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Enhanced Ethanol Production by High Temperature-Tolerance Mutant *Pichia kudriavzevii* T-T2 in various Carbon and Nitrogen Sources

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Previously, we had constructed proline accumulating yeast *Pichia kudriavzevii* mutant (T-T2) that exhibited high temperature tolerance phenotype. In this study, we then analyzed the ethanol production of the particular mutant isolate in various carbon (C) and nitrogen sources (N). Fermentation rate of T-T2 either in 4% glucose or substrate mixture (SM = 2%glucose : 2% xylose) was higher than WT strain. The highest CO_2 evolving rate by the T-T2 in glucose and SM was 57.0 ml/hour and 51.4 ml/hour, respectively, suggesting that the T-T2 was capable of using xylose as C-source. The T-T2 resulted significant increase of total evolved CO_2 than WT strain in glucose as substrate. The T-T2 isolate was capable of producing higher ethanol than its WT strains in various C-sources, including glucose, maltose, sucrose, xylose and SM. However, glucose was found as the most preferable C-source to produce ethanol (3.2%) whereas maltose was the least preferable one. T-T2 was found capable in using inorganic sources ($NaNO_3$) to produce ethanol (2.4%) as compared to other N-sources (urea, $(NH_4)_2SO_4$, NH_4Cl). Ethanol production by T-T2 strain in $NaNO_3$ was 16% higher than its WT strain. This data indicates that $NaNO_3$ is potentially applied as N source alternative to produce ethanol. Based on our study, mutant strain T-T2 shows higher fermentation rate than its WT strain in various sugar and N-sources. The fact that T-T2 could produce ethanol from SM in a similarfermentation rate to single glucose as substrate, suggesting its potential application in the 2nd generation of bioethanol productions.

Key words: bioethanol, carbon sources, fermentation, *Pichia kudriavzevii*

Pada penelitian sebelumnya, kami telah mengkonstruksi mutan khamir *Pichia kudriavzevii* T-T2 pengakumulasi prolin, yang toleran terhadap cekaman suhu tinggi (45^0C). Dalam penelitian ini, dilakukan analisis produksi etanol khamir mutan T-T2 pada berbagai sumber karbon (C) dan nitrogen (N). Laju fermentasi T-T2 pada 4% glukosa atau campuran sumber karbon (SM = 2% glukosa : 2% xilosa) lebih tinggi dari tipe liarnya (WT). Laju pelepasan CO_2 oleh isolate T-T2 pada subtract glukosa dan SM masing-masing sebesar 57.0 ml/jam dan 51.4 ml/jam. Hal tersebut menunjukkan kemampuan T-T2 dalam menggunakan substrat xilosa. Total pelepasan CO_2 oleh T-T2 lebih tinggi dari WT pada substrat glukosa. T-T2 mampu memproduksi etanol lebih tinggi dari WT pada berbagai sumber karbon termasuk glukosa, maltose, sukrosa, xilosa dan SM. T-T2 menghasilkan etanol tertinggi dengan glukosa (3.2%) dan terendah pada sumber karbon maltose. T-T2 mampu menggunakan substrat N anorganik ($NaNO_3$) untuk memproduksi etanol (2.4%) dibandingkan sumber N lain (urea, $(NH_4)_2SO_4$, NH_4Cl). Produksi etanol oleh T-T2 pada substrat $NaNO_3$ lebih tinggi 16% dibandingkan WT. Dari hasil penelitian ini diketahui bahwa mutan T-T2 menunjukkan laju fermentasi yang lebih tinggi dari WT-nya pada berbagai sumber C dan N. Kemampuan T-T2 dalam memproduksi etanol dari substrat SM pada laju yang sama dengan glukosa menunjukkan potensinya dalam produksi etanol generasi kedua.

Kata kunci: bioetanol, fermentasi, *Pichia kudriavzevii*, sumber karbon

Currently, the demand of alternative energy is massively increasing worldwide, in order to overcome the energy crisis. Indeed various approaches have been developed including the optimization of bioethanol production via fermentation. To date, almost all bioethanol production is produced by edible sources such as sugars and starch (Naik *et al.* 2010; Duraisam *et al.* 2017). A substrate alternative for bioethanol production by using lignocellulose hydrolysate is believed as one of the strategy to increase ethanol production (Frigon and Guiot 2010; Gupta and Verma 2015). Thus, fermentative microbes that able to use both pentose and hexose sugars are needed to perform efficient conversion of lignocellulose hydrolysate to ethanol (Unrean *et al.* 2010; Zhang *et al.* 2015).

The common industrial yeast *Saccharomyces cerevisiae*, however, could only produce ethanol from glucose e(Azhar *et al.* 2017), thus limiting its activity in lignocellulose-based ethanol productions, or popularly known as 2nd generation of bioethanol productions (Sims *et al.* 2010). In addition, *S. cerevisiae* is sensitive to the elevated temperature occurs during fermentation, as revealed by numerous study (Azhar *et*

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al. 2017; Favaro *et al.* 2019). Thus, *S. cerevisiae* is not applicable for simultaneous saccharification and fermentation (SSF) process in which enzymatic hydrolysis reactions happen at 40–50 °C (Yuan *et al.* 2017). Currently, the search of potential high temperature tolerance yeast strains is gaining attention amongst researcher, as one of the strategies to overcome fermentation barrier. In addition, development of yeast strains through mutagenesis to construct fermentation-related stresses has been demonstrated as one of the potential approach in improving ethanol fermentation rate (Astuti *et al.* 2018; Deparis *et al.* 2017; Lam *et al.* 2014).

In our previous study, we have isolated a non-conventional yeast, *Pichia kudriavzevii* that capable in using both pentose sugar (xylose) and glucose (Astuti *et al.* 2018). We managed to construct proline accumulating mutant of *P. kudriavzevii* isolate T via random mutagenesis. Among mutant strains obtained, T-T2 exhibited high-temperature (45 ^{0}C) tolerance phenotype. Such yeast-phenotype is essential, since during fermentation yeast are exposed to various fermentation-associated stresses, including high temperature, hyperosmotic, as well as oxidative stresses (Zhang *et al.* 2015; Kitichantaropas *et al.* 2016).

In this study, we performed fermentation rate analysis of mutant T-T2 in hexose sugar and sugar mixture (SM/glucose and xylose) based on gas CO_2 evolving rate. Furthermore, quantification of ethanol yield in various sugar sources was also conducted to observe the preferable carbon and nitrogen sources for optimum ethanol productions. From our study, we found that fermentation rate of mutant T-T2 was higher than that WT strain both in glucose and SM substrate. Amongst sugar sources tested, glucose was the most preferable sugar sources while maltose was the least one. Inorganic nitrogen sources, $NaNO_3$, were potentially used to substitute organic nitrogen source to produce ethanol.These data are important for further application of the mutant isolate in a complex substrate, including lignocellulose hydrolysate.

MATERIALS AND METHODS

Isolates and Medium. *P. kudriavzevii* isolates, both WT and mutant (T-T2), was routinely main *yeast peptone dextrose* (YPD) at room temperature (Astuti *et al.* 2018).Fermentation medium was prepared by using YP medium (1% yeast extract and 2% peptone)with modified carbon source concentration (4%) and sugar mixture (2% each). Carbon sources used in this study was glucose, maltose, sucrose, and xylose. Sugar mixture-fermentation was performed in 2% glucose: 2% xylose. Various nitogen sources ($NaNO_3$, urea, $(NH_4)_2SO_4$, NH_4Cl) was used to substitute peptone in glucose-containing YPD medium.

Measurement of Rate of Fermentation. Fermentation medium was prepared in different carbon sources i.e 4% of glucose, xylose, maltose, and sucrose. YPD medium containing 2% glucose and 2% xylose was also prepared for sugar mixture-based ethanol productions. Both WT and mutant strains was pre-cultured in YPD medium containing glucose for overnight in shaker at room temperature. Absorbance of culture was then measured by using spectrophotometer atλ600nm. Pre-culture was then transferred to new fermentation medium with various sugar treatments at initial OD600= 0.1. Prior transfer to fermentation medium, pre-culture was pelleted and washed in designated fermentation medium at initial pH medium 6.8. Fermentation culture was then incubated for 56 hours in fermenter at room temperature with shaking at 120 rpm. Fermentation was monitored by measuring the volume of evolved carbon dioxide using Fermograph II (Atto) per hour.

Ethanol Production in Various Sugar Sources. Ethanol production was measured based on previous study by Astuti *et al.* (2018) with modification in sugar concentrations. Pre-culture of both WT and T-T2 mutant strain was prepared as describe previously. Fermentation medium with various sugar compound (2%) i.e. glucose, xylose, maltose, sucrose. For SM treatment, 2% glucose and 2% xylose was used as carbon sources. SM was used in this analysis. Ethanol content was measured via densitometry approach by using pycnometer. Ethanol content was measured for each 6 hour in 48 hours of fermentations. Ethanol content was measured using formula as described by Avicor *et al.* (2015).

Ethanol Production in Various Nitrogen Sources. Fermentation medium for alternative nitrogen sources analysis was prepared by substituting peptone in YP medium to various inorganic sources. Glucose was used as carbon source in this analysis. Inorganic sources used in this assay including 2% $NaNO_3$, 0.2% urea, 0.02% $(NH_4)_2SO_4$, 1% NH_4Cl, as described elsewhere (Nadeem *et al*. 2015). Ethanol content was measured at each 6 hours during 48 hours of fermentation, via densitometry approach by using pycnometer. Ethanol content was measured using formula as described by Avicor *et al.* (2015).

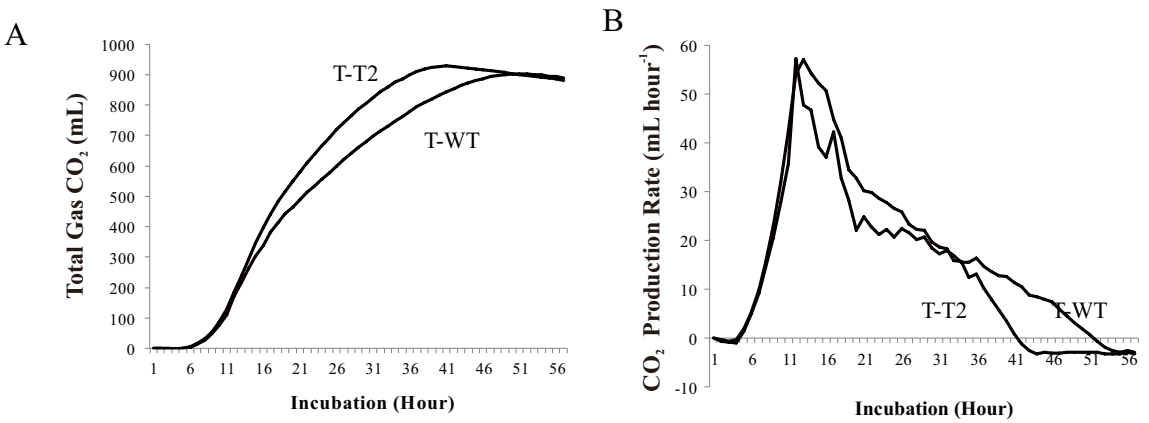

Fig 1 Generation of CO_2 (gas carbondioxide) during small-scale fermentation using 4% glucose as carbon source of *P. kudriavzevii* mutant T-T2 and its WT strains (A) The total amount of CO_2 (B) CO_2 evolving rate during 56 hours of incubation. Yeast was grown in fermentation medium with 4% glucose. Fermentation was monitored by measuring the volume of evolved carbon dioxide using Fermograph II (Atto) per hour for 56 hours.

RESULTS

Fermentation Rate of T-T2 is Higher than WT Strains in Glucose -based Fermentation. To investigate the fermentation rate of T-T2 isolates, we measured the evolved carbon dioxide during 56-hours of fermentation. Interestingly, T-T2 mutant strain showed significant increase of total CO_2 production at early fermentation compared to WT strains. Indeed, T-T2 mutant accumulated highest volume of total gas CO_2 after 40 hours (928.9 ml), while WT strains took 50 hours to accumulate the highest volume of CO_2 (904.0 ml) (Fig 1). Higher fermentation performance in glucose as substrate was also supported based on gas CO_2 evolving rate. In instance, highest CO_2 production rate by T-T2 was recorded after 12 hours of incubation in a value of 57.0 ml/hour, which was 19% higher than WT strains. Our data indicates that mutant T-T2 perform better fermentation rate in glucose as carbon source than the WT strain.

Fermentation Rate of T-T2 is Higher than WT Strains in Sugar Mixture-based Fermentation. Similar pattern of total gas CO_2 and CO_2 evolving rate was found in sugar mixture-based fermentation between mutant T-T2 and WT strains (Figure 2). In general, the fermentation rate of T-T2 strain in sugar mixture was lower than that in glucose. However, this result clarifies the capability of mutant T-T2 to ferment xylose in addition to glucose, in a relatively similar rate.

Ethanol Production in Various Sugar Sources. The production of ethanol in various sugar compounds was conducted to find the most preferred fermentable sugarsby isolates T-T2. In general, the production of ethanol by T-T2 was higher than its WT strain. The most preferable hexose sugar for T-T2 ranged from glucose (3.2%), sucrose (2.4%), and maltose (1.5%) (Fig 3A, C-D). Ethanol production from xylose (2.7%), however, was conducted in 96 hours of incubation (Fig 3B). As expected, T-T2 could ferment sugar mixture to produce ethanol (2.9%) (Fig 3E).

Ethanol Production in Various Nitrogen Sources. In this assay we used glucose as carbon sources, since glucose was the most preferable carbon sources for T-T2 isolates. Production of ethanol in mutant strains was higher than its WT strain. The highest ethanol production was shown in $NaNO_3$ treatment (2.4%), followed with urea (1.1%), $(NH_4)_2SO_4$(1.4%), NH_4Cl (1.6%)(Fig 4A-D). These data indicate that inorganic nitrogen, $NaNO_3$, could be used as substitute for peptone, which was used in the fermentation medium as organic N-source.

DISCUSSION

High temperature tolerance yeast has been gaining serious attention amongst researchers as this particular yeast is applicable in various industrial fermentation processes. In this study, we attempted to evaluate the fermentation ability of high-temperature tolerance non-conventional yeasts *P. kudriavzevii* T-T2. T-T2 has been evaluated for being tolerance against 45^{0}C stress conditions (Astuti *et al.* 2018). Based on our data, T-T2 performed better fermentation rate compared to WT strain both in glucose and sugar mixture (glucose and xylose) as carbon source thus yielding higher ethanol content. Previous study by Chamnipa *et al.* (2018) revealed that thermotolerant yeast *P. kudriavzevii* RZ8-1 could produce high ethanol yield (35.51 g/L) in glucose due to up-regulation of genes involved in

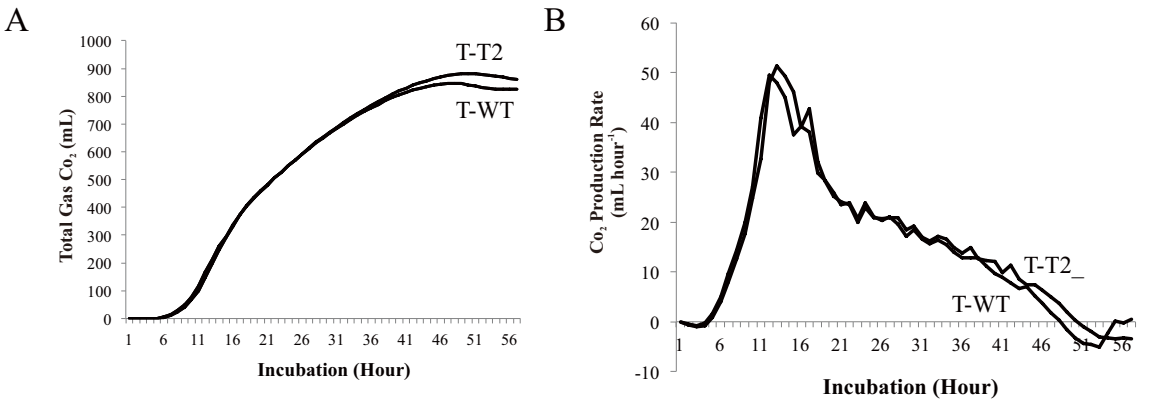

Fig 2 Generation of CO_2 during small-scale fermentation using 4% sugar mixture as carbon source of *P. kudriavzevii* mutant T-T2 and its WT strains(A) The total amount of CO_2 (B) CO_2 evolving rate during 56 hours of incubation. Yeast was grown in fermentation medium with 4% sugar mixture medium. Fermentation was monitored by measuring the volume of evolved carbon dioxide using Fermograph II (Atto) per hour for 56 hours.

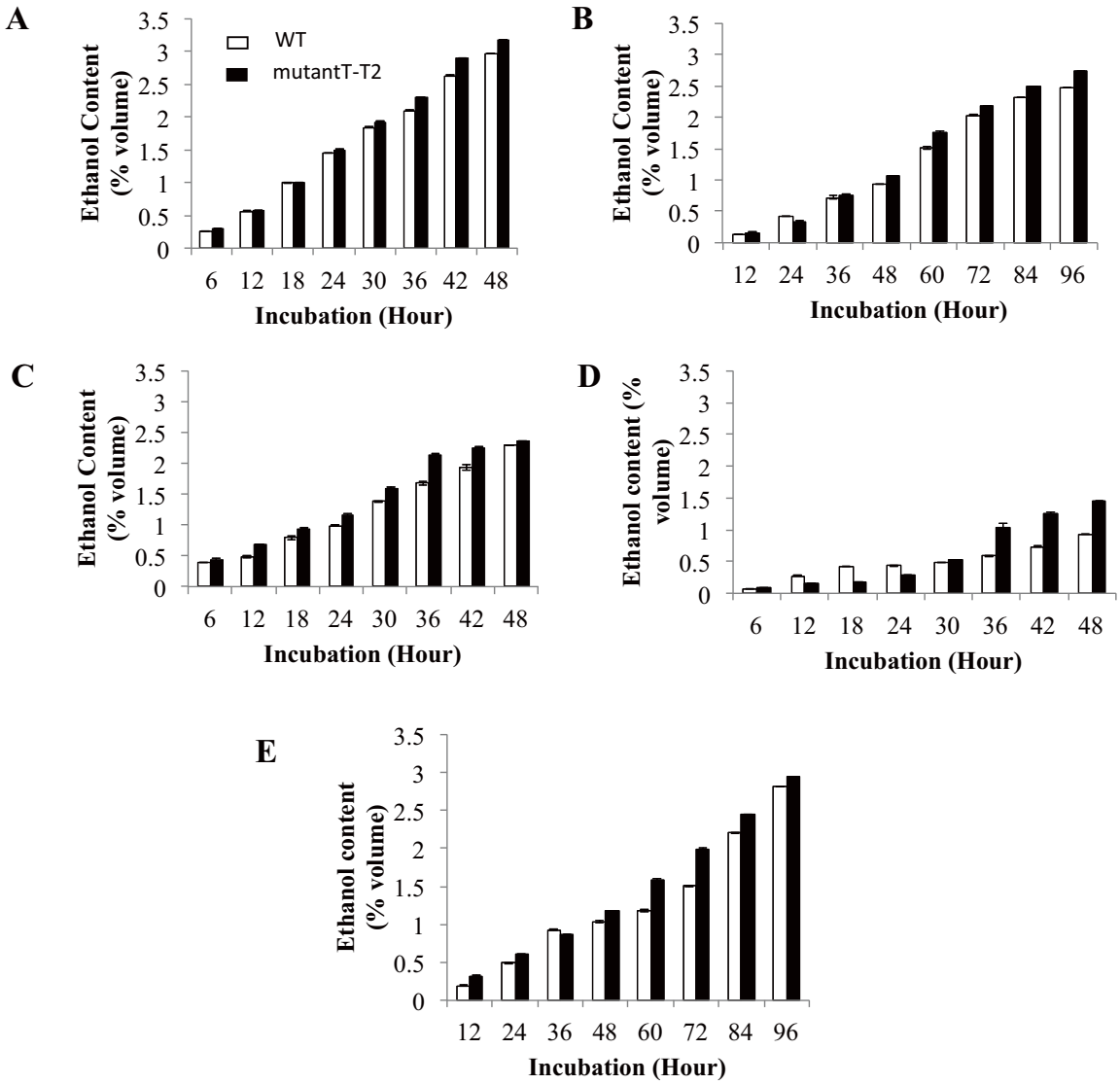

Fig 3 Ethanol production of *P. kudriavzevii* T-T2 mutant strains compared to WT strain in sugar source (A) Glucose (B) Xylose (C) Sucrose (D) Maltose (E) Sugar mixture (glucose:xylose). Yeast was grown in YPD medium with various carbon sources (2%) and incubated for 48 hours, while xylose fermentation was conducted for 96 hours. Ethanol content was measured at designated time by using Pycnometer.

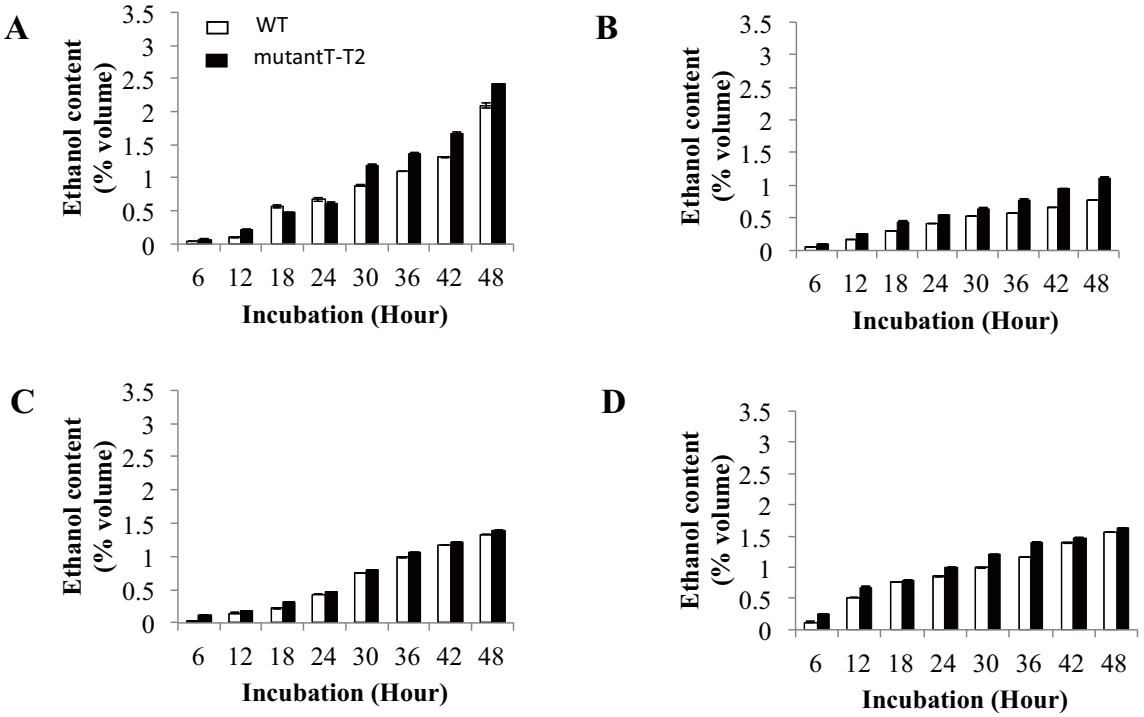

Fig 4 Ethanol production of *P. kudriavzevii* T-T2 mutant strains compared to WT strain in different inorganic nitrogen sources (A) $NaNO_3$ (B) Urea (C) $(NH_4)_2SO_4$(D) NH_4Cl. Yeast was grown in YD medium with various concentration of inorganic nitrogen sources, as indicated in method section. Inorganic nitrogen sources were added to substitute peptone. Cultures were then incubated for 48 hours. Ethanol content was measured at designated time by using Pycnometer.

ethanol production and heat stresses including heat shock proteins (*ssq1* and *hsp90*), alcohol dehydrogenases (*adh1*, *adh2*, *adh3* and *adh4*) and glyceraldehyde-3-phosphate dehydrogenase (*tdh2*). Further study in the cellular and molecular mechanism in T-T2 mutant strains is needed to reveal the underlying mode of actions of the particular isolates compared to the WT strains. Total CO_2 gas produced by T-T2 isolates is considered high as compared to certain other yeast isolates. In instance, Sake yeast K7 produced 1 L of CO_2 after more than one day in 20% glucose-based fermentation (Watanabe *et al.* 2011). To maintain stabile fermentation rate, certain physiological modifications can be conducted. It is reported that excess availability of a certain yeast assimilable amino acid, arginine, could result in a more sustained CO_2 production rate throughout alcohol fermentation (Butzke and Park 2011). Such phenomenon was observed in *Saccharomyces bayanus*-mediated alcohol fermentation (Butzke and Park 2011).

In this study, we found that T-T2 mutant strain was able to utilize pentose sugar as shown in sugar mixture fermentation treatment. Different to that study by Silva *et al.* (2011), yeast *P. stipitis* NRRL Y-7124 showed higher fermentation rate in glucose than xylose, based on glucose-consumption and ethanol productions. The slower fermentation rate shown by T-T2 might be due to low xylose consumption rate of the particular isolates. Previous studies confirmed that *P. stipitis* has a slower sugar consumption rate compared to *Saccharomyces cerevisiae* and requires microaerophilic condition for ethanol productions (Rivera *et al.* 2011). It is therefore, analysis on the aeration during batch fermentation of T-T2 isolates should be conducted to find the optimum fermentation conditios.

Currently, pentose-utilizing yeast has been constructed via genetic engineering as one of the strategy for an effective sugar conversion into fermentation products (Gao *et al.* 2019; Sharma *et al.* 2018)). Common industrial yeast *S. cerevisiae* has been engineered to heterologously express xylose isomerase genes from fungal *Piromyces sp.* E2 (Chomvong *et al.* 2016) or bacteria *Clostridium phytofermentans* (Demeke *et al.* 2013), so that the enzyme could convert xylose to xylulose. The later sugar form can be used by *S. cerevisiae* as fermentation substrates. In this study, we found that T-T2 could ferment both glucose and xylose. This data is important for further applications of the mutant strain in a sugar mixture-based fermentation, including complex sugar substrate such as

lignocellulose.

Based on the sugar source treatments, amongst sugar sources used, glucose was the most preferable substrate for ethanol productions both by WT and T-T2 mutant. However, longer incubation time was needed in xylose-based fermentation, as xylose is slowly used by *Pichia* isolates, as described elsewhere (Rivera *et al.* 2011). In sugar mixture, it was likely that T-T2 utilized glucose prior to xylose. It is due to the repression of xylose utilization by glucose that occurs in various pentose-utilizing yeast including *K. marxianus* (Hua *et al.* 2019), and *Spathaspora passalidarum*(Rodrussamee *et al.* 2018). Ethanol productions by T-T2 is considered high as compared to previous study by Koutinas *et al.* (2015) that showed the capability of *P. kudriavzevii* KVMP10 in producing 0.19% ethanol from 1% xylose.

The concentration of sugar could affect the ethanol productions. In instance, Koutinas et al (2015) reported that *P. kudriavzevii* KVMP1 produced 0.45% and 0.49% ethanol from 1% glucose and sucrose, respectively. Among other *P. kudriavzevii* isolates, T-T2 showed markedly potential fermentation agent, as T-T2 could use maltose as substrate. Other study by Yuangsaard *et al.* (2013) mentioned null productions of ethanol from maltose by *P. kudriavzevii* DMKU 3-ET15. Based on our study, 2% of $NaNO_3$, could be used as substitute for peptone, which was used in the fermentation medium as organic N-source. Further study in the optimization of inorganic N-source is needed to further clarify the potential used of other inorganic N-sources such as urea, $(NH_4)_2SO_4$ and NH_4Cl. Previous study by Nadeem *et al.* (2015) indicate that yeast *S. cerevisiae* gave gave a comparatively better yield of ethanol, in medium containing inorganic nitrogen source, $(NH_4)_2SO_4$ than other N-sources such as NH_4NO_3 and NH_4Cl.

In conclusion, mutant T-T2 performed higher fermentation rate in various sugar and nitrogen sources than WT strains. Glucose and $NaNO_3$ are the most preferable carbon and inorganic nitrogen source for ethanol productions, respectively. Interestingly, T-T2 could ferment sugar mixture of glucose and xylose, thus indicating its potential in further application in more complex sugars as fermentation substrates, such as lignocellulose.

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