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Ethanol Productivity of Ethanol-Tolerant Mutant Strain *Pichia kudriavzevii* R-T3 in Monoculture and Co-culture Fermentation with *Saccharomyces cerevisiae*

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ABSTRACT

We previously developed ethanol-tolerant *P. kudriavzevii* R-T3 (*P.ku* R-T3) mutant from its parental strain *P.ku* R-WT by evolutionary adaptation method. Hence, we further analyze the ethanol productivity of the particular isolates in a monoculture and co-culture with industrial yeast *Saccharomyces cerevisiae* BY4741 at various inoculum ratio. Based on the spot assay, R3 mutant yeast showed better cell viability under 10% ethanol stress than the wild type, potentially due to the high expression level of *PKINO1* gene involved in the synthesis of inositol. In the monoculture fermentation, *S. cerevisiae* could use glucose, while *P.ku* could use mixed glucose and xylose as carbon sources for ethanol fermentation. *P.ku* R-T3 performed the most potential ethanol kinetics parameters, including the highest ethanol production (10.10 g/L), ethanol productivity (0.21 g/L/h), and fermentation efficiency (84.36%). Upscaling the inoculum of *P.ku* R-T3 by ten times resulted in 10% higher ethanol production. However, the highest substrate utilization rate did not indicate an increase in ethanol production. Indeed, *P.ku* R-T3 showed a low mixed substrate use but produced higher ethanol production than *S. cerevisiae*, as much as 21-31%, depending on the initial inoculum. Interestingly, the co-culture of *P.ku* R-T3 and BY4741 did not substantially produce higher ethanol production than the monoculture technique. About 30% reduction of ethanol production was found by co-culturing BY4741 with *P.ku* R-T3 than *P.ku* R-T3 alone. Taken together, the monoculture fermentation of *P.ku* R-T3 remains the promising fermentation technique than that of the co-culture with industrial yeast *S. cerevisiae*.

1. Introduction

Bioethanol is one of the important chemicals which can be used in various industrial fields, particularly as an alternative renewable fuel (Fivga *et al.* 2019). In the industrial sectors, ethanol is used in blends various products such as cosmetics, hairsprays, window cleaners, and various pharmaceutical products. In addition to its renewable production, ethanol as an alternative fuel also has advantages for the environment, including low toxicity, biodegradability, and lower pollutants than petroleum fuels (John *et al.* 2011). Indeed, ethanol as an additive material can increase the octane number in gasoline and produce lower carbon monoxide (CO) and Unburnt Hydro-Carbon (UHC) emissions than pure gasoline (Elfasakhany 2015). An increase in ethanol production must accompany the high demand for bioethanol. Efforts to increase bioethanol production are currently being developed to find efficient methods through development in bioprocess (fermentation) strategy, application of potential microbe for fermentation and utilization of available waste as substrate, including lignocellulose biomass.

Lignocellulose biomass is now potentially be used as an alternative substrate source in second-generation bioethanol productions because it contains high carbohydrates (Geddes *et al.* 2011). Agricultural waste such as rice straw, bagasse, corn cobs, and oil palm empty bunches has been considered potential lignocellulose biomass for bioethanol productions (Binod *et al.* 2010; Gutierrez-Rivera *et al.* 2015; Sewsynker-Sukai and Gueguim Kana 2018; Sukhang *et al.* 2020). Hydrolysis of lignocellulose produces sugars such as glucose, xylose, and other by- products fermented into ethanol by

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microorganisms (Binod *et al.* 2010). Industrial yeast such as *Saccharomyces cerevisiae* has been long used for glucose-based substrate fermentation due to its high ethanol yield and high tolerance to ethanol (up to 10% v/v) (Parapouli *et al.* 2020). However, this yeast could not use five carbon sources as fermentation substrates, which are present in lignocellulose-based ethanol fermentation. Thus, the search for potential yeast isolates capable of utilizing both six and five carbon sources would provide a potential strategy in increasing ethanol productivity in such advanced ethanol production.

We have previously isolated ethanologenic yeast, *Pichia kudriavzevii* R (*P.ku* R-WT), from rotten tropical fruit (Astuti *et al.* 2018a) which capable of utilizing both glucose and xylose as substrates (Rahmadhani *et al.* 2020). We constructed the mutant strain of *P.ku* R-T3 from the parental strain of *P.ku* R by using the mutagenesis approach. *P.ku* R-T3 was found tolerant against 10% ethanol stress, similar to that industrial yeast, *S. cerevisiae* (Astuti *et al.* 2018b). The application of fermentation-related stress-tolerant yeast strains in a fermentation process is markedly essential. Indeed, during fermentation, yeasts are exposed to various environmental stresses such as high osmotic pressure due to high substrate content, high temperature as resulted from exergonic fermentation reactions, and accumulation of the product, ethanol. Such stresses would therefore alleviate yeast viability which in turn resulting low ethanol productivity.

In addition to utilization of stress tolerant-yeast isolates, ethanol production can also be increased via the co-culture technique. Indeed, a previous study indicates that co-culture techniques can provide an opportunity to increase ethanol production through maximum substrate utilization compared to a single culture system (Chen *et al.* 2018). For instance, co-culture of *S. cerevisiae* OVB and *P. stipitis* NCIM 3498 can increase ethanol production by 5% compared to single culture (Srilekha Yadav *et al.* 2011). Thus in this study, we evaluated the ethanol productivity of co-culture of *P.ku* R-WT and *P.ku* R-T3 with industrial yeast *S. cerevisiae* BY4741 compared to the monoculture. In order to find the optimum fermentation efficiency, we have also employed different ratio of each yeast isolate used. This study is important to confirm the most potent strategy to improve ethanol productivity from potential ethanologenic yeast *P. kudriavzevii.* It is worth noting that the ability of *P.ku* in utilizing both glucose and xylose may benefit the second generation of bioethanol productions, which employs complex substrates from residual biomass, such as forest, industrial, or municipal wastes (Branco *et al.* 2019).

Indeed, in this study we found that the newly ethanol stress tolerance mutant *P.ku* R-T3 performed higher ethanol productivity and fermentation efficiency than its wildtype cells and *S. cerevisiae*, thus suggesting its potential application in larger fermentation scale.

2. Materials and Methods

2.1. Yeast Isolates and Growth Medium

Yeasts isolates, *P.ku* R-WT, *P.ku* R-T3, and *S. cerevisiae* BY4741 (*S.c* BY4741) were routinely maintained in Yeast Peptone Dextrose (YPD) agar medium and incubated for two days at room temperature (28-30°C). Fermentation test was done in Oxidative Fermentative (OF) medium (g/100 ml) composed by 1 gram carbon source, 0.2 gram peptone, 0.03 gram K_2HPO_4, 0.2 gram NaCl, and 0.003 gram bromthymol blue (pH 7.1). Fermentation in the mixed substrate was prepared in Yeast Peptone medium containing 2% (w/v) glucose and 2% (w/v) xylose.

2.2. Cell Viability Assay

Cell viability test under ethanol stress conditions was evaluated using the spot assay method (Cheng *et al.* 2016; Astuti *et al.* 2018b). Yeast cultured in YPD broth medium (OD_{600} = 1) was transferred into a sterile microplate (Nunc 96) with a total volume of 200 µL, then 20 µL of the previous culture was transferred into 180 µL of YPD broth media for serial dilutions of 10-1 to 10-4. Each dilution (2 µL) was spot on the YPD agar consisting of various concentrations of ethanol (0% (control), 8%, and 10%). Plates were then incubated for 48 h at ±28°C in a incubator (Memmert, Schwabach, Germany).

2.3. Gene Expression Analysis

Analysis of *PKINO1* and *TDH2* genes expression began with harvesting yeast cells after 4 h incubation in YPD broth medium at room temperature (±28°C). The mRNA was extracted by using the *RNeasy Mini Kit* (Qiagen, USA). cDNA synthesis was done using the *SensiFAST cDNA Synthesis Kit* (Bioline, USA). qPCR analysis was performed using the *THUNDERBIRD SYBR® qPCR Mix* (Toyobo, Japan). Primers used for Quantitive Real-Time PCR (qRT-PCR) analysis are shown in Table 1.

2.4. Growth Curve

Each yeast isolate were inoculated into 50 ml YPD broth medium (initial OD_{600} = 0.1). Each culture was then incubated in a shaker incubator (Memmert, Schwabach, Germany) at room temperature (±28°C).

Table 1. Primers used for Quantitive Real-Time PCR (qRT-PCR) analysis

Primer	Primer sequence (5'-3')	References
Actin-F	CATTCAAGCCGTTTTGTCCT	(Chamnipa *et al.* 2018)
Actin-R	GGAAATCACTGCTTTGGCTC	
PKINO1-IF-o7	AACAAACACAATTACAAAAAAT GCCATCCATTAAGGTCAA	(Sugiyama *et al.* 2018)
PKINO1-IF-o8	CAGGTCGACTCTAGAGGATCTC CATGTATGGCTTCAGTTG	
TDH2-F	TTTCCAACGCTTCCTGTACC	(Chamnipa *et al.* 2018)
TDH2-R	CGTCAAGTTGGTTTCCTGGT	

The number of viable cells was then calculated using Total Plate Count (TPC) method using YPD agar medium. Prior to plating, sufficient serial dilution in sterile 0.85% (w/v) NaCl solutions was performed. Spread plate method was applied in this assay. The number of yeast colonies in each agar plate was then calculated following incubation for 48 h at room temperature.

2.5. Interaction Test between Isolates

Interaction test between isolates was determined using the agar plate method. This assay was used to determine the interaction between isolates. In this regards, negative interaction (antagonism) between isolates suggesting that they could not be used in co-culture fermentation. Yeast *S. cerevisiae* BY4741 was incubated for 24 h in 5 ml YPD broth medium as main culture (OD_{600} = 1). 1% of main culture was then inoculated in 50 ml of liquid YPD agar (±40°C), and mixed with gently shaking. Liquid YPD agar was poured into a Petri dish and waited until the agar was completely firmed. *P.ku* R-WT and *P.ku* R-T3 isolates were then streaked on the particular YPD agar. Nystatin (50 µg/ml) was used as a positive control. 20 µL nystatin solution was dropped in a paper disc (diameter 0.22 µm). Agar plate was incubated for 48 h at 30°C. Observation of clear zone around colony in agar plate indicated negative interaction between isolates while absence of a zone corresponded to harmless interaction between *S. cerevisiae* BY4741 and both *P.ku* isolates.

2.6. Fermentation Test on Various Carbon Sources

The fermentation test was carried out based on previous study (Hugh and Leifson 1953). Fermentation OF medium was prepared with modified carbon sources i.e glucose, sucrose, maltose, and xylose. Each yeast isolates was cultured on YPD broth medium as main culture for overnight (OD_{600} = 1). Main culture was then then inoculated into OF medium in starting OD_{600} = 0.1. Alteration of pH was calculated between initial and final culture following 48-72 h of incubation.

2.7. Sugar Consumption Assay

Fermentation medium was prepared by using YP medium with a modified carbon source containing a mixture of 2% (w/v) glucose and 2% (w/v) xylose, as described previously (Rahmadhani *et al.* 2020). Fermentation was conducted in monoculture and co-culture at different initial concentration of inoculum. Each yeast isolate was cultured in YPD medium as main culture (final OD_{600} = 1). Prior to inoculation in mixed sugar fermentation medium, 0.1 ml of culture (1X inoculum) or 1 ml (10X) inoculum was centrifuged at 3,000 rpm for 5 min. Pellet cells were then collected and suspended with 100 µL fermentation medium. Culture suspension was then transferred in 10 ml fermentation medium to measure sugar utilization by using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The reducing sugar levels was evaluated every 8 h by taking out 0.5 ml of sample then centrifuged at 3,000 rpm for 10 min. The supernatant was diluted using sterile distilled water and added with 1 ml of DNS reagent. Suspension was then heated in boiling water for 10 min and waited to cool at room temperature. Absorbance of reducing sugar was measured using a spectrophotometer at 540 nm wavelength (Gusakov *et al.* 2011). Reducing sugar content is calculated based on the standard sugar-xylose curve.

Substrate consumption rate was then calculated using the formula:

$$\text{Substrate consumption rate} = \frac{\Delta S \times 100\%}{S0}$$

ΔS = ubstrate consumption = final [glucose] - initial [glucose]

2.8. Ethanol Production Assay

Each yeast isolates at a different inoculum ratio was prepared as described above in sugar consumption assay. Pre-cultures were prepared in 250 ml Erlenmeyer flasks containing 25 ml of modified fermentation media as described earlier with of 2% (w/v) glucose and 2% (w/v) xylose as carbon source. Measurement of ethanol product was carried out after 48 h incubation using gas chromatography (GC). The ethanol content was determined with a GC 17A Shimadzu Gas Chromatograph. A Rt Qbond plus column was used at a starting temperature of 40°C and raised to 160°C after sample injection at a rate of 20°C/min. It was then raised to 200°C at a rate of 50°C/min and kept at this temperature for 8 min. Nitrogen was used as carrier gas at a flow rate of 28

ml/min and at a pressure of 61 kPa. For each sample, a volume of 1 µL was automatically injected onto the GC column using a split syringe. The ethanol in the samples was measured by comparing it with known ethanol standards. Duplicate cultures were prepared for each experiment. Kinetics parameter of ethanol productions were then calculated using the following formula:

$$\text{Fermentation efficiency} = \frac{P}{\text{Theoritical ethanol}}$$

$$\text{Ethanol productivity} = Qp = \frac{\text{Ethanol concentration}\left(\frac{g}{L}\right)}{\text{Time (h)}}$$

P = Ethanol concentration

3. Results

3.1. Mutant *P.ku* R-T3 Shows Ethanol Stress Tolerance Phenotype

The mutant *P.ku* R-T3 strain used in this study exhibited ethanol stress tolerant phenotype as shown in Figure 1. A Slightly better ethanol stress tolerance phenotype was shown by the particular mutant isolate compared to the WT strain. On the other hand, *S.c* BY4741 was not capable to cope with 8% (v/v) ethanol stress. It is worth noting that the *P.ku* R-T3 mutant used in this study has been kept for more than a year in a cryopreserved culture. Hence, *P.ku* R-T3 mutant still maintained its ethanol stress tolerance phenotype as described earlier (Rahmadhani *et al.* 2020).

3.2. *PKINO1* Gene is Highly Expressed in Mutant *P.ku* R3 Strain

The qRT-PCR analysis showed that the expression level of the *PKINO1* gene which encodes key enzyme on the inositol biosynthesis, in *P.ku* R3 was four times higher than *P.ku* WT (Figure 2A). Interestingly, we found that the expression level of *TDH2* gene which encodesglyceraldehyde-3-phosphatedehydrogenase important in the glycolysis was slightly decreased in the *P.ku* R3 mutant strain (Figure 2B).

3.3. Yeast Growth in Different Carbon Sources

Yeast *P.ku* R-T3 and its corresponding WT could ferment xylose as carbon source. A color change due to development of acid as resulted during fermentation in xylose-base oxidative fermentation medium was observed (Figure 3A). Based on the growth assay, *P.ku* R-WT and *P.ku* R-T3 mutant strain entered stationary phase earlier (6-9 h) than that of *S.c* BY4741 in the fermentation medium (Figure 3B). Thus, suggesting the rapid growth of both *P.ku* R-WT and *P.ku* R-T3 strains. Interestingly, the *P.ku* R-T3 showed higher number of viable cells during log phase as compared to its wild type strain and *S. cerevisiae*. However, all strains maintained a relatively similar number of viable cells within the stationary phase until 48 h of incubation.

As *P.ku* R-WT and *P.ku* R-T3 were designed for co-culture fermentation with *S.c* BY4741, thus interaction assay between these isolates were conducted. The result showed no clear zone around the *P.ku* R-WT and *P.ku* R-T3 colony against the *S.c* BY4741 isolate.

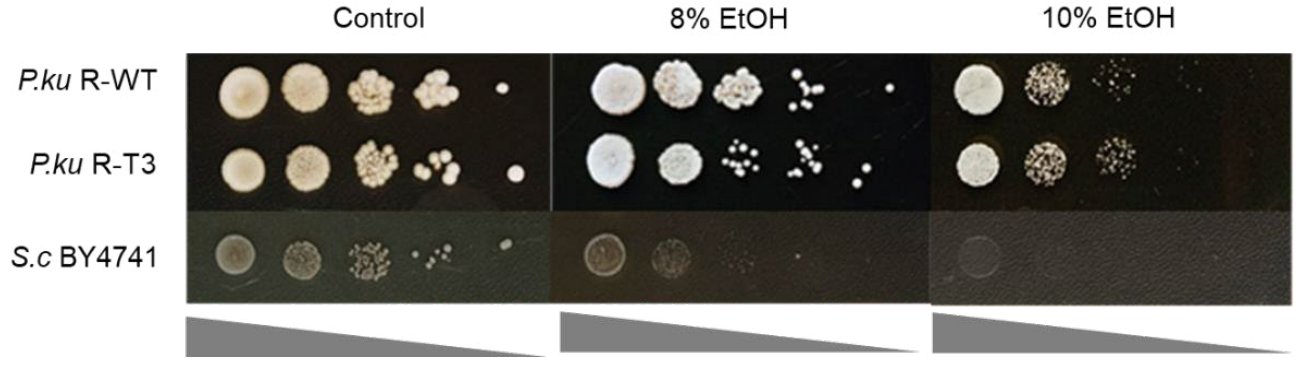


Figure 1. Stress tolerance assay of yeast *P. kudriavzevii* wild type (*P.ku* R-WT) and mutant (*P.ku* R-T3) isolates against ethanol stresses (8 and 10% (v/v) ethanol) by using spot assay. Yeast *S. cerevisiae* (S.c BY4741) was used as control of ethanol sensitive yeast. Yeasts grown in YPD agar medium without ethanol supplementation was used as control. Agar plates were incubated for 48 h at 30°C

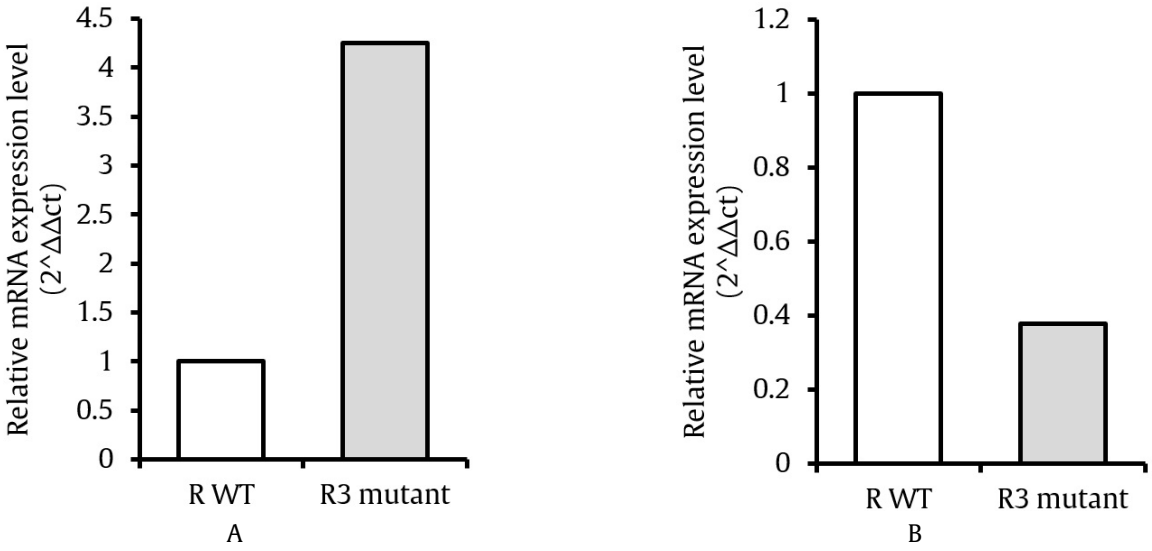


Figure 2. The expression of gene (A) *PKINO1* and (B) *TDH2* of *P.ku* WT and *P.ku* R-T3 strains by using qRT-PCR method. The expression level of each target gene was normalized by act1 gene

On the other hand, positive control (*Nystatin*) formed a clear zone around the paper disc against *S.c* BY4741 as cultured in overlay agar (Figure 3C). This assay shows *P.ku* R-WT and *P.ku* R-T3 could be co-cultured with *S.c* BY4741.

3.4. Ethanol Fermentation Productivity

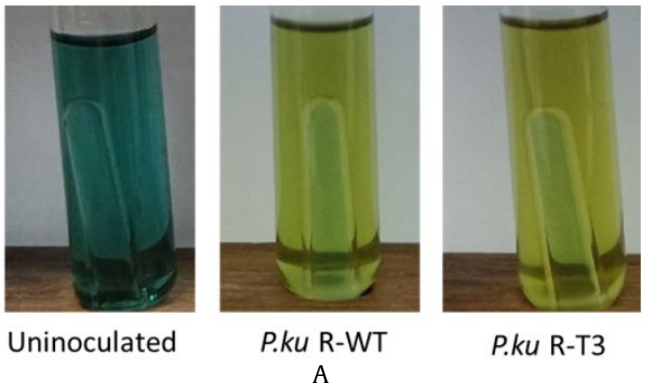
Amongst the fermentation experiment, the isolate *P.ku* R-T3 resulted in the highest ethanol production (10.10 g/L) in monoculture technique with a 10X inoculum ratio compared to its wild type strain or *S. cerevisiae*, either in monoculture or co-culture techniques (Table 2). A relatively similar pattern of substrate consumption was found in both monoculture and co-culture fermentation at different initial inoculum ration (Figure 4A and B). Yet, the substrate consumption rate in *P.ku* R-T3 (Figure 4A).

Interestingly, the higher substrate consumption rate of yeast isolate did not substantially correlate positively with high ethanol production, as shown by the particular *P.ku* R-T3 isolate. Indeed, in our study, substrate consumption rate likely has negative correlation with ethanol production, both in mono- and co-culture technique (Figure 4C and D). Surprisingly, co-culture of *P. ku* R-T3 with *S.c* BY4741 did not result elevated ethanol productions than that of *P.ku* R-T3 monoculture fermentation. It is worth noting that higher initial inoculum concentration might not necessarily increase ethanol production, as shown in *P.ku* R-WT and *S.c* BY4741, but *P.ku* R-T3 in monoculture technique (Figure 4C). Interestingly, although co-culture of *P.ku* R-WT with *S.c* BY4741 exhibited higher consumption rate than that co-culture of *P.ku* R-T3 with *S.c* BY4741, the production ethanol of the former treatment was conversely lower, especially in the 1X initial inoculum of *P.ku* R-T3 (Figure 4D).

In addition to that higher ethanol production, the ethanol productivity (Qp) and fermentation efficiency (Ey) of *P.ku* R-T3 was found the highest amongst all fermentation conditions (Table 3). However, the co-culture of *P.ku* R-T3 with *S.c* BY4741 did not significantly increase the Qp and Ey. It is worth noting that, from our experiment, the fermentation efficiency was positively correlated with ethanol productivity both in mono- and co-culture technique (Figure 5A and B). However, the fermentation efficiency was not found higher towards increased in initial inoculum (Figure 5A). Indeed, utilization of 10X initial inoculum of *S.c* BY4741 relatively caused lower fermentation efficiency than 1X, especially in co-culture fermentation (Figure 5B).

4. Discussion

The yeast *Pichia kudriavzevii* has gaining serious attention recently due to its markedly relevant physiological characters in fermentation-related industries. The ability of *P. kudriavzevii* to ferment five carbon sources such as xylose (Nweze *et al.* 2019; Martha *et al.* 2020) further supports its utilization in lignocellulose-based fermentation industries. Another potential traits such as ethanol tolerance (Radecka *et al.* 2015), thermotolerance (Yuangsaard *et al.* 2013; Koutinas *et al.* 2016; Ndubuisi *et al.* 2018), and lignocellulose hydrolysate inhibitor tolerant (Dandi *et al.* 2013) have also been reported in *P. kudriavzevii* isolates. Indeed, previous study reported



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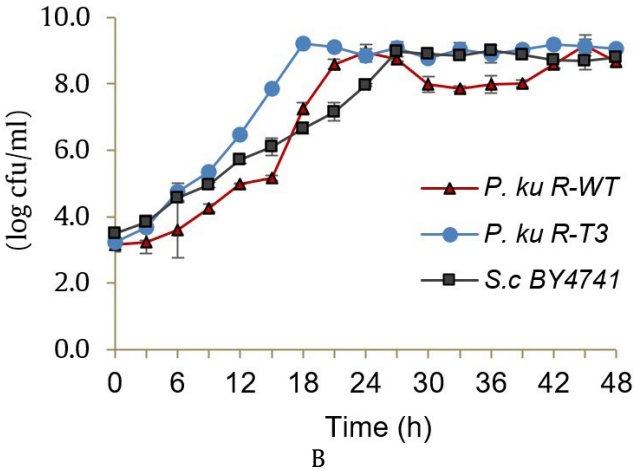

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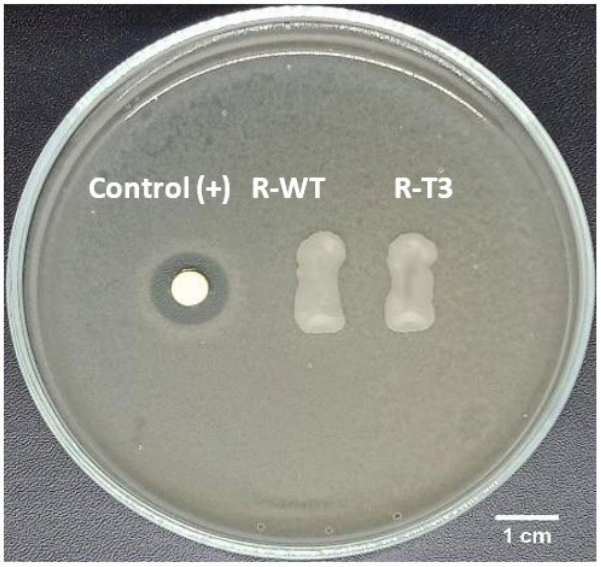


C

Figure 3. (A) Both isolate yeasts *P. kudriavzevii* wild type (*P.ku* R-WT) and mutant (*P.ku* R-T3) could utilize xylose as sole carbon source in oxidative fermentative medium as shown by color change in *P.ku* treatment due to lower pH. Treatment was conducted for 24 h of incubation at room temperature, (B) growth curve of yeast *P.ku* R-WT, *P.ku* R-T3 and *S. cerevisiae* (*S.c* BY4741) in liquid YPD medium at room temperature for 48 h of incubation. Viable cells was calculated by using pour plate method on YPD agar medium and incubated for 48 h at room temperature, (C) interaction between *P.ku* R-WT, *P.ku* R-T3 toward *S.c* BY4741 by dual culture method by using YPD agar medium. *S.c* BY4741 was used as culture in overlay YPD agar. Observation of clear zone was conducted following 48 h incubation at room temperature (±27°C). Treatment of Nystatin was used as positive control

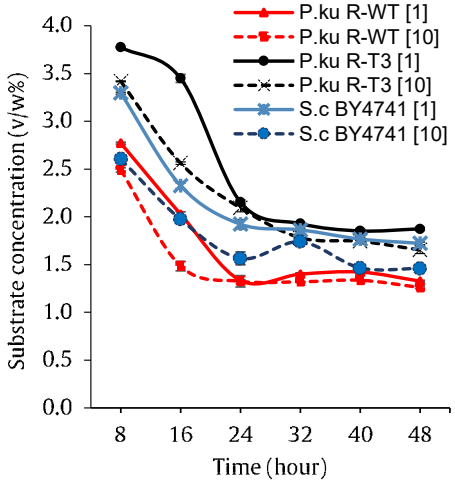
the potential activity of thermotolerant *P. kudriavzeii* in ethanol production RZ8-1 using sugarcane bagasse hydrolysate (Chamnipa *et al.* 2018).

As shown in this study, *P.ku* WT and its developed mutant strain *P.ku* R-T3 showed the ability to use xylose as sole carbon source (Figure 3A). *P.ku* R-T3 showed better ethanol tolerance phenotype than WT and *S.c* BY4741. Our data suggest that higher expression of *PKINO1* in *P.ku* R-T3 in non-ethanol stress conditions likely support the adaptive tolerance mechanism in this particular mutant strain. Indeed, overexpression of *PKINO1* gene in *P. kudriavzevii* N77-4 improves ethanol and acetic acid resistance and most importantly increased its ethanol production compared to WT (Sugiyama *et al.* 2018). *PKINO1* gene encodes the key enzyme for inositol biosynthesis which critical for ethanol resistance (Sugiyama *et al.* 2018).

As in glucose-containing medium, *P.ku* R-T3 isolates were markedly showed rapid growth compared to its WT strain in *S.c* BY4741. Interestingly, in mixed substrate (glucose-xylose) (Table 2), *P.ku* R3 has a lower substrate consumption rate than its

Table 2. The substrate consumption rate and ethanol production both in mono- and co-culture techniques of *P.ku* R-WT, *P.ku* R-T3 and *S.c* BY4741

Fermentation type	Yeast isolate [inoculum]	Substrate consumption rate (%) ± SD	Ethanol production (g/l) ± SD
Monoculture	*P.ku* R-WT [1]	66.82±0.78	9.80±0.06
	P.ku R-WT [10]	68.45±1.11	5.85±0.28
	P.ku R-T3 [1]	53.15±1.46	9.20±0.04
	P.ku R-T3 [10]	58.65±3.40	10.10±0.17
	S.c BY4741[1]	62.48±0.63	7.63±0.08
	S.c BY4741[10]	66.14±2.08	7.66±0.06
Coculture	*S.c* BY4741: *P.ku* R-WT [1:1]	68.78±0.58	8.35±0.05
	S.c BY4741: *P.ku* R-WT [1:10]	67.77±0.18	7.82±0.27
	S.c BY4741: *P.ku* R-WT [10:1]	70.42±3.16	7.03±0.16
	S.c BY4741: *P.ku* R-T3 [1:1]	58.10±1.06	8.95±0.12
	S.c BY4741: *P.ku* R-T3 [1:10]	61.03±1.61	8.80±0.16
	S.c BY4741: *P.ku* R-T3 [10:1]	61.57±0.44	7.09±0.20



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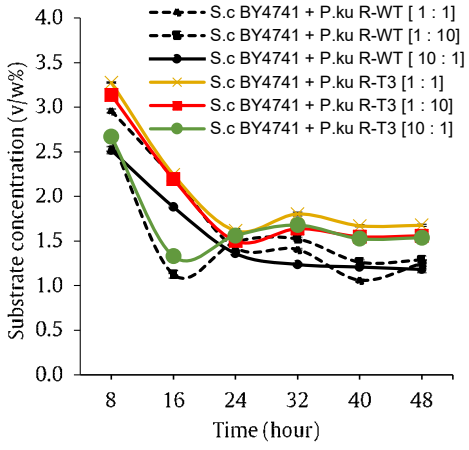

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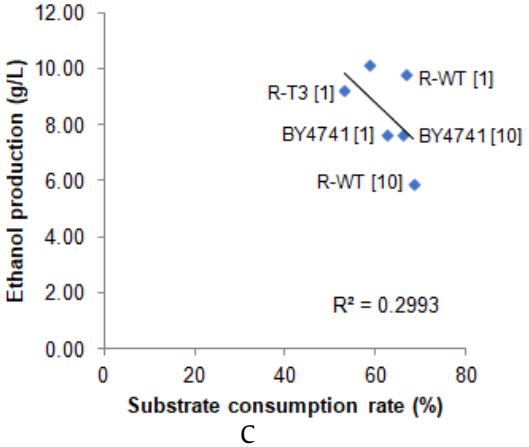

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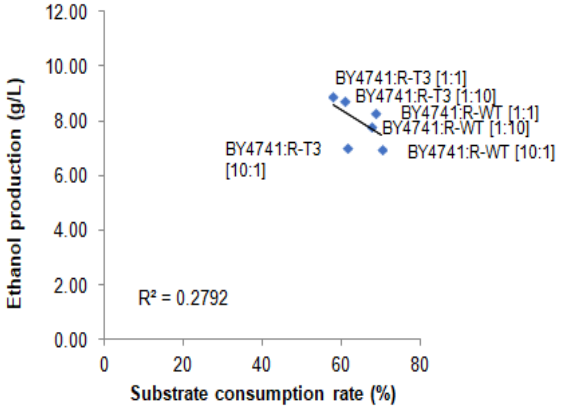

D

Figure 4. Substrate consumption rate of (A) monoculture (B) co-culture fermentation by using isolate *P. kudriavzevii* wild type (*P.ku* R-WT), *P. kudriavzevii* mutant (*P.ku* R-T3) and *S. cerevisiae* (*S.c* BY4741) at different initial inoculum concentration (as shown in bracket) and ratio. Fermentations were done in room temperature for 48 h. Correlation pattern between ethanol production and substrate consumption of (C) monoculture (D) co-culture fermentation

Table 3. The ethanol productivity (Qp) and fermentation efficiency (Ey) from the mono- and co-culture of *P.ku* R-WT, *P.ku* R-T3, and *S.c* BY4741

Fermentation type	Yeast isolate [inoculum]	Qp (g/L/h) ± SD	Ey (%) ± SD
Monoculture	*P.ku* R-WT [1]	0.20±0.01	71.70±0.84
	P.ku R-WT [10]	0.12±0.06	41.78±0.68
	P.ku R-T3 [1]	0.19±0.01	84.66±2.37
	P.ku R-T3 [10]	0.21±0.04	84.36±4.76
	S.c BY4741 [1]	0.16±0.02	59.72±0.60
	S.c BY4741 [10]	0.16±0.01	56.63±1.70
Coculture	*S.c* BY4741: *P.ku* R-WT [1:1]	0.17±0.01	59.35±0.50
	S.c BY4741: *P.ku* R-WT [1:10]	0.16±0.06	56.39±0.15
	S.c BY4741: *P.ku* R-WT [10:1]	0.15±0.03	48.87±2.19
	S.c BY4741: *P.ku* R-T3 [1:1]	0.19±0.03	75.32±1.37
	S.c BY4741: *P.ku* R-T3 [1:10]	0.18±0.03	70.52±1.89
	S.c BY4741: *P.ku* R-T3 [10:1]	0.15±0.04	56.32±0.40

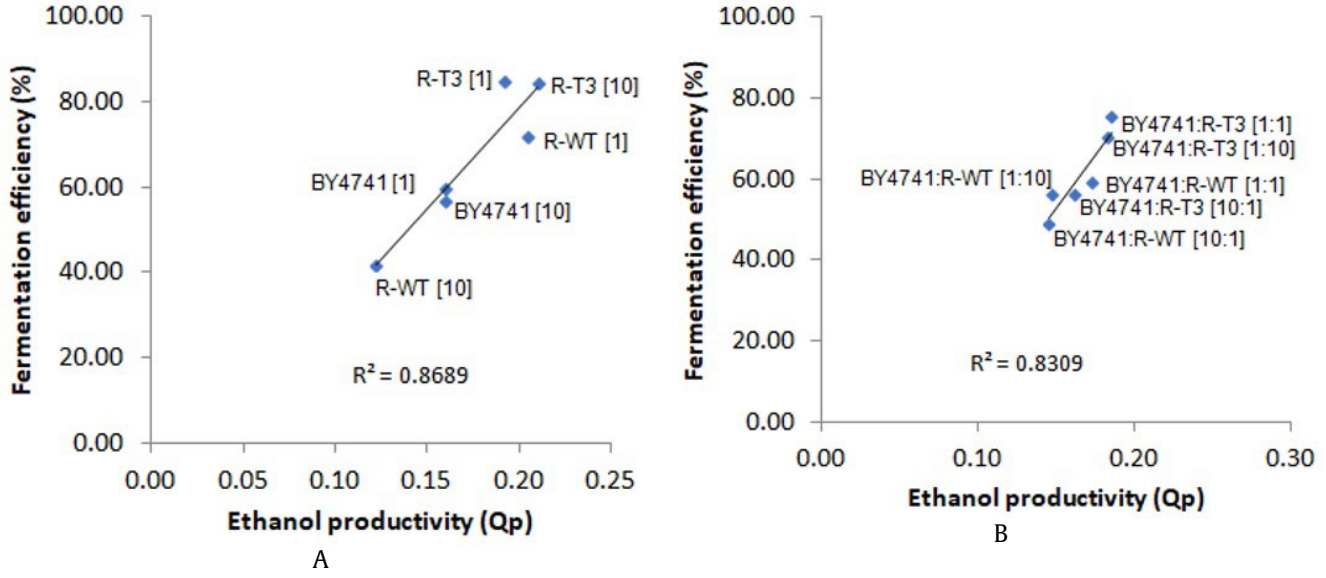

Figure 5. Correlation pattern between ethanol productivity (Qp) and fermentation efficiency (Ey) in (A) monoculture (B) co-culture fermentation. Isolates used and initial inoculum ratio are indicated in the graph

WT and *S.c* BY4741 yet produces the highest ethanol content (10.10 g/L ethanol). This data indicates that *P.ku* R3 has the highest fermentation efficiency (84.36%) and ethanol productivity (0.21 g/L/h), as confirmed in this study (Table 3). Such ethanol production of *P.ku* R3 was markedly higher than previously reported ethanol production from other genera of *Pichia*, such as *P. stipitis* NCIM 3498 (4.52 +/- 0.23 g/L) (Naseeruddin *et al.* 2019), *Pichia stipitis* CBS 5773 (9.6 g/L) (Domínguez *et al.* 2000), *Pichia kudriavzevii* MBY1358 (8.35±0.03 g/L) (Choi *et al.* 2017). In addition, higher ethanol production of *P.ku* R-T3 than *P.ku* R-WT was likely unaffected by lower *TDH2* gene (encoding glyceraldehyde-3-phosphate dehydrogenase) expression levels in the particular mutant strains. As in *S. cerevisiae*, glyceraldehyde-3-phosphate dehydrogenase is encoded by three genes, including *TDH1, TDH2,* and *TDH3* (Linck *et al.* 2014). Thus, potentially redundant *TDHs* genes might occur in yeast *P.ku*. In addition, in other yeast strains, enzyme synthesis by the particular genes appears to be constitutive (McAlister and Holland 1985).

Co-culture of *P.ku* R-T3 isolate with *S.c* BY4741, however, resulted lower ethanol productivity (0.15-0.19 g/L/h) and fermentation efficiency (56.32-75.32%) than monoculture fermentation using *P.ku* R-T3 only (Table 3). It might be that in co-culture fermentation, *S.c* BY4741 mostly used glucose as substrate for growth in early time of incubation, leading to low glucose available for *P.ku* R-T3 strain. Indeed, *S.c* BY4741 took a longer log phase than either *P.ku* R-T3 or *P.ku* R-WT (Figure 3B). Such phenomenon has also been described by former study using laboratory engineered *S. cerevisiae* (Papapetridis *et al.*

2018). On the other hand, the availability of glucose in the medium causes catabolite repression in *P.ku* thus inhibiting its xylose-fermenting activity. A previous study indicated that carbon catabolite repression can limit the industrial application of co-cultures with xylose-fermenting yeasts because ethanol produced from glucose may decrease the yield due to the inhibition of the xylose fermentation by glucose (Chen 2011). Previous study confirmed that xylose-fermenting isolates need different concentrations of substrate which allow xylose consumption. In instance, *P. stipitis* required the glucose concentration in the medium to be below 2% (w/v) before significant xylose utilization occurred. The other xylose utilizing yeasts, such as *Candida steatolytica*, and *C. shehatae* was found to utilize xylose effectively in the presence of approximately 5% and 3% (w/v) glucose respectively (Panchal *et al.* 1988).

In this study, single culture fermentation of isolate *P.ku* R-T3 showed promising ethanol productivity in a fermentation by using mixed substrate of glucose and xylose. However, it is worth noting that the ethanol productivity resulting from co-culture of *P.ku* R-WT or *P.ku* R-T3 with *S.c* BY4741 (0.15-0.19 g/L/h) (Table 3) was found to be comparable to previous study. For instance, co-culture of restricted catabolite repressed mutant *P. stipitis* CCY39501 (P5-200-16) and respiratory deficient mutant *S. cerevisiae* showed Qp value of 0.17 g/L/h (Kordowska-Wiater and Targo ski 2002). The co-culture of *P.ku* R-T3 with *S.c* BY4741 in our study resulted higher ethanol productivity compares to other co-culture of *Zymomonas mobilis* and *Pachysolen tannophilus* (0.60 g/L/h) (Ferreira *et al.* 2018). Thus our data indicate that newly mutant isolate *P.ku* R-T3 potentially be applied for larger scale ethanol fermentation either in monoculture or co-culture with *S. cerevisiae*.

This study shows the potential application of newly engineered mutant strain *P.ku* R-T3 in ethanol production using mixed substrate of glucose and xylose. Although, co-culture of the particular isolate with *S.c* BY4741 did not significantly increase the ethanol productivity and fermentation on efficiency, the promising kinetic parameter of *P.ku* R-T3 ethanol productivity may support its further research and development. Further bioprocess engineering such as continuous fermentation in maintaining more xylose in the medium may potentially be conducted. In addition, restricted catabolite repressed mutant may also be developed via genetic engineering of the *P.ku* strain as a strategy in promoting ethanol production.

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