of fermentation process. The concentration of reducing sugars in foodstuffs can be determined based on their ability to reduce other reagents. The analysis of reducing sugars was done using the DNS method (Miller, 1959).

Analysis of total sugar was performed using the phenol-sulfate method (Dubois et al., 1956). The total sugar was measured at hour - 0 (before fermentation after pretreatment) and hour to – 48 (end of fermentation before distillation).

Total dissolved solids (TDS) was measured on molasses before pretreatment, after pretreatment, and fermentation at 0 and 48 hours. TDS were measured using a refractometer with the unit % Brix.

The optical density (OD) measurement was carried out based on the absorbance value of the sample using a spectrophotometer. The OD was measured at 8, 24, 33, and 48 hours by taking 1 mL of sample and placed into a 2-mL microtube, then centrifeged at 14,000 rpm for 5 minutes.

Ethanol content was analysed at the 48th-hour of fermentation process using an alcohol meter. Ethanol yield was calculated to evaluate the substrate's efficiency in producing ethanol.

Statistical analysis

The two-way analysis of variance (ANOVA) was used in this study as it simultaneously tested the effect of two factors on the dependent variable. Duncan's Multiple Range Test (DMRT) test was carried out to determine the significant difference in the effect on each treatment. The DMRT test was also used to determine the best treatment by selecting the highest average results . The statistical data analysis software used was SPSS 16.0.

Best treatment selection

The best treatment was determined using the multiple attribute method (Zeleny, 1982). Test multiple attributes used to clarify the results of the ANOVA and DMRT tests based on value ideal both maximum and minimum of all parameters tested (Retnaningtyas et al, 2014).

Results and Discussion

Growth Profiles of Kluyveromyces marxianus

Figure 1 shows that the culture of *K. marxianus* UB-5 might have undergone an adaptation phase from 0 to 5 hours. This was followed by a logarithmic phase at 5 hours to 24 hours, indicated

by the highest increase in the OD value of cells between 12-24 hours. Then, it entered the stationary phase at 36 – 72 hours. While the death phase was still not visible as the curve was still on a stagnant pattern. Therefore, the starter age was selected based on the highest OD increase in the cell before reaching the stationary phase. The figure indicates the increase in cell OD peak before the stationary phase was at 24 hours. Thus, the age of the starter used was the 18th hour, as it is still within the logarithmic phase and has the fastest growth rate. This aligns with Rodrussamee et al. (2011), who stated that the exponential phase in *K. marxianus* in various media occurs between 12 – 24 hours. According to Khongsay et al. (2010), determining the appropriate starter age helps the cell to adapt to the fermentation medium, thus improving the efficacy of fermentation.

pH

pH measurement was carried out by dipping the pH meter into the sample solution to be measured. Ensure all the pH meter probes are submerged and wait for the pH number to appear. The working principle of the pH meter is to measure the amount of H_3O^+ ions in the solution by a glass electrode (Mujadin et al, 2017). Figure 2 shows a decrease in pH after 48 hours of fermentation, indicating the presence of CO_2 and organic acids from cell metabolism during fermentation. Similarly, Fadilah et al. (2018) stated that a decrease in pH occurs due to the presence of acidic released CO_2 gas. Itelima et al. (2013) also stated that a decrease in pH might also occur due to organic acids accumulation during fermentation. Moreover, Suryawati et al. (2008) proved that *K. marxianus* could produce acetic acid during fermentation. The high acetic acid can lower the pH of the fermentation medium and become an inhibitor of cell growth during fermentation. A decrease in pH could affect enzyme denaturation, thus decreasing the effectiveness of enzyme production.

On this parameter, two-way ANOVA was carried out to determine the effect of inoculum concentration and speed of agitation on decreasing pH during the molasses fermentation process by Kluyveromyces marxianus. The results show that the pH decreased during the fermentation. This indicates that the interaction between both factors had a significant effect (sig<0.05) on the decrease in pH during fermentation.

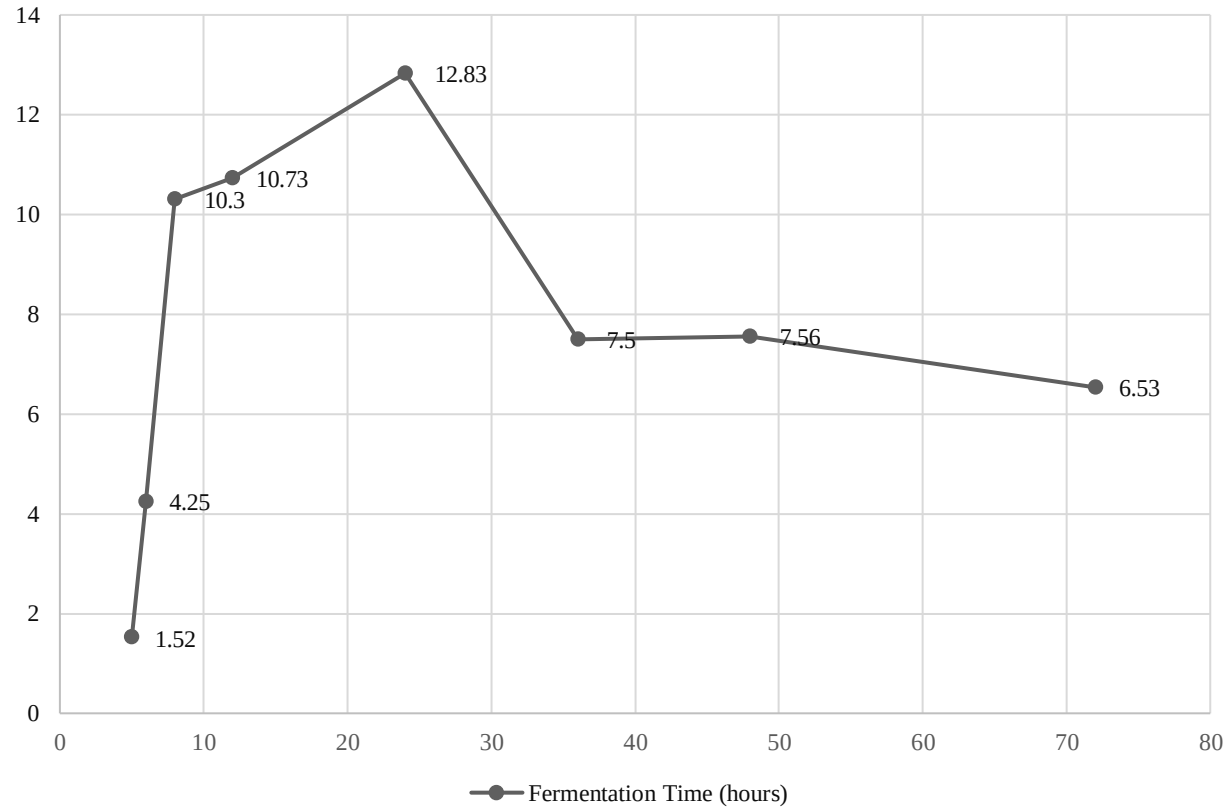

Figure 1. *Kluyveromyces marxianus* UB-5 culture growth curve

Based on Figure 2, increasing inoculum concentration and agitation speed was parallel to a decrease in pH. However, at an agitation speed of 200 rpm only a slight decrease in pH was observed. According to Rodmui et al. (2008), agitation speed affects the pH of fermentation, in which the higher the agitation speed, the higher the pH value results at the end of the fermentation. This could be due to a high dissolved oxygen concentration in the fermentation medium. In addition, the higher the inoculum concentration, the higher the number of cells, leading to an increase in organic acids and carbon dioxide and a significant decrease in pH value.

Based on Table 1, the treatment combinations have different subset notations, indicating a significant difference between treatments. The treatment with the highest pH reduction (0.87) was from treatment with a combination of 10% inoculum concentration and agitation speed of 150 rpm. This result was significantly different compared with other treatments. While, the treatment with the lowest pH reduction (0.47) was from treatment with a combination of 10% inoculum concentration and agitation speed of 100 rpm. The treatment with the same notation indicated that no significant difference was observed.

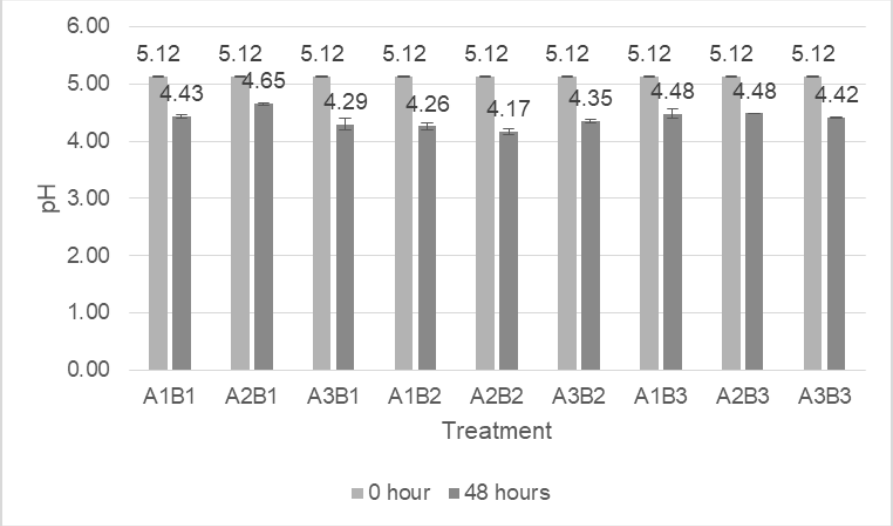

Figure 2. Effect of inoculum concentration and agitation speed on pH during fermentation. Error bars represent standard deviation of three measurement.

Table 1. Effect of inoculum concentration and agitation speed on reduction rate of pH, TGR, and TDS, OD, and ethanol concentration

Agitation speed (rpm)	Inoculum concentration (%)	pH reduction rate*	TGR reduction rate (%w/v)**	TDS reduction rate (%w/v)***	OD increase rate	Ethanol concentration (%)
100	5	0.69 ± 0.012^{bc}	3.19 ± 0.178^{a}	5.40 ± 0.200^{a}	1.05 ± 0.070^{a}	2.44 ± 0.103^{b}
	10	0.47 ± 0.012^{a}	3.45 ± 0.143^{b}	5.77 ± 0.351^{b}	1.23 ± 0.291^{ab}	2.95 ± 0.026^{d}
	15	0.83 ± 0.087^{d}	3.91 ± 0.232^{cd}	6.10 ± 0.173^{c}	1.22 ± 0.367^{ab}	3.06 ± 0.113^{e}
150	5	0.87 ± 0.067^{de}	4.08 ± 0.074^{d}	6.40 ± 0.173^{c}	1.59 ± 0.225^{b}	3.30 ± 0.138^{g}
	10	0.95 ± 0.038^{e}	3.83 ± 0.131^{c}	5.50 ± 0.100^{ab}	1.19 ± 0.070^{ab}	3.44 ± 0.052^{h}
	15	0.77 ± 0.049^{cd}	3.85 ± 0.190^{c}	7.30 ± 0.300^{d}	2.69 ± 0.095^{c}	3.18 ± 0.051^{f}
200	5	0.64 ± 0.091^{b}	4.38 ± 0.348^{e}	9.00 ± 0.200^{g}	4.35 ± 0.306^{d}	2.20 ± 0.035^{a}
	10	0.64 ± 0.020^{b}	4.43 ± 0.283^{e}	8.00 ± 0.200^{e}	6.03 ± 0.261^{f}	2.71 ± 0.071^{c}
	15	0.70 ± 0.015^{bc}	4.82 ± 0.180^{f}	8.53 ± 0.058^{f}	5.01 ± 0.042^{e}	2.44 ± 0.121^{d}

*****The reduction rate in pH was obtained from the result of reducing the pH value at -48 hours with a pH value at -0. ** The reduction rate of TRS was obtained from the result of reducing the TRS value at -48 hours with the TRS value at -0. *** The reduction rate of TDS was obtained from the result of reducing the TDS value at -48 hours with the TRS value at -0. Different alphabetical letter indicates significant differences at $P<0.05$

Total sugar

Based on Figure 3, it can be seen that there was a decrease in total sugar during fermentation for 48 hours. A decrease in total sugar indicated sugar consumption by *K. marxianus* UB-5 to be converted into energy and ethanol. According to Hawusiwa et al. (2014), a decrease in total sugar concentration occurs due to the breakdown of glucose and fructose by yeast into pyruvic acid. This pyruvic acid is later transformed through a decarboxylation process to acetaldehyde, which then undergoes dehydrogenation into ethanol. This is evidenced by Rodrussamee et al. (2011), that Kluyveromyces marxianus can utilize various sugars, including glucose, mannose, galactose, and xylose. The ANOVA results also showed a significant effect (P<0.05) from a combination of inoculum concentration and agitation speed in decreasing total sugar during fermentation.

Figure 3 shows that the higher the inoculum concentration and agitation speed, the lower the total sugar. According to Rodmui et al. (2008), agitation speed affects total sugar consumption during fermentation, where the higher the agitation speed, the higher the cell growth rate and biomass formation. In the duplication process, yeast cells need sugar as a carbon source to produce energy. Zhang et al. (2011) also stated that increasing inoculum concentration causes an increase in cell number and fastens the substrate utilization process.

Table 1 shows that the treatment combinations have different subset notations, indicating a significant difference between treatments. The treatment with the highest reduction in total sugar (9.03% w/v) was from a combination of 5% inoculum concentration and an agitation speed of 200 rpm. While treatment with a combination of 5% inoculum concentration and 100 rpm agitation speed had the lowest reduction in total sugar of 5.33% w/v.

Total reducing sugar

Figure 4 indicates a decrease in TRS after 48 hours of fermentation, which could be due to its consumption by microorganisms to convert sugar molecules into energy and ethanol. This is in accordance with Putri (2016) that a decrease in TRS in the fermentation media can occur due to the continuous use of the reducing sugar by yeast for cell growth and conversion to ethanol. The figure also shows that increasing inoculum concentration and agitation speed was parallel to reduced TRS. A previous study by Putri (2016) has proven that increasing the number of cells in the fermentation media decreases TRS values and increases ethanol yields. Similarly, Rodmui et al. (2008) also found that agitation speed positively affects better media utilization. Thus, it could shorten cells' adaptation phase and increase biomass formation from the conversion of reducing sugars. The ANOVA results showed that all treatments significantly reduced TRS during fermentation (P<0.05). Therefore, a DMRT test was carried out to investigate the interaction of inoculum concentration and agitation speed. The results showed significant differences, as shown in Table 1. Again, treatment with a combination of 15% inoculum concentration and 200 rpm agitation speed had the highest TRS value (4.82% w/v). While, the lowest was generated from treatment with a combination of 5% inoculum concentration and 100 rpm agitation speed, giving the value of 3.19% w/v.

Total dissolved solids

Figure 5 shows a decrease in TDS after 48 hours of fermentation, directly proportional to a reduction of TRS value. This trend could be due to sucrose consumption, which is the highest sugar component in molasses (Jung et al., 2013). According to O'Hara and Mundree (2016), molasses contains water, vitamins, organic and inorganic compounds, with sucrose in a high percentage. The amount of sucrose is often calculated as TDS in % Brix units. Also, prior to fermentation, pretreatment has been subjected to molasses to remove contaminants and inhibitors, thus reducing the TDS values. The results indicate that TDS values were approximately 2% higher than TRS values, indicating the presence of non-sugar solids in molasses, which could inhibit bioethanol fermentations. The ANOVA results also show similar trends of a decrease in TDS during fermentation. Increasing inoculum concentration and agitation speed reduces the TDS values, as shown in Figure 5.

This result is in line with Rodmui et al. (2008) that an increase in the dissolved oxygen content increased the cell's number of cells, triggering a higher substrate utilization process to form energy and ethanol. The study also confirmed a significant difference between treatments. As shown in Table 1, treatment with 5% inoculum concentration and 200 rpm agitation speed had the highest TDS value of 9% w/v. While the lowest TDS value (5.40 %w/v) was obtained from treatment with 5% inoculum concentration and 100 rpm agitation speed.

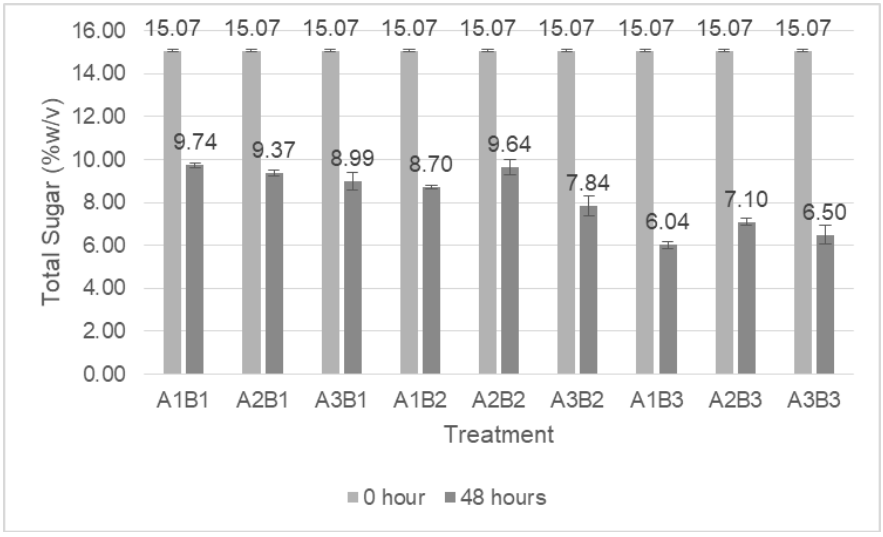

Figure 3. Effect of inoculum concentration and agitation speed on total sugar during fermentation. Error bars represent standard deviation of three measurement.

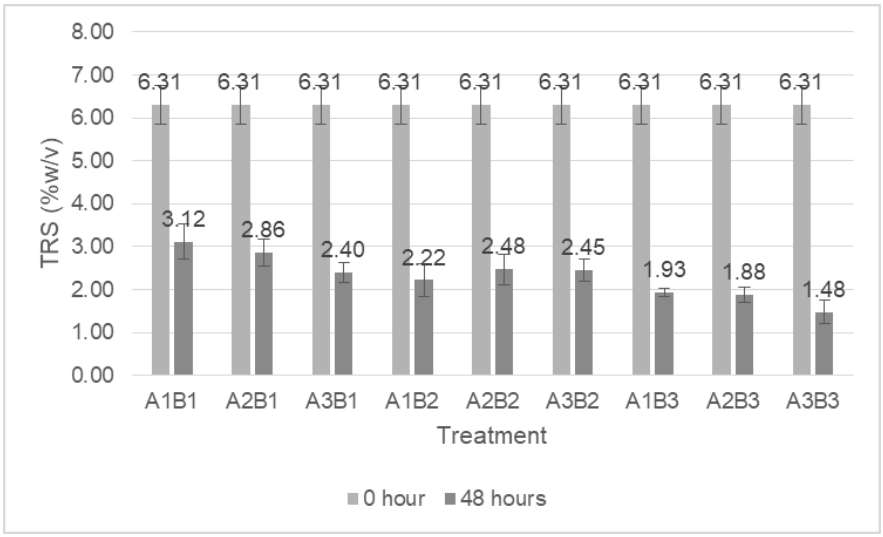

Figure 4. Effect of inoculum concentration and agitation speed on total reducing sugar (TRS). Error bars represent standard deviation of three measurement.

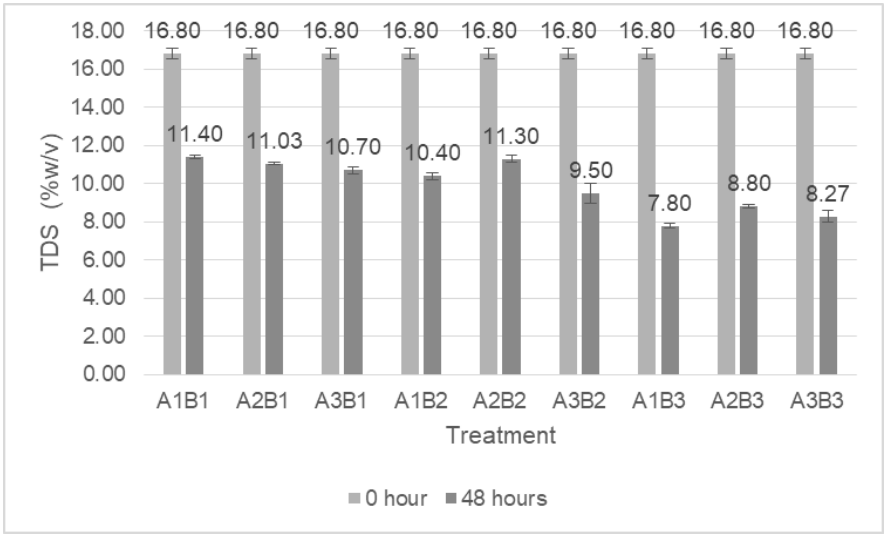

Figure 5. Effect of inoculum concentration and agitation speed on total dissolved solids (TDS) during fermentation. Error bars represent standard deviation of three measurement.

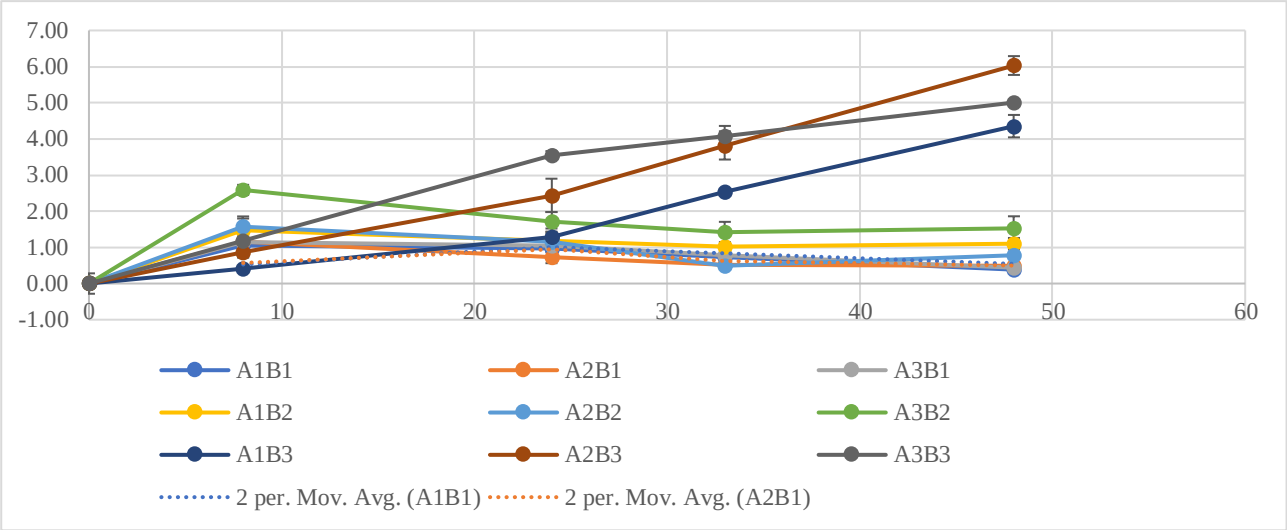

Figure 6. The trends in optical density (OD) cells during fermentation. Error bars represent standard deviation of three measurement.

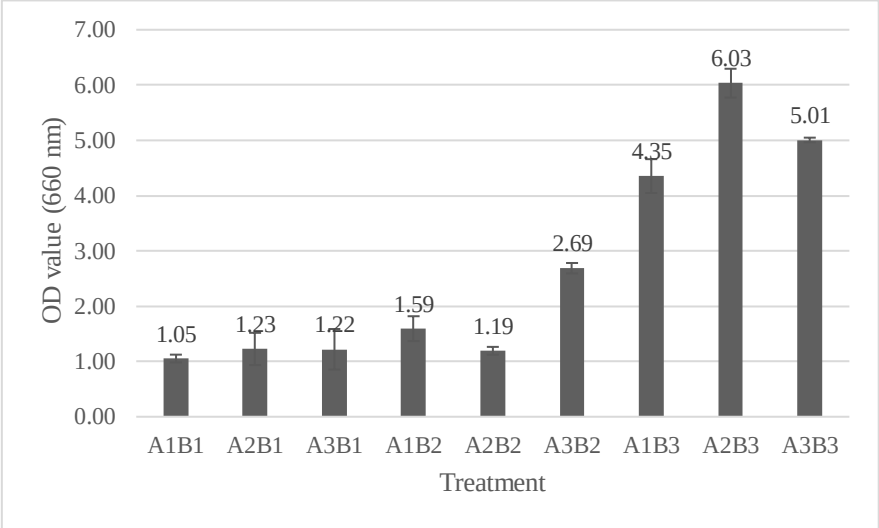

Figure 7. The average value of optical density (OD) cells. Error bars represent standard deviation of three measurement.

Optical Density (OD) of cells

Figure 6 shows that increasing inoculum concentration was parallel to an increase in the OD cells. Koutsoumanis et al. (2005) stated that high inoculum concentrations allow better growth initiation than low inoculum concentrations. They found that high inoculum concentrations may give microorganisms a higher survival ability under certain environmental conditions. However, an increase in agitation speeds was found to lengthen the logarithmic phase. For example, at 100 rpm of agitation speed, the logarithmic phase occurred until 8 hours of fermentation. Similarly, at 150 rpm agitation speed, the logarithmic phase also occurred until 8 hours of fermentation but with a slightly higher rate of cell increase. However, at 200 rpm agitation speed, the cells still experienced a rapid increase until 48 hours of fermentation. According to Rodmui et al. (2008), agitation speed positively affected cell growth by increasing the mass transfer of substrate, by-products, and oxygen. In addition, the agitation speed also affected the dissolved oxygen of the substrate. High dissolved oxygen is beneficial for cell growth, promoting the yeast to increase ATP production (or energy) through the aerobic respiration pathway for cell duplication.

Figure 7 shows the average values of OD cells, which support the findings that increasing agitation speed increases the cell's numbers. Diana (2013) reported that the standard TPC curve regression could show the relationship between

OD and the number of cell colonies. Their study found that the regression equation for the standard curve of OD to TPC was y = 459.98x - 106.64; thus, the culture has an OD value of 2.284 and a colony number of $94.41x10^{11}$ CFU/mL. Furthermore, based on the ANOVA results (Table 1), the interaction between the inoculum concentration and the agitation speed was evident and had a significant effect on increasing the OD cells during the fermentations (P<0.05). The results demonstrate that treatment with 10% inoculum concentration and 200 rpm agitation speed gave the highest increase rate in the OD cells value (6.03 %). While the lowest increase rate of OD cells (5.33 %) was generated from treatment with 5% inoculum treatment and 100 rpm agitation speed.

Ethanol production

Figure 8 shows that an increase in inoculum concentration from 5% to 10% positively impacts increasing bioethanol content. However, if the inoculum concentration was above 15%, it may reduce the bioethanol content. This is in accordance with Sar et al. (2019) that the inoculum concentration of 10% produces optimum bioethanol content and continues to decrease when the inoculum concentration increases between 20-30%. On the other hand, increasing agitation speed from 100 rpm to 150 rpm positively increased bioethanol content, and a different trend was observed at 200 rpm. Rodmui et al. (2008) stated that increasing agitation speed was correlated to an increase in dissolved oxygen essential for cell growth but not for bioethanol production. According to Mo et al. (2019), when ethanol concentration increases up to the tolerance limit, it can inhibit cell growth and even kill the cells. Therefore, it is essential to identify the ethanol tolerance limit in each culture to determine the ethanol production capacity. *Kluyveromyces marxianus* UB-5 has an ethanol tolerance limit of 6% on YPD media.

Table 1 shows that the interaction between inoculum concentration and agitation speed significantly affected the bioethanol content (P<0, 05). Therefore, a further test of DMRT was carried out, indicating a significant difference between treatments was observed. The treatment with the highest ethanol content (3.44% v/v) was from a combination of 10% inoculum concentration and 150 rpm agitation speed. While the lowest ethanol content (2.20% v/v) was from treatment with 5% inoculum treatment and 200 rpm agitation speed. These findings indicate that both inoculum concentration and agitation speed influence the efficiency of fermentation, thus affecting the bioethanol content.

Best treatment selection

Table 2 indicates that treatments with an agitation speed of 150 rpm at all concentrations perform better than other counterparts. Specifically, treatment at an inoculum concentration of 10% gave the lowest score value, thus given as rank 1 and selected as the best treatment. This was followed by treatment with the same speed at inoculum concentrations of 15% and 5%, respectively. The lowest ranks were from treatment at an agitation speed of 200 rpm with 5% and 20% concentrations. The selected best treatment has a higher ethanol content of 3.44% and gave the highest increase rate in OD cells and the highest reduction rate in total sugar, TRS, and TDS, respectively.

Table 2. Determination of the best treatment

Agitation Speed (rpm)	Inoculum Size (%)	Score				
		L1	L2	L3	Result Total	Rank
100	5	0.210	0.014	0.103	0.327	5
	10	0.218	0.014	0.100	0.332	6
	15	0.239	0.013	0.100	0.352	7
150	5	0.200	0.010	0.092	0.303	3
	10	0.126	0.011	0.100	0.236	1
	15	0.210	0.008	0.069	0.287	2
200	5	0.365	0.017	0.055	0.437	9
	10	0.264	0.010	0.041	0.315	4
	15	0.335	0.015	0.055	0.404	8

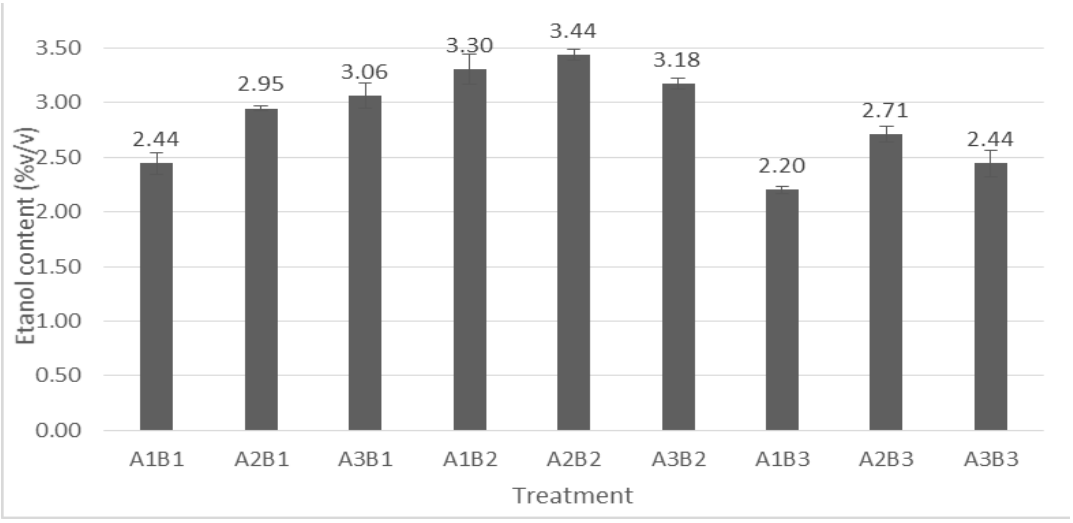

Figure 8. The ethanol content after 48 hours of fermentation. Error bars represent standard deviation of three measurement.

Conclusions

This study confirmed that variations in inoculum concentration and agitation speed affected the fermentation efficiency using *Kluyveromyces marxianus* UB-5 strain. The best treatment was obtained from 10% inoculum concentration with 150 rpm agitation speed. This treatment had the highest ethanol content and yields of 3.44% and 89.91%, respectively. The selected best treatment also had the highest reduction rate in total sugar, TRS, and TDS, as well as the highest increase rate in OD cells. Further in-depth study is required to evaluate the optimum fermentation condition using *Kluyveromyces marxianus* UB-5 strain for optimum bioethanol production.

Declarations

Conflict of interests The authors declare no competing interests.

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