Substrate utilization of ethanologenic yeasts co-cultivation of
_Pichia kudriavzevii_ and _Saccharomyces cerevisiae_

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Abstract. Bioethanol is one of the renewable alternative energies and can be used as a substitute for fossil fuels. Bioethanol is produced from a fermentation process, which is assisted by microbial yeast groups (ethanologenic yeast). Yeast _Pichia kudriavzevii_ is capable of converting both carbon source pentose and hexose sugars to ethanol. In the previous study, _P. kudriavzevii_ ethanol stress - tolerant mutant strains T5 was successfully constructed via directed mutagenesis thus suggesting their potential as fermentation agent. One strategy to increase ethanol production is by applying co-cultivation techniques. Thus, this study aimed to examine the ability of sugar substrate utilization by ethanologenic yeast _S. cerevisiae_ and _P. kudriavzevii_ at various inoculum ratio. The results of the study indicated that no antagonist interaction detected between _S. cerevisiae_ with all of _P. kudriavzevii_, both mutant and wild type strains. Based on sugar consumption analysis, both yeast isolates could be used in ethanol production simultaneously to maximize mixed substrate utilization. Our study revealed that the best sugar consumption found on co-cultivation of _S. cerevisiae_ with wild type _P. kudriavzevii_ T (wt) in 1: 1 inoculum ratio.

Keywords: Bioethanol, co-cultivation, fermentation, reducing sugar

1. Introduction

Increased demand of fossil fuel causes a decline in petroleum reserves. This is because of petroleum is a non-renewable natural resource, it has limited amount and the production of petroleum oil requires up to million years. These conditions can not be solved by conserving the usage of fuel, instead by utilizing alternative fuels that can be produced sustainably. Currently, one of the alternative fuels that has been developed is bioethanol. Based on research, ethanol is utilized as a substance for gas mixture that is called biogasoline. Biogasoline has several advantages, such as increase in octane number, produce a more efficient combustion, and also reduce CO2 gas emission [1].

The efforts to increase the production of ethanol have been carried by utilizing lignocellulose hydrolysate obtained from agricultural wastes. Liquefaction and hydrolysis treatment are required to convert the complex lignocellulose into monomers [2]. Microbes can then utilize the resulted simple sugars as a fermentation substrate to produce ethanol. Ethanologenic yeast is a microbe that has a significant role in ethanol fermentation. According to the research, industrial yeast such as _Saccharomyces cerevisiae_ has proven to be efficient and rapid in fermenting hexose sugar (C6) into ethanol [3]. Amongst ethanologenic yeasts, _Pichia_ spp. is gaining serious attention by scientist, as this group of yeast has several advantages, such as utilizing pentose sugar (C5) and hexose as substrates for ethanol fermentation, where not all yeast able to utilize pentose sugar for its metabolic process.
During the fermentation process, yeast experiences stress condition, such as high ethanol content stress. Therefore, application of ethanol stress-resistant yeast is a strategy to increase the production of ethanol. T5 mutant yeast was previously constructed through adaptive mutant selection, so it has superior trait to survive in high ethanol stress condition (15%) [4]. The application of co-cultivation ethanol stress-resistant mutated yeast is expected to increase cell viability and optimize substrate usage during the ethanol fermentation process.

The yeasts that were used in this research including wild type S. cerevisiae, wild type Pichia kudriazvveii (T(wt)) [5], and P. kudriavzveii T5 mutants [4]. Based on the research, fermentation with co-cultivation method provides an opportunity for simultaneous conversion of glucose and xylose substrates [6]. Therefore, the ratio of inoculum in yeast co-cultivation of S. cerevisiae and P. kudriaevzei needs to be determined to maximize the use of sugar substrates. The increase of yeast cells in fermentation media will also result in increased substrate consumption, thus resulted in ethanol production [7]. The objective of this research was to test the ability of ethanologenic yeasts including S. cerevisiae and P. kudriavzevii in consuming sugar substrate at various inoculum ratios in a co-cultivation treatment.

2. Materials and methods

2.1. Interaction test between isolates

Interaction test between isolates started by enrichment of S. cerevisiae in Yeast Peptone Dextrose Broth (YPDB) media and incubated for 24 hours. YPD media (1 L) composed by 10 g yeast extract, 20 g peptone, 20 g D-glucose, and 20 g agar. S. cerevisiae as much 500 µL (1%) was inoculated inside of liquefied Yeast Peptone Dextrose Agar (YPDA) media at ±50 °C, then poured into sterile petri dish. After the media has been solidified, P. kudriaevzei T wild type (wt) and T5 mutant were streaked on the agar plate surface using inoculation loop in room temperature for 48 hours. Observation was conducted by observing the presence, of clear zone around P. kudriaevzei colonies. The presence of clear zones indicated antagonistic interaction between two isolates, while the absence of clear zones indicated potential of synergistic interaction between two isolates.

2.2. Fermentation test using oxidative fermentative media

Fermentation test was conducted based on a method described elsewhere [8]. Each of P. kudriaevzei T(wt), T5 mutant, and S. cerevisiae yeasts were inoculated in various sugar substrate in oxidative fermentative (OF) media. The treatments consisted of co-cultivation of P. kudriaevzei and S. cerevisiae in 1 OF media and yeast isolate inside 1 OF media (control). Observation was conducted after inoculation and incubation for 48 hours by observing the color change of agar media from blue to yellow, which indicated decrease of pH inside of the media.

2.3. Effect of inoculum ratio on reducing sugar content

Starter culture was produced by enrichment of P. kudriaevzeii T(wt), T5 mutant, and S. cerevisiae in YPDB media. After 16 hours of incubation, optical density (OD) measurement was done using spectrophotometer at 600 nm wavelengths. Isolates with OD of 0.6-0.8 were used as starter culture. As much as 1% starter inoculated inside of YPDB medium that contained mixture of 2% glucose and 2% xylose. The ratios of S. cerevisiae and P. kudriaevzei were 1:10, 1:1, and 10:1 in each media. Reducing sugar content measured for every 8 hours with dinitro salicylic acid (DNS) method (Miller 1959). DNS reagent (1 L) composed of 6.3 g DNS, 182 g Rochelle salts (potassium sodium tartrate), 5g phenol, 5g sodium bisulfite, and 21.4 g sodium hydroxide. Sugar content was measured by pipetting 0.5 mL of the sample then put inside of 1.5 mL micro tube and centrifuged at 10.000 rpm for 10 minutes. The amount of 0.01 mL supernatant was pipetted, then added 0.99 mL sterile water and 2 mL DNS. Test tubes were homogened using vortex and heated at 100 °C for 10 minutes. Then, the test tubes were cooled at room temperature and sugar content was measured using spectrophotometer at 540 nm wavelength.
3. Results

3.1. Interaction test between isolates

The result of interaction test between isolates of *S. cerevisiae* with *P. kudriavzevii* is shown at figure 1. The result showed that there were no clear zones around the colonies. This result indicated that between *S. cerevisiae* and two isolates of *P. kudriavzevii* in this research were not antagonistically interacted with each other.

![Figure 1. The interaction between *S. cerevisiae* yeast isolates (spread on media) and *P. kudriavzevii* T (wt) and T5 mutants (arrow).](image)

3.2. Fermentation test using oxidative fermentative media

Fermentation test treatment was conducted to determine yeast ability in utilizing various carbon sources as a fermentation substrate to produce ethanol. Carbon sources that were used in this research were glucose, sucrose, maltose, xylose, and dextrose. The result of fermentation test showed that each yeast isolate had different ability in utilizing carbon source (table 1). The ability depends on metabolism type of each yeast genera. Treatment using single isolate and co-cultivation was conducted to determine the fermentation rate based on colour change in agar media (table 1) and final pH after incubation for 48 hours (table 2).

![Table 1. Color changes in OF media with various carbon sources after 48 hours fermentation by *S. cerevisiae* and *P. kudriavzevii* (T (wt) and T5).](image)

| Isolate                  | Carbon Source | Glucose | Sucrose | Maltose | Xylose | Dextrose |
|--------------------------|---------------|---------|---------|---------|--------|----------|
| T (wt)                   | +++++         | ++      | +++++   | ++      | +++    |
| T5                       | +++++         | +       | +++     | +       | ++     |
| *S. cerevisiae*          | +++++         | +       | +++     | +       | +      |
| *S. cerevisiae* + T (wt) | +++++         | +       | +++     | +       | +++    |
| *S. cerevisiae* + T5     | +++++         | +       | +++     | +       | +++    |

(*) Indicates that there is a change in blue to yellow, more + that can be more yellow

Beside of color change of the media, pH change could also be an indicator of yeast’s ability to do fermentation. The results of difference in reduced initial pH (6.0) to end pH were showed in table 2.

3.3. Effect of inoculum ratio on reducing sugar content

Measurement of reduced substrate content was done to observe the amount of glucose-xylose content used by yeast at a certain time and to compare the most potential inoculum ratio. Substrate used in this research was 2% glucose and 2% xylose on YPDA medium with inoculum ratios of 1:10, 1:1, and
10:1. Measurement result of glucose-xylose in media with single yeast treatment and co-cultivated *P. kudriavzevii* T (wt) showed in figure 2. The results showed that co-cultivation treatment of *P. kudriavzevii* T (wt) and *S. cerevisiae* indicated that there was a higher substrate utilization compared to single culture treatment (figure 2).

**Table 2.** Changes of pH value in the treatment of single isolates and co-cultivation of *S. cerevisiae* with *P. kudriavzevii* T (wt), and T5 on various carbon sources after 48 hours incubation.

| Isolates               | Carbon source | Glucose | Sucrose | Maltose | Xilose | Dextrose |
|------------------------|---------------|---------|---------|---------|--------|----------|
| T (wt)                 |               | 5.0     | 6.0     | 6.0     | 5.0    | 5.5      |
| T5                     |               | 4.5     | 5.5     | 6.0     | 5.2    | 6.0      |
| *S. cerevisiae*        |               | 4.5     | 6.0     | 6.0     | 6.0    | 6.0      |
| *S. cerevisiae* + T (wt) |             | 4.0     | 5.5     | 5.8     | 5.0    | 5.7      |
| *S. cerevisiae* + T5   |               | 4.0     | 5.3     | 6.0     | 5.0    | 6.0      |

**Figure 2.** Measurement of reducing sugar on co-cultivation of *S. cerevisiae* and *P. kudriavzevii*T (wt) (a) ratio of 1:10 (b) ratio of 1:1 (c) ratio of 10:1 at 2% glucose + 2% xylose mixed substrate.
Ratio comparison between 1:10 (figure 2a) and 1:1 (figure 2b) showed a very promising result, because utilization of total substrate content in co-cultivation treatment is higher than single isolate, respectively 65.6% and 66.6%. The high activity of glucose-xylose substrate consumption was shown during the 0-24-hour period, while during 24-48-hour period there was a decrease in substrate consumption.

Measurement result of glucose-xylose in single isolate treatment and co-cultivation of S. cerevisiae and P. kudriavzevii mutant T5 showed in figure 3. The result showed that the utilization of substrate for 48 hours in co-cultivation treatment was lower than single isolate treatment. Sugar substrate content that were used in co-cultivation were 1:10, 1:1 and 10:1, respectively 51.6%, 54.2%, and 55%. The sugar contents were not significantly different with substrate content that was used by single isolate S. cerevisiae with the 10 times concentration, which was 62%. The results indicated that substrate consumption activity on mutant T5 treatment with inoculum ratio 10:1 was mostly carried out by S. cerevisiae.

4. Discussion
Interaction test between S. cerevisiae with P. kudriavzevii was done to determine the interaction between two isolates. Based on the test result, both of yeast isolates could be applied in co-cultivation method for ethanol production. Yeast co-cultivation method in ethanol production needs cooperation
of both microbial communities in optimizing the use of sugar substrate that will be converted to ethanol. Certain microbes need other microbes to grow optimally (synergistic) and vice versa. The presence of other microorganisms in the particular environment could actually inhibit the growth of these microbes (antagonistic) [9]. Co-cultivation method was also reported previously between \textit{P. kudriavzevii} or \textit{S. cerevisiae} yeast with brewer’s yeast in brewing process [10].

The research result showed that all single isolates treatment of \textit{P. kudriavzevii} and all of co-cultivation treatment of both yeasts could utilize carbon sources such as glucose and maltose. \textit{P. kudriavzevii} yeast was able use xylose as carbon source, but needed a longer incubation time to show colour change in OF media. Based on result, \textit{S. cerevisiae} could utilize glucose and maltose substrate [8]. The ability of fermentation was showed by colour change in OF media from blue to yellow and gas formation inside the Durham tube. The gas bubble was formed from respiration process when using sugar substrate. Sugar then converted to CO$_2$ and H$_2$O. The increase of CO$_2$ gas also lowered pH level [11].

Colour change in media after incubation time is one of the indicators of fermentation and production of acidic compounds. Addition of bromo thymol blue (BTB) indicator in OF media resulted in pH decline and colour change of BTB indicator into yellow [12]. The research result showed the differences in single isolate treatment and co-cultivation treatment influenced the end pH of ethanol fermentation process. During the fermentation process, there was a decrease in the initial pH of the media (6.0) in certain treatments (table 2). It is showed that during the fermentation process there were other compounds aside from alcohol [13]. The lowest pH declines happened in all co-cultivation treatments using glucose substrate with end pH 4.0. Few treatments showed that there were no significant pH differences in OF media. This indicated that yeast isolate could only utilize certain substrates as its carbon sources. The higher variety of substrates that could be utilized by yeasts, the more likely the yeast to utilize substrates optimally for ethanol production.

Based on the research, the combination of 2% glucose and 2% xylose substrate was done to determine sugar consumption in ethanol production by \textit{P. kudriavzevii} T (wt) and mutants (R-T1 and R-T2). The Mutants used had been constructed to withstand high temperature and ethanol stress [14]. Ratio modification of inoculum was used to determine the suitable ratio for the highest ethanol production. Ratios of used \textit{S. cerevisiae} and \textit{P. kudriavzevii} were 1:10, 1:1, and 10:1. The ratios had been used in ethanol production of co-cultivated \textit{Cyberlindnera fabianii} with brewers’ yeast [15].

Based on the research result, the highest substrate content showed by co-cultivation treatment of \textit{S. cerevisiae} and \textit{P. kudriavzevii} T (wt) with inoculum ratio of 1:1 compared to its single isolate treatment. According to the literature, co-cultivation treatment could increase the production of ethanol by using \textit{S. cerevisiae} (OVB 11) and \textit{Pichia stipitis} (NCM 3498) compared to the single isolate treatment. Closed ethanol fermentation using single isolate of \textit{S. cerevisiae} (OVB 11) could result in maximum ethanol production of 7.5 g/L during incubation time of 36 hours. Most of the available xylose was not converted by yeast in lignocellulose hydrolysate rice straw media containing glucose and xylose. On the other side, maximum ethanol production in co-cultivation of \textit{S. cerevisiae} (OVB 11) with \textit{P. stipitis} (NCM 3498) showed a higher ethanol production of 12 g/L during incubation time of 36 hours [16]. Substrate used was still occurred in the treatment of yeast single isolates of \textit{P. kudriavzevii} (figure 2a and figure 2b). It could be possible that both yeasts utilized most of the available glucose in the first 24 hours, while xylose could only be utilized by \textit{P. kudriavzevii}. Glucose was utilized first then followed by xylose by \textit{P. kudriavzevii}. Yeast group of pentose sugar users such as \textit{Pichia} had a gene for the conversion of xylose to xylose-5-phosphate and then converted to ethanol through the pentose phosphate pathway [17].

Substrates that were used in co-cultivation treatment of \textit{P. kudriavzevii} mutant T5 (figure 3) was much lower compared to co-cultivation of its wild type strain (wt) isolate (figure 2). \textit{S. cerevisiae} had a sufficient ability in utilizing hexose sugar [3], while pentose-user yeast was slower in converting hexose and pentose into ethanol [18]. Based on previous reports, \textit{S. cerevisiae} yeast NCIM 3570 could utilize glucose optimally by measuring the residue of fermented glucose (0.96 g/L) after being fermented for 24 hours and the concentration of ethanol was 9.83±0.20 g/L, however the yeast could
not use xylose as a carbon source. The produced ethanol was higher than *P. stipitis* NCIM 3499 with glucose residue of 7.43 g/L, xylose residue of 1.59 g/L, and ethanol results respectively 3.97±0.21 g/L and 9.94±0.19 g/L with fermentation time for 40 hours [19].

The usage of glucose-xylose in co-cultivation of mutant yeast was much lower compared to co-cultivation of its wild type (WT) T. The results showed that the T5 mutant performance in fermenting ethanol was declined. Based on a study, yeast resulted from mutagenesis of *P. kudriavzevii* that could withstand high temperature and ethanol stress had a better performance compared to its wild type (WT) isolate. *P. kudriavzevii* R-T1 mutant could produce 20% and 190% of ethanol higher than its WT strain, while R-T2 mutant could produce 22% and 172% of ethanol higher than its WT strain [14].

5. Conclusion

Interaction of *S. cerevisiae* with both isolates of *P. kudriavzevii* showed that there was no antagonistic interaction, thus both of the yeast isolates could be applied for co-cultivation method. Both yeasts could be used in ethanol production simultaneously to maximize available substrate usage for ethanol production. Utilization of sugar substrate xylose-glucose best performed by co-cultivation treatment between *S. cerevisiae* and *P. kudriavzevii* T (wt) with inoculum ratio of 1:1.

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