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The Ethanol Production Activity of Indigenous Thermotolerant Yeast *Pichia kudriavzevii* 1P4

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Pichia kudriavzevii 1P4 is a thermotolerant-ethanologenic yeast potential for application in ethanol industry. In this study we evaluated the stress tolerance phenotype of *P. kudriavzevii* 1P4 in dealing with fermentation related-stresses, including high temperature stress, high sugar content, ethanol content and the fermentation capacity of the particular isolate. Based on spot assay, 1P4 showed stress tolerance phenotype against high sugar concentration for up to 30% sugar content and 10% ethanol stress. In addition, 1P4 was capable to show temperature-stress tolerance phenotype for up to 42°C, suggesting that 1P4 belong to thermotolerant yeast isolate. Fermentative activity was measured by using glucose consumption and ethanol production assay. We evaluated the fermentative and growth rate of 1P4 at various temperature condition which were 27°C, 37°C and 42°C using YPD media (at initial glucose of 2%, 10% and 20%). Interestingly, 1P4 consumed the highest glucose in 20% of concentration at 37°C (15.29%), simultaneously with the highest concentration of ethanol (32.05 g/L ethanol and 0.67 g/L/h ethanol productivity). Cell growth analysis showed that growth of 1P4 isolate increased with higher initial glucose condition yet decreased as temperature during fermentation was raised. The growth rate of 1P4 was found high in 20% initial glucose at 37°C than 2% and 10% at same temperature. In addition, 1P4 exhibited short lag phase at high-temperature fermentation. Our data indicate that 1P4 can potentially be applied as fermentation agent especially in high-temperature ethanol fermentation.

Key words: bioethanol production, fermentation related-stress, thermotolerant yeast

Pichia kudriavzevii 1P4 merupakan khamir etanologenik-termotoleran yang berpotensi diaplikasikan dalam industri etanol. Dalam penelitian ini kami mengevaluasi karakter ketahanan cekaman isolat 1P4 terhadap cekaman fermentasi, meliputi ketahan terhadap suhu tinggi, kadar gula tinggi, kadar etanol tinggi dan aktivitas fermentasi isolat tersebut. Berdasarkan spot tes analisis, 1P4 menunjukkan fenotipe ketahanan terhadap cekaman gula tinggi hingga 30% dan etanol 10%. Lebih lanjut, 1P4 mampu menunjukkan ketahanan terhadap suhu hingga 42°C, yang mengindikasikan bahwa 1P4 termasuk dalam isolat khamir termotoleran. Aktivitas fermentasi diukur menggunakan metode konsumsi glukosa dan produksi etanol. kami mengevaluasi laju fermentasi dan pertumbuhan 1P4 pada berbagai kondisi media yaitu, 27°C, 37°C dan 42°C, menggunakan media YPD (dengan kadar gula awal 2%, 10% dan 20%). Menariknya, 1P4 mengkonsumsi konsentrasi gula tertinggi pada media dengan kadar gula 20% pada suhu 37°C (15.29%), simultan dengan produksi konsentrasi etanol tertinggi pada kondisi tersebut (32.05 gl^{-1} etanol dan 0.67 g l^{-1} h produktivitas volumetrik etanol). Analisis pertumbuhan sel menunjukkan bahwa pertumbuhan isolat 1P4 meningkat dengan naiknya konsentrasi glukosa namun menurun seiring meningkatnya suhu fermentasi. Laju pertumbuhan 1P4 tinggi pada media dengan kadar gula awal 20% dibandingkan media 2% dan 10% pada suhu yang sama. 1P4 juga menunjukkan fase lag yang pendek pada fermentasi dengan suhu tinggi. Hasil penelitian kami mengindikasikan bahwa 1P4 potensial diaplikasikan sebagai agen fermentasi terutama pada fermentasi etanol dengan suhu tinggi.

Kata kunci: cekaman fermentasi, khamir termotoleran, *Pichia kudriavzevii*

The improvement of consumption fossil fuel and environmental pollution led to the finding of environmental sustainable energy sources (Azhar *et al*. 2017). Bioethanol is a promising alternate for fossil fuel that can be produced through yeast fermentation (Branco *et al*. 2019). However during bioethanol fermentation, yeast cells are confronted with many different fermentation related-stresses including high temperature stress, high osmotic stress and ethanol stress (Saini *et al*. 2018). Highly concentrated medium containing high sugar concentration may lead to higher ethanol product at the end of fermentation, yet high sugar and ethanol content during fermentation could influence the growth, viability and fermentative activity of ethanologenic yeast (D'Amore 1992). In addition, bioethanol fermentation generated heat in the process due to exothermic reaction and thereafter raised the temperature of fermentation broth (Kumar *et al*. 2013). Elevated temperature during fermentation was significantly inhibited growth of yeast in due time affects bioethanol production (Divine *et al*. 2016).

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Thus, in order to meet the requirement of ethanologenic yeast for high-temperature ethanol fermentation (HTEF), we need yeast isolates with high tolerance against fermentation related-stresses while capable to produce higher ethanol yields in high temperature and sugar concentration conditions.

Using thermotolerant yeast for bioethanol production in high temperature fermentation could gain some advantages such as higher saccharification and fermentation rates, energy saving through reduction cooling cost and minimalize risk of contamination (Arora *et al*. 2015). *Saccharomyces cerevisiae* is industrial ethanologenic yeast which is most commonly used for bioethanol production worldwide. *Saccharomyces cerevisiae* had known as a good ethanol producer with high ethanol tolerance phenotype (Chi *et al*. 2010). However as mesophilic yeast, most of *S. cerevisiae* strains could not grow and produce ethanol in high temperature fermentation (Kitagawa *et al*. 2010). Moreover, stressful condition like an increase in ethanol concentration, temperature, osmotic stress and bacterial contamination are the reason why the yeast cannot survive during fermentation (Basso *et al*. 2008). For these reasons, exploration and investigation of nonconventional yeast for bioethanol production in high temperature still gained significant interest worldwide.

Pichia kudriavzevii had received major interest as an ethanol fermentation agent for more than a decade due to its capability to grow at high temperature and high sugar concentration (Isono *et al*. 2012, Kaewkrajay *et al*. 2014, Diaz-nava *et al.* 2017, Martha *et al*. 2020). Previous study reported many strains of *P. kudriavzevii* had good ability to grow and produce ethanol at high concentration temperature of over 40°C (Pongcharoen *et al.* 2018). Then, it is important to investigate characteristic of newly isolated *P. kudriavzevii* strain as potential fermentation agent for HTEF. Yeast strain 1P4 is *P. kuriavzevii* isolated from cacao fermentation that able to grow at 40°C which is potential as an ethanol fermentation agent at HTEF (Inderiani 2017). In this study, we evaluated the stress tolerance phenotype of *P. kudriavzevii* 1P4 in dealing with fermentation related-stresses, including high temperature stress, high sugar content and ethanol content. Furthermore, we performed fermentative activity analysis using glucose consumption and ethanol production assay at different initial glucose and temperatures. These data are key determinant to evaluate yeast fitness as a fermentation agent for HTEF.

MATERIALS AND METHODS

Yeast Strains and Morphology Characterization. Yeast strain had been isolated and identified previously as *P.kudriavzevii* 1P4 at Microbiology Lab IPB University (Inderiani 2017). For this study, this strain was regrown from freeze-dried isolate into YPD liquid media (1% Yeast Extract, 2% Peptone, 2% glucose) for 24 h at room temperature (27°C). The culture from YPD liquid media were aseptically streaked into YPD solid media. Pure colonies of *P. kudriavzevii* 1P4 were used as working culture for all experiments. For morphology characterization, 1P4 isolate was grown at YPD media for 24 h at room temperature then observed using light microscope to identify cell shape, cell size and budding cell type. For stress tolerance experiment, *Saccharomyces cerevisiae* BY4741 was used as a comparison isolate (Riles and Fay 2019).

Stress Tolerance Assay. Stress tolerance of *P. kudriavzevii* 1P4 at YPD media with high sugar content, high ethanol content at high temperatures was evaluated by spot assay method (Astuti *et al*. 2018). In this regards, yeast *S. cerevisiae* was applied as control. Yeasts were cultured in YPD media for 18-24 h as the main culture. Serial dilution was performed with starting OD of 0.5 OD_{600nm}. About 2 µL of each dilution was spotted at YPD solid media with 2% initial glucose and incubated at temperatures 27°C, 37°C, 40°C and 42°C for evaluating temperatures tolerance. YPD solid media with 2%, 10%, 20% and 30% glucose concentration were used as spot media for sugar tolerance assay while YPD without ethanol, with 5%, 10% and 15% ethanol were used as spot media for ethanol tolerance assay. The experiment was carried out in triplicate for 72 h observation.

Glucose Consumption. Yeast 1P4 was cultured in YPD liquid media with optical density 0.9-1 at 600 nm as the main culture. About 5% of an active inoculum was transferred into fresh liquid YPD media with initial glucose of 2%, 10% and 20% then incubated at 27°C, 37°C and 42°C with shaking 120 rpm. The experiment was carried out in 15 mL reaction tubes with a working volume of 10 mL YPD liquid media and conducted in triplicate. The sugar concentration was determined per 8 h during 48 h observation by DNS method (Miller 1959).

Growth Cell. The procedures of preparation to evaluate growth cell *P. kudriavzevii* 1P4 were same as measuring glucose consumption as described earlier. Yeast growth was monitored by measuring optical density of the culture at YPD media with different

initial glucose of 2%, 10% and 20% and temperatures (27°C, 37°C and 42°C) at 600 nm (OD_{600nm}) per 3 h during 48 h of fermentation.

Ethanol Production. The fermentation culture of 1P4 isolate was prepared in YPD media with different initial glucose of 2%, 10% and 20% at 27°C, 37°C and 42°C using the same procedures as evaluating glucose consumption and growth cell. Ethanol concentration was measured at 48 h of fermentation using gas chromatography. Experiment was conducted in duplicate for each condition.

RESULTS

Morphological Identification. Morphological characteristic of 1P4 isolate was identified using light microscope after 24 h incubation at YPD media. As macroscopic identification, colony of *P. kudriavzevii* 1P4 was found round and had white cream color (Figure 1a). Based on microscopic observation, single cell 1P4 was found ovoid with size 4 – 10 µM and monopolar budding cell type (Figure 1b).

Stress Tolerance Assay. Yeast *P. kudriavzevii* 1P4 was more tolerant to fermentation-related stresses than *S. cerevisieae* BY4741 (Figure 2). Growth of yeast *P. kudriavzevii* 1P4 was unchanged following high temperatures stress conditions (Figure 2a-d) while *S. cerevisiae* could not grow at temperatures of 40°C and 42°C (Figure 2c-d). Interestingly, both of isolates of *P. kudriavzevii* 1P4 and *S. cerevisiae* BY4741 exhibited high sugar stress tolerance up to 30% initial glucose (Figure 2e-h). The difference was yeast 1P4 had wider and thicker colony isolate than comparison isolate BY4741. 1P4 isolate showed good growth at control condition and spot media with 5% ethanol (Figure 2i-j). However, high ethanol concentration affected the growth of 1P4 isolate at ethanol stress media. Growth of 1P4 isolate was slightly decreased in media containing 10% ethanol compared to control condition while growth of BY4741 was severe at those stress media (Figure 2k). Both isolates exhibited incapability to grow against 15% ethanol stress conditions (Figure 2l).

Glucose Consumption. Glucose utilization of *P. kudriavzevii* 1P4 was measured in different initial glucose (2%, 10% and 20%) and temperature levels (27°C, 37°C and 42°C) with 2% of initial glucose at 27°C as a control condition (Figure 3). The reducing sugar was rapidly consumed by 1P4 isolate during the first 24 h at media with 2% initial glucose at all temperatures (27°C, 37°C and 42°C). 1P4 isolate utilized glucose almost completely at this temperature, with only 0.15%-0.08% (w/v) glucose remaining at 48 h. The similar pattern in glucose consumption was showed at condition of 10% at 37°C and 42°C. However, the reducing sugar rate consumption was faster at 37°C than at 42°C. 1P4 assimilated reducing sugar almost completely at 36 h, respectively 4 h faster than at condition of 42°C. Indeed, 1P4 isolate showed higher glucose consumption rate of all initial glucose concentration at 37°C compared other conditions. 1P4 consumed the most glucose content of 20% initial glucose at 37°C (15.29%) than 2% initial glucose (1.8%) and 10% initial glucose (9.9%), respectively.

Cell Growth of Yeast 1P4. Cell growth of yeast *P. kudriavzevii* 1P4 at YPD media with initial glucose 2%, 10% and 20% were grouped in three different temperatures (27°C, 37°C and 42°C), with condition of 2% sugar at 27°C was designated as control experiment. Our data shows that OD_{600nm} of yeast 1P4 increased with higher initial glucose condition yet decreased as temperature during fermentation was raised (Figure 4). 1P4 had higher final OD value at 27°C than both of 37°C and 42°C conditions. In addition, the final OD value was higher at media of 20°C initial glucose than of 2% and 10% initial glucose at 37°C though extremely low at 2% initial glucose at 42°C.

As found in this study, the growth pattern of 1P4 isolate differs depending on availability of glucose on the media and levels of temperatures. Based on OD_{600nm} data, yeast 1P4 showed lag phase up to 6 hours and followed by exponential growth for up to 24 h at control condition. It is worth nothing that 1P4 isolate also showed *diauxic* phenomenon after 24 h. However, growth pattern of 10% and 20% initial glucose at 27°C and all conditions at 37°C showed lag phase then followed by exponential phase until 48 h. In contrast, cell growth of 10% and 20% initial glucose showed progressive increase of turbidity value for up to 24 hours as exponential growth.

The duration of lag phase of 1P4 isolate was affected by initial glucose and temperature. The higher initial glucose at media prolonged the lag phase although raising the temperature shortened the duration. Interestingly, 1P4 isolate grown at media with 10% initial glucose at 27°C, all initial glucose at 37°C and 20% initial glucose at 42°C had lag phase as same duration as control condition. However, lag phase became longer than control at 20% initial glucose at 27°C hence shorter than control at 2% and 10% initial glucose at 42°C.

Ethanol Production. The kinetic parameters of ethanol production 1P4 in media YPD with different

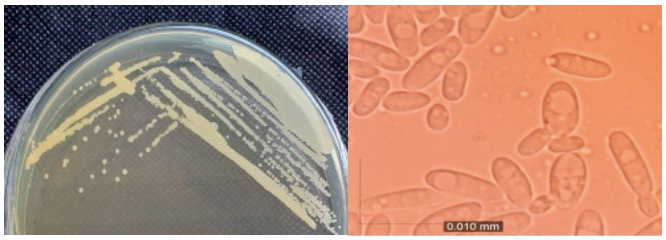

Fig 1 Morphological characterization of *P. kudriavzevii* 1P4 grown at YPD solid media after 24 h incubation. (a) Colony of 1P4 isolate. (b) Cell of 1P4 isolate under light microscope (1000X).

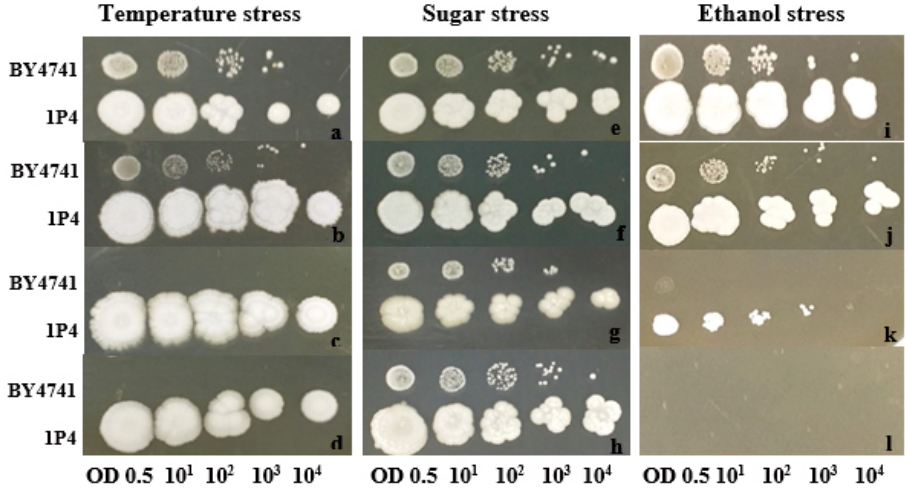

Fig 2 Effect of temperature, sugar and ethanol stresses on the cell growth of *P. kudriavzevii* 1P4 after 72 hours incubation. *S. cerevisiae* BY4741 was used as control isolate. **Temperature stress**: a. 27°C (control), b. 37°C, c. 40°C, d. 42°C. **Sugar stress**: e. 2% (control), f. 10%, g. 20%, h. 30%. **Ethanol stress**: i. 0% (control), j. 5%, k. 10%, l. 15%. BY4741: isolate *S. cerevisiae* BY4741, 1P4: isolate *P. kudriavzevii* 1P4.

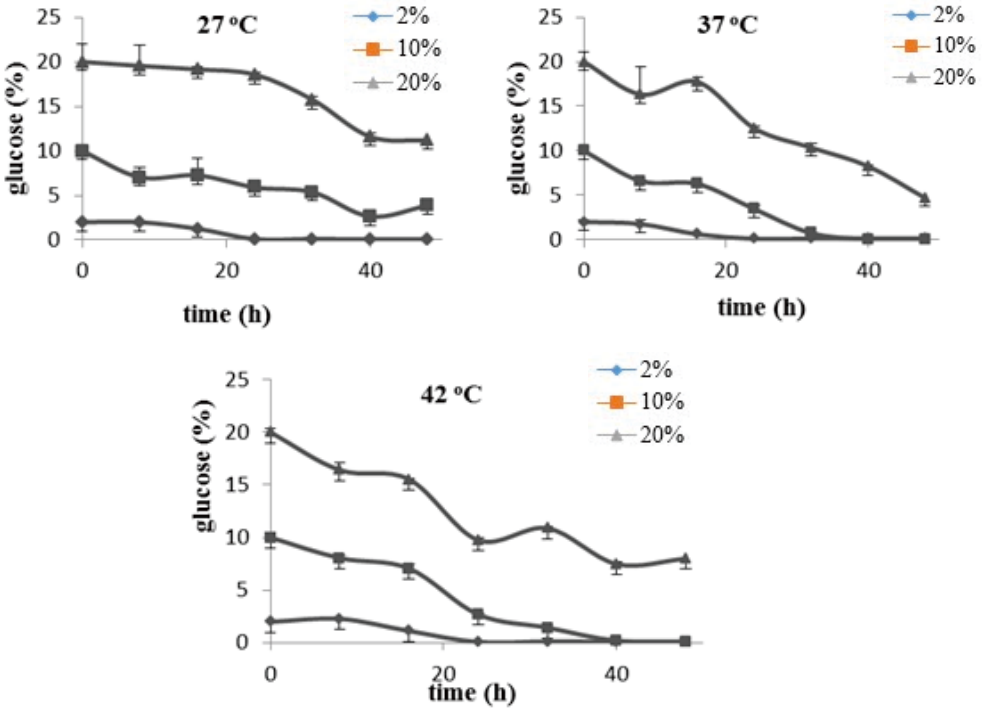

Fig 3 Glucose consumption of 1P4 isolate at media YPD of 2%, 10% and 20% initial glucose at various temperatures (27°C, 37°C and 42°C) during 48 h fermentation. Glucose concentration was measured using DNS method. Condition of 2% initial glucose at 27°C was used as control experiment. —◆— YPD media with 2% initial glucose. —■— YPD media with 10% initial glucose. —▲— YPD media with 20% initial glucose.

initial glucose and temperatures was shown at Table 1. The ethanol concentration of 1P4 isolate increased as temperature of fermentation was raised. As found in this study, 1P4 isolate produced significantly higher ethanol concentrations at 37°C and 42°C than those at control condition. Moreover, 1P4 isolate yielded higher ethanol concentration at 37°C (32.05 g/L) than 42°C (14.80 g/L), respectively. On the other hand, the availability of glucose at fermentation media affected the ethanol production of 1P4 isolate. Ethanol concentration was found increasing as more glucose was added at fermentation media. The ethanol production of 1P4 was found high in 20% initial glucose (14.80 g/L) at 42°C than 2% (4.15 g/L) and 10% (9.55 g/L) at same temperature, respectively. The highest ethanol concentration of 1P4 was showed of 20% initial glucose at 37°C (32.05 g/L). This suggests that 1P4 isolate is potential for good candidate of fermentation agent at high-temperature ethanol fermentation.

1P4 isolate showed the highest ethanol yield, ethanol productivity and fermentation efficiency at condition of 37°C (Table 1). Furthermore, the highest ethanol yield and fermentation efficiency were exhibited of 2% initial sugar at 37°C (0.44 g/g and 87.62%, respectively) whereas the highest ethanol productivity was found of 20% initial glucose at same temperature (0.67 g/L/h). As found in this study, the ethanol yield of 1P4 and fermentation efficiency was decreased at higher initial glucose available at fermentation media. The ethanol yield and fermentation efficiency of 1P4 of 2% initial glucose at 42°C were 0.22 g/g and 42.85%, respectively, compared to the condition 20% initial glucose (0.12 g/g and 12.10%, respectively). In contrast, ethanol productivity of 1P4 was shown increasing at higher initial glucose. The ethanol productivity of 1P4 of 2% initial glucose at 37°C was 0.17 g/L/h compared to the condition of 20% initial glucose (0.67 g/L/h).

DISCUSSION

Competent thermotolerant yeast as a fermentation agent is one of important key for successful producing ethanol in HTEF (Yuangsaard *et al*. 2013). As found in this study, 1P4 showed high tolerance phenotype against 42°C and 30% initial glucose. However, severe growth of 1P4 at 10% ethanol concentration indicated that ethanol tolerance mechanism was independent pathway from temperature and sugar tolerance at 1P4 isolate.

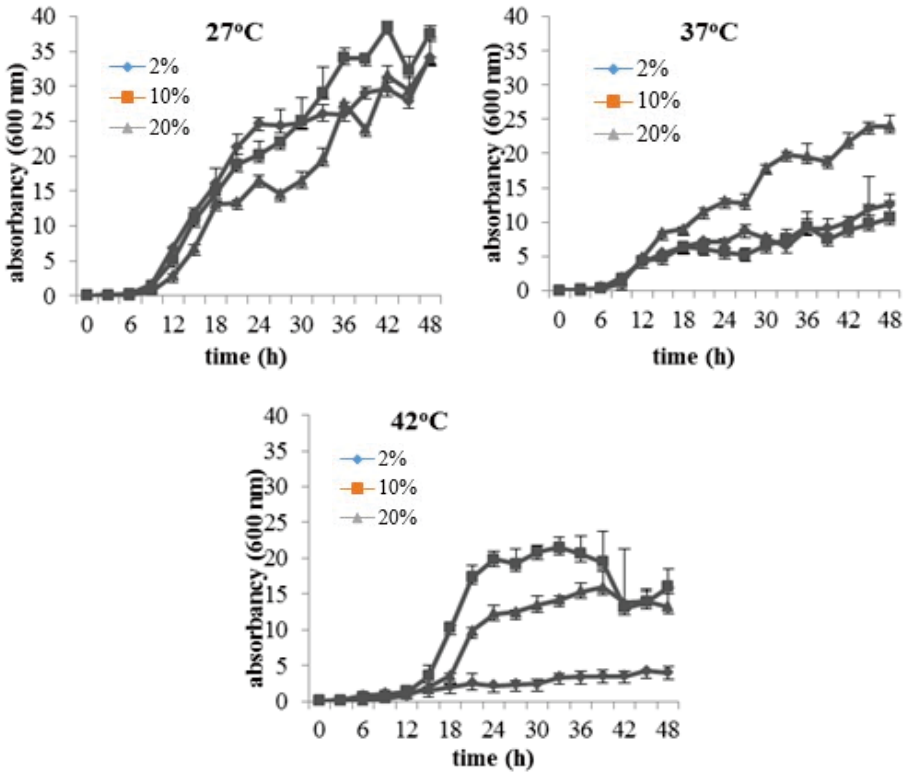

Fig 4 Growth curve of *P. kudriavzevii* 1P4 in different initial glucose (2%, 10% and 20%) and temperatures (27°C, 37°C and 42°C) during 48 h fermentation. Cell growth was measured using spectrophotometer at OD_{600nm} absorbance. Condition of 2% initial glucose at 27°C was used as control experiment. —◆— YPD media with 2% initial glucose. —■— YPD media with 10% initial glucose. —▲— YPD media with 20% initial glucose

Table 1 Kinetic parameters of ethanol production by *P. kudriavzevii* 1P4 with different initial glucose and temperatures conditions

Temperatures	Initial sugar	P (g/L)	Yp/s (g/g)	Qp (g/L/h)	Ey (%)
27°C	2%	5.05±3.32	0.26±0.17	0.11±0.07	51.46±0.34
	10%	12.90±1.84	0.21±0.03	0.27±0.04	41.11±0.06
	20%	12.30±2.40	0.14±0.03	0.26±0.05	27.20±0.05
37°C	2%	8.25±0.63	0.44±0.03	0.17±0.01	87.62±0.07
	10%	23.10±2.26	0.23±0.02	0.48±0.05	45.49 ±0.04
	20%	32.05±7.42	0.20±0.49	0.67±0.15	40.96±0.09
42°C	2%	4.15±2.19	0.22±0.12	0.09±0.05	42.85±0.22
	10%	9.55±2.80	0.10±0.03	0.20±0.06	18.83±0.05
	20%	14.80±8.77	0.12±0.07	0.31±0.18	24.10±0.14

P: ethanol concentration (g/L), Yp/s: ethanol yield (g/g), Qp: ethanol productivity (g/L/h), Ey: fermentation efficiency (%).

Temperature has been reported causing protein damage and yeast responding to this condition by inducing of HSPs and trehalose (Techaparin *et al*. 2017, Chamnipa *et al*. 2018). HSPs played role as molecular chaperones to protecting protein cells from thermally damage (Saini *et al*. 2018). Previous studies reported that trehalose is an important marker for potential stress resistance in yeast, since yeast with high trehalose accumulation showed temperature and ethanol tolerance (Lahiri *et al*. 2014, Gibson *et al*. 2007). However, it was reported that the phenotype of high tolerance against fermentation related-stresses depends on several factors including yeast strain, composition of fermentation medium, intracellular ethanol accumulation, incubation temperature and osmotic pressure (Banat *et al*. 1998).

The important characteristics of yeast in order to be applied for successful fermentation agent are capable to grow and produce high ethanol yield during HTEF. According to the growth curve of 1P4, cell density of 1P4 changed due to different glucose concentration and temperature. High cell density of 1P4 was presented in control condition, although the final OD at all initial glucose was almost similar in 27°C. As expected, the cell density was gradually decreased as temperature of fermentation was raised, although higher cell density was found at higher initial glucose. This might be related to temperature stress that inhibited the cell growth of 1P4. Previous study reported that elevated temperature suppressed several proteins that involve in various metabolism pathways and affected protein transport and vesicle organization (Choudary *et al*. 2016). Interestingly, control condition showed diauxic phenomenon which is related to availability of glucose concentration in the media.

In this study, we also found that concentration of glucose and temperatures affected duration of lag phase. Lag phase of 1P4 became shorter at HTEF although higher initial glucose extended the duration. This characteristic might be an advantage for 1P4 as a fermentation agent for HTEF, considering that 1P4 isolate can adapt more quickly at high temperature. Duration of lag phase is obviously an important of yeast phenotype in industrial fermentation, since short lag time allows yeast cells to grow more quickly in substrate fermentation (Varelas *et al*. 2017).

Fermentation was conducted in nine conditions of initial glucose and temperatures. From our study, we found that 1P4 showed markedly better fermentation activity at HTEF than control. Amongst all condition, 1P4 consumed the most substrate of 20% initial glucose at 37°C simultaneously with the highest ethanol production at same condition. The ethanol production of 1P4 was higher compared to *S. cerevisiae* RL-11 that produced 11.7 g/L ethanol concentration and *Scheffersomyces stipitis* CBS 6054 that obtained 8.2 g/L ethanol concentration (Mussatto *et al*. 2012, Scordia *et al*. 2012). Moreover, the ethanol production of 1P4 decline at 42°C. This pattern was similar to ethanol production of *P. kudriavzevii* strains that showed its ethanol production decreased gradually as the temperature was raised (Techaparin *et al*. 2017, Ndubuisi *et al*. 2018). It is important to note that 1P4 showed highest fermentation rate at media with high glucose at 37°C, suggesting that this temperature is optimum condition for 1P4 to produce ethanol. This finding is considered high than that of *S. cerevisiae* strains which had 30°C of optimum temperature for ethanol production (Choi *et al*. 2010), and of *P.*

kudriavzevii strain reported previously which had 25°C of optimum condition (Agrawal *et al*. 2019).

In conclusion, *P. kudriavzevii* 1P4 performed higher fermentative activity at HTEF. 1P4 consumed the highest concentration of glucose of 20% at 37°C (15.29%), simultaneously with the highest concentration of ethanol (32.05 g/L ethanol and 0.67 g/L/h ethanol productivity). 1P4 isolate also showed high tolerance against sugar stress up to 30% glucose and temperature stress up to 42°C. Interestingly, the cell growth analysis exhibited short lag phase of 1P4 as temperature of fermentation was raised. This indicates that *P. kudriavzevii* is potentially applied as ethanol fermentation agent for high temperature ethanol fermentation. Thus further study in large scale fermentor is required.

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