

# Ethanol Production by Novel Proline Accumulating *Pichia kudriavzevii* Mutants Strains Tolerant to High Temperature and Ethanol Stresses

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## Article history

Received: 7-08-2018

Revised: 1-09-2018

Accepted: 10-09-2018

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**Abstract:** Accumulation of osmoprotectant molecule, proline, has been reported to induce a stress tolerance phenotype in yeast cells. In this study, two ethanologenic isolates of yeast, *Pichia kudriavzevii* (R and T), both capable of using both five and six sugar compounds to produce ethanol, were mutated via ethyl methanesulfonate (EMS) treatment. Proline-accumulating mutant strains were selected by using proline-analogue (*L-azetidine-2-carboxylic acid*). Mutant strains were confirmed to accumulate proline in various level ranging from 11% to 154% compared to the wild type cells. Selected mutant strains were more resistant to high ethanol concentration (15%) and high temperature stress (45°C) compared to their Wild Type (WT) cells and industrial bioethanol yeast, *Saccharomyces cerevisiae*. Interestingly, the fermentation rate of isolate R-T1 and T-T2 was higher than its WT, based on quantitation of reducing sugar and ethanol content in both glucose and mixed glucose-xylose fermentations. The highest ethanol production was shown by strain R-T1 (3.3g/100 mL) that produced 7% and 20% higher ethanol compared to its WT in glucose and glucose-xylose as fermentation substrate, respectively. It is worth noting that ethanol production activity of T-T2 was 22% and 172% higher than its WT and industrial yeast *S. cerevisiae*, respectively. Our study indicates that proline accumulation in yeast *P. kudriavzevii* may promote ethanol production, especially in mixed substrate fermentations.

**Keywords:** *Pichia kudriavzevii*, ethyl Methanesulfonate, AZC-Tolerance, Proline, Sugar Mixture Fermentations

## Introduction

Yeasts that belong to the genera of *Pichia* have been known for their capability of using both hexose and pentose sugars as substrates for bioethanol productions (Mussatto *et al.*, 2012). On the other hand, *S. cerevisiae*, the commonly used industrial yeast, is unable to use pentose as a substrate, thus suggesting superiority of *Pichia* in its application for the 2nd generation of bioethanol productions (Radecka *et al.*, 2015). Microbe used in fermentation is one of the limiting factors for a maximum production of bioethanol. During fermentation, yeasts are exposed to various environmental stresses, including an elevated temperature, osmotic pressure and high ethanol stresses, causing depletion upon ethanol production (Ansanay-Galeote *et al.*, 2001). Thus, for more effective and valuable ethanol production, yeast strain

developments are needed, particularly to construct stress tolerance-mutant strains.

Various approaches have been employed to construct mutant yeast capable of combating fermentation-related stresses. In *S. cerevisiae*, modifications of proline metabolism pathway results in mutant strains with enhanced stress-tolerant phenotypes against high temperature stresses, ethanol, freeze drying, dehydration, high salinity (Sekine *et al.*, 2007; Sasano *et al.*, 2012a). For instance, a proline accumulating mutant strain has been successfully constructed through put1-gene deletion (Takagi *et al.*, 2000). Put1, a proline oxidase enzyme, is responsible for converting proline to pyrroline-5-carboxylate or glutamic-gamma-semialdehyde (Morita *et al.*, 2003). Furthermore, two various amino acid substitutions of  $\gamma$ -glutamyl kinase (PRO1), Asp154Asn and Ile150Thr, significantly

enhance intracellular proline content in *S. cerevisiae*, through desensitization of feedback inhibition of that PRO1 activity (Tatehashi and Takagi, 2013). The amino acid substitution was constructed formerly through random mutagenesis by ethyl methane sulfonate treatment and mutant selection via AZC tolerance assay. The toxicity of AZC can be tolerated through intracellular proline accumulation (Shichiri *et al.*, 2001). Proline has been known for its biological and biotechnological purposes (Sasano *et al.*, 2012b). Indeed, proline plays an important role in maintaining the stability of proteins and membrane structure following stress exposure. In addition, proline has also been reported to inhibit the development of protein aggregate and to reduce reactivity of radical molecules (Takagi *et al.*, 2008).

Development of *Pichia*-mediated bioethanol production has been reported. Both physiological and genetic engineering approaches have been employed to enhance the capability of yeast to ferment sugars. For instance, supplementation of CaCO<sub>3</sub> and CaCl<sub>2</sub> in sugar mixture (glucose and xylose) fermentation could increase ethanol production by *Pichia* for nearly 200% (Okonkwo *et al.*, 2016). Spontaneous mutation of *Pichia stipitis* NRRL Y-7124 has also been employed to construct acetic acid tolerant strains, which is important for fermentation using lignocellulose hydrolysate as substrate (Nigam, 2001). To date, development of *Pichia* yeast strain through modifications of proline metabolism pathway has never been reported yet. Thus in this study we constructed proline-accumulating *Pichia* mutant strains and investigated its phenotype following stress exposures. Two isolates of *P. kudriavzevii* were used in this study. We managed to employ random mutagenesis to construct proline-accumulating strains. Interestingly, the viability of proline accumulating mutant strains under stress exposures was higher compared to that of wild type strains. Further analysis on the ethanol productions showed that mutant strains were able to produce higher ethanol yield in sugar mixture substrate, compared to wild type strains.

## Materials and Methods

### *Strains and Culture Media*

Two isolates of *Pichia kudriavzevii* (isolate R and T) formerly isolated from rotten tropical fruits were routinely maintained in a Yeast Peptone Dextrose medium. Both yeast isolates were used as our former study revealed their capability to ferment glucose and xylose in oxidative-fermentative medium.

### *Random Mutagenesis*

Construction of mutant was performed as described earlier (Takagi *et al.*, 1997). Mutations were induced by

treatment of 4% with ethyl methane sulfonate in each mid-log phase *P. kudriavzevii* culture for five hours. The culture was then serially diluted and the mutagenized cells were then cultured in Synthetic Dextrose (SD) medium (0.17% Yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose and 0.1% allantoin) containing 5 mg/ml proline analogues, L-Azetidine-2-carboxylic acid (AZC, Sigma). After incubation at 30°C for four days, the resulting colonies were picked up and spread to the SD medium with increased AZC concentrations (7mg/ml). The growing colonies were then once again spread in higher AZC treatment (10mg/ml). Mutagenized yeast colonies with a larger diameter than other growing colonies were chosen for further analysis.

### *Proline Content Analysis*

Selected mutant colonies were cultured in Yeast Peptone Dextrose (YPD) medium for 24 h to mid log phase in 5 ml culture. Collected cells were then washed with 0.9% NaCl twice and re-suspended in 0.5 mL distilled water. Cells were then incubated in boiling water for 10 min to extract cells lysate. Cell suspensions were then centrifuged (5 min, 15,000 rpm). The collected supernatants were then concentrated with evaporator to 100 µL. The Intracellular content of the proline was measured by amino acid analyzer (L-8500A-Hitachi, Japan).

### *Stress Tolerance Assay*

This assay was performed by a simple spot assay method in media containing different stress conditions. Both mutant and wild type yeast strains were cultured in a YPD medium and incubated for 24 h at room temperature as the main culture. Serial dilution was then performed. About 3 µL of culture from each dilution was spotted on top of the YPD media containing various ethanol concentrations (5%, 10%, 12% and 15%). For high temperature stress, the YPD medium containing spotted culture was incubated in 37, 40 and 45°C. Plates were incubated for 3 days.

### *Reducing Sugar Content Measurement*

Selected mutant colonies were evaluated for the sugar consumption rate during fermentation. Yeast colonies of both mutant and wild type strains were cultured in a fermentation medium containing different carbon source, 2% glucose and xylose. Reduced sugar content was calculated by using the 3,5-Dinitrosalicylic acid (DNS) method as described earlier (Miller, 1959). Each independent glucose and xylose standard curve was used as standards. For sugar mixture fermentations, a glucose standard curve was used to calculate the reducing sugar content.

### *Quantitation of Ethanol Production*

The fermentation culture of yeast colonies of both mutant and wild type strains was prepared by using a

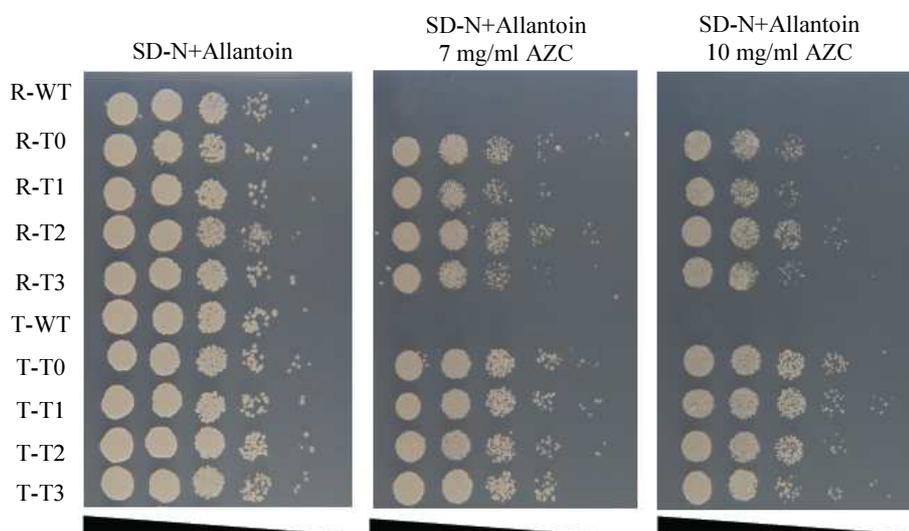
fermentation medium containing either glucose or xylose only and a glucose-xylose mix (50% each). The composition of the fermentation medium, which was YP medium, contained 1% yeast extract and 2% peptone with addition of 20g/L sugar (glucose or xylose). Ethanol content was measured by piknometer following 6, 30 and 48 h of fermentation. Ethanol content was measured using formula as described by Avicor *et al.* (2015).

## Results

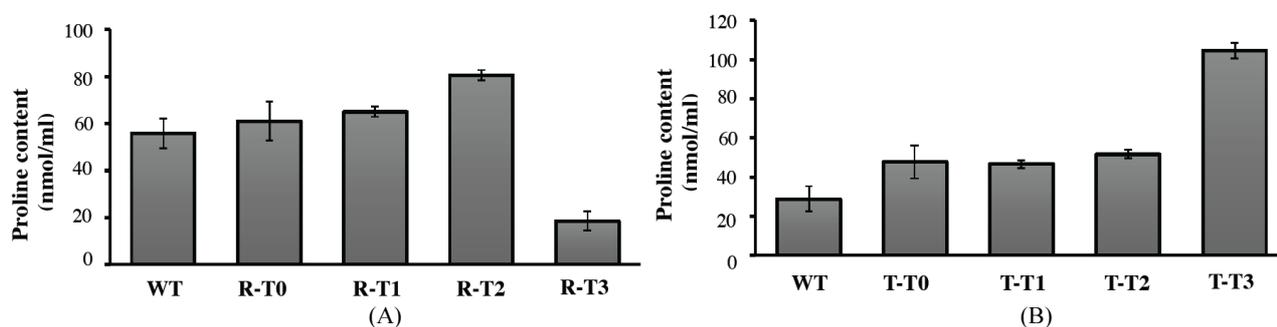
### Construction of Proline Accumulating Mutant Strains

Random mutagenesis through EMS treatment resulted in numerous mutant colonies. However, the only mutants that showed viability better than the wild type cells in SD medium containing 7mg/ml and 10mg/ml

AZC (Fig. 1) were selected to use for further analysis. It is worth noting that wild type strains showed severe growth under AZC-mediated proteotoxic stress. Thus emerging the possibility of proline accumulation in the mutant cells. Interestingly, the intracellular proline content of *P. kudriavzevii* isolate R- derived mutants (R-T0 and R-T1) to its wild type was not significantly different, yet resulted in essentially different phenotype in corresponding to the capability of mutants to grow in AZC stress-medium. A contrarily phenomenon was found in R-T3 which had the lowest proline content of all mutant tested (18.3nmol/ml) (Fig. 2A); however, the viability of this mutant strain in AZC containing medium was higher than its WT (Fig. 1). On the other hand, all of the mutant strains of that isolate T showed higher intracellular proline content compared to their WT strains (Fig. 2B).



**Fig. 1:** Spot test assay of *P. kudriavzevii* mutant and wild type strains in medium containing proline analogue, AZC. RWT: isolate R-wild type; T-WT: isolate T-wild type; T1-T3: EMS-derived mutant strains of each isolate R and T. Colonies were grown for 2 days in 30°C



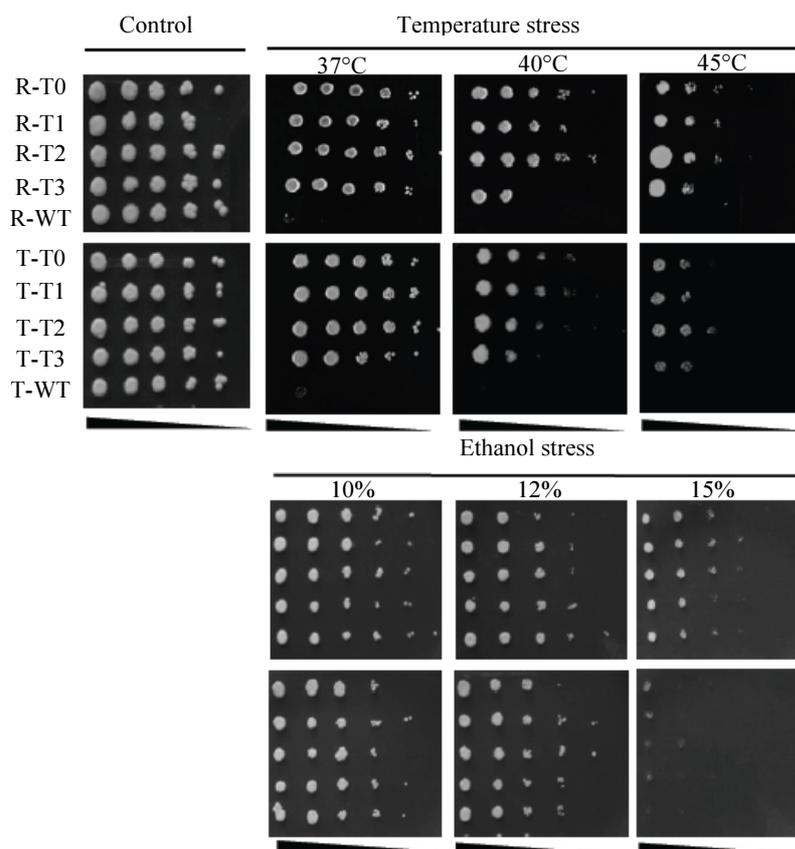
**Fig. 2:** The intracellular proline content of wild type and mutant strains of *P. kudriavzevii* (A) isolate R and (B) isolate T. Each yeast strains were grown in SD medium for 24 h until mid log phase, 5mg/ml AZC was then subjected to the culture for 3 h. Cells were then harvested and extracted to produce cells lysate. Intracellular proline from cell lysate was measured three times by Amino Acid Analyzer

### Stress Tolerance Assays

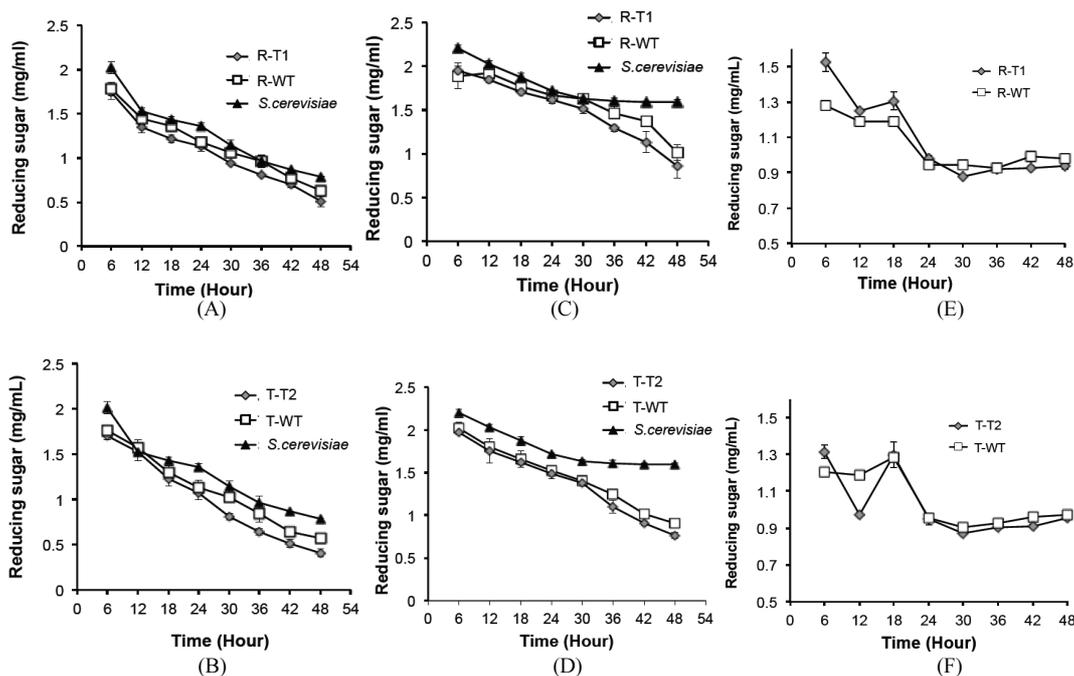
As expected, mutant strains were more tolerant to high temperature stress compared to their wild type strains (Fig. 3). Wild type strains of both isolate R and T exhibited severe growth following high temperature stress conditions (Fig. 3). In high ethanol stress (15%), mutant R-T1 and R-T2 as well as T-T2 showed a higher survival rate than their respective wild type strains. Interestingly, mutant strains that exhibited high temperature stress tolerance were not essentially resistant to ethanol stress treatment, as shown by most of the isolate T-derived mutant strains. These results indicate that the EMS-based random mutagenesis likely mediates the stress tolerance mechanism in *P. kudriavzevii*, essential for combating high temperature stress conditions but does not protect against ethanol stress. Thus, further analysis of high temperature stress tolerance pathways of those mutant strains is needed. Based on the spot test assay, one mutant strain from each wild type isolate which showed stress tolerance phenotype were chosen for further analysis, including RT1 and T-T2.

### Reducing Sugar Measurement

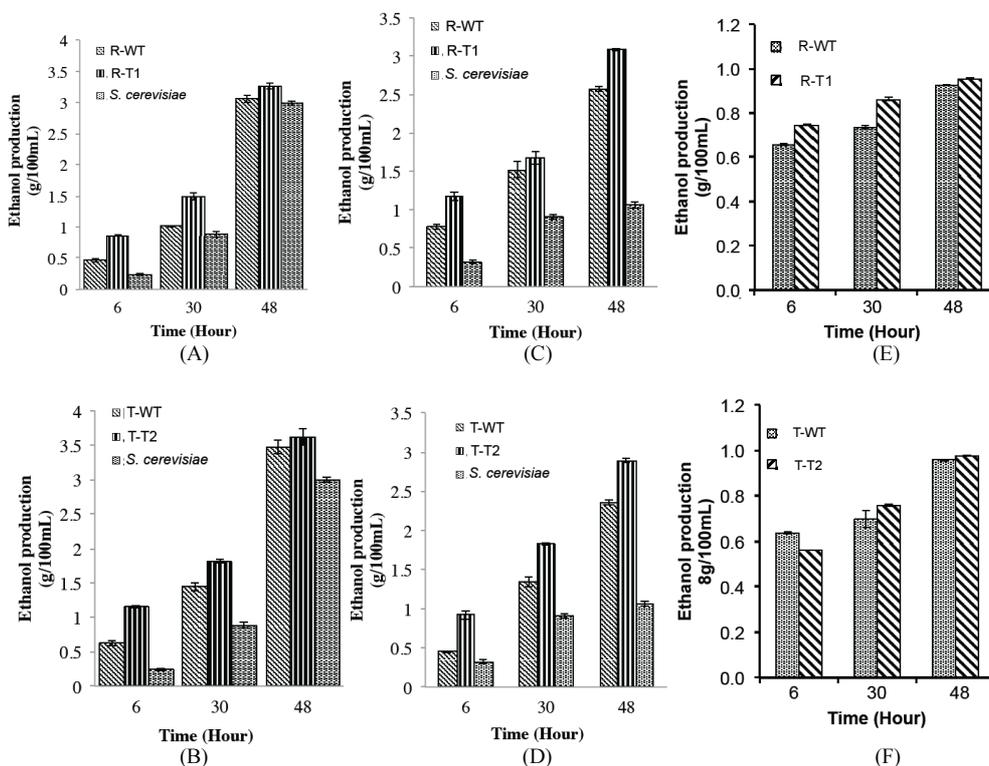
The reducing sugar content was measured in a fermentation culture with glucose, xylose and glucosexylose sugar mixtures as substrate (Fig. 4). Mutant strains R-T1 and T-T2 showed relatively similar patterns in glucose consumption to their wild type strains (Fig. 4A-B). Interestingly, mutant strains were capable of using glucose in a relatively higher rate compared to *S. cerevisiae*, the commonly used industrial yeast (Fig. 4A-D). Both mutant R-T1 and TT2 were capable of effectively using both glucose and xylose within 48 h of sugar mixture fermentation, compared to *S. cerevisiae* (Fig. 4C-D). Indeed, the content of reducing sugar of *S. cerevisiae* culture in the glucose-xylose mixture medium remain stable after 30 h on incubation, while in *P. kudriavzevii* of both WT and mutants strains continued to decrease in a time dependent manner (Fig. 4C-D). For xylose containing medium, the reducing sugar content within 48 h culture remained similar between WT and mutant strains (Fig. 4E-F).



**Fig. 3:** Spot test assay of *P. kudriavzevii* mutant and wild type strains in high temperature stress (37°C, 40°C, 45°C), and ethanol stress conditions (10, 12 and 15% (v/v) ethanol). Incubation at 30°C in YPD medium was used as control. All treatment was incubated for three days. R-WT: isolate R- wild type; T-WT: isolate T-wild type; T1-T3: EMS-derived mutant strains of each isolate R and T



**Fig. 4:** Changes in reducing sugar content during fermentation using various substrate (A-B) glucose, (C-D) sugar mixture glucose:xylose and (E-F) xylose by each mutant R-T1 and T-T2. Reducing sugar content was measured by using DNS method. Wild type strain (R and T) and *S. cerevisiae* were used as control.



**Fig. 5:** Ethanol production by mutant strain R-T1 and T-T2 compared to WT strain and *S. cerevisiae* in different carbon source (A-B) glucose, (C-D) sugar mixture glucose:xylose and (E-F) xylose. Fermentation was conducted for 48 h. Wild type strain and *S. cerevisiae* were used as control

## Ethanol Production

Production of ethanol in the glucose of mutant strain R-T1 and T-T2 slightly increased as compared to corresponding wild types (Fig. 5A-B). For instance, RT1 produced 3.3g/100 mL ethanol which was 7% and 9% higher than its wild type strain and *S. cerevisiae*, respectively (Fig. 5A). On the other hand, mutant T-T2 produced an 11% higher ethanol yield compared to R-T1 mutant after 48 h of the fermentation period and approximately 21% higher ethanol production than *S. cerevisiae* (Fig. 5B). This suggests its strong potential as a fermentation agent among mutant strain tested.

A slight decrease in ethanol production was found in mutant strains when using sugar mixture (xyloseglucose) as a substrate compared to glucose fermentation (Fig. 5C-D). Here, we found that at most 15% lower ethanol production was found in sugar mixture fermentation than glucose fermentation of both mutant strains. However, mutant R-T1 and T-T2 were capable of producing higher ethanol yields than their respective WT strains and *S. cerevisiae* in sugar mixture fermentations (Fig. 5C-D). Indeed, production of ethanol by R-T1 was 20% and 190% higher than its WT strain and *S. cerevisiae*, respectively (Fig. 5C). A relatively similar fermentation rate was also shown by mutant TT2 which was capable of producing 22% and 172% higher ethanol content compared to its WT and *S. cerevisiae* after 48 h of the fermentation period (Fig. 5D). We noticed that production of ethanol by both mutant strains R-T1 and T-2 was not significantly different when using sugar mixture as substrate. It is worth noting that there was 50% depletion of ethanol production by *S. cerevisiae* in sugar mixture fermentation compared to glucose fermentation.

Ethanol production in xylose as the main carbon source resulted a relatively similar value between wild type and mutant strains of *P. kudriavzevii* (Fig. 5E-F). Interestingly, 60% depletion of ethanol production was found in xylose fermentation compared to that of glucose fermentation. This suggests that glucose may likely serve as the preferred carbon source for *P. kudriavzevii*'s mutant and wild type strains in producing ethanol.

## Discussion

During fermentation, yeasts are exposed to various types of fermentation-related stresses, including high temperature, osmotic, oxidative and ethanol stress. Thus, observation and discovery of novel stress tolerance of yeast from potential samples, such as fermented food and beverages as well as agricultural waste management system,s are currently conducted for further application in the bioethanol industry (Steensels and Verstrepen, 2014; Zabed *et al.*, 2017). Modifications upon yeast metabolic pathways to optimize ethanol production is gaining significant interest worldwide (Alper *et al.*,

2006; Ekberg *et al.*, 2013). Various studies regarding genetic and physiological engineering upon yeast isolates as a fermentation agent has been conducted (Zhao and Bai, 2009; Ha *et al.*, 2011; Lam *et al.*, 2014; Harner *et al.*, 2015). Advanced research in omics also provide significant development in yeast engineering to support an efficient ethanol production in various types of substrates (Marks *et al.*, 2008). *Pichia* is one of the major interests of biofuel-related research, since this yeast genera can deliver both hexose and pentose sugar fermentations (Steensels and Verstrepen, 2014). This suggests its potential application in 2nd bioethanol production.

In this study, we objected to modify the proline metabolic pathway of non-conventional yeast *P. kudriavzevii* to enhance stress tolerance phenotype against high temperature and ethanol stresses. Random mutagenesis was applied to construct *Pichia* mutant strains since this genera of yeast has not been widely explored particularly its genetic properties of stress tolerance mechanisms. Thus random modifications upon genetic sequence via EMS was used to construct the targeted mutant instead of target mutagenesis.

Proline accumulating strains of *P. kudriavzevii* was constructed by random mutagenesis. In this study, the proline accumulating mutant strains derived from directed evolution in AZC treatment showed potential characters as fermentation agent. For instance, both mutant R-T1 and T-T2 mutants were able to survive in 45°C and 15% ethanol stresses. Following those potential characters, the ethanol production of mutant in glucose and sugar mixture fermentation was also higher than its wild type and industrial yeast strains, *S. cerevisiae*. This suggests that genetic changes through directed evolution is involved in modulating ethanol synthetic pathway. Previous studies have confirmed that proline may act as a cytoprotective molecule that increases cells' viability during fermentation and significantly increases fermentation yield. Both exogenous and genetic modifications-derived proline accumulation may induce a stress tolerance mechanism in yeast (Takagi *et al.*, 2005; Kaino *et al.*, 2008; Sasano *et al.*, 2012a; 2012b).

Interestingly, mutant strains of *P. kudriavzevii* isolate R and T both showed high AZC-resistant phenotypes against 10mg/ml AZC. It is worth noting that such high AZC-tolerance phenotypes exhibited by yeast *P. kudriavzevii* mutant strains is quite remarkable, since most of the yeast cells could tolerate relatively lower amount of AZC (0.5-2mg/ml). Mutant R-T3 showed distinct character to the other mutant tested, since its intracellular proline content was significantly lower than its WT strains, yet showed an AZC-resistant phenotype. It is likely that an AZC-tolerance mechanism in R-T3 may occur in a proline-independent pathway. A previous study had reported that yeast may remain viable following exposure of AZC-proteotoxic stresses by

inducing ubiquitinating ligase Rsp5 activity (Hiraishi *et al.*, 2006). Mutation of the general amino acid permease encoding gene, GAP1, in *S. cerevisiae* also induced an AZC-tolerant phenotype (Haitani *et al.*, 2009).

It is worth noting that, in sugar mixture fermentations, mutant strains, primarily T-T2, produced lower ethanol concentrations than in glucose fermentations. Such phenomenon had been previously reported as “glucose repression”. Shi *et al.* (2014) also reported that the production of ethanol of mutant *P. stipitis* TJ2-3 was relatively similar to that WT cells in mixture sugar fermentations. The presence of glucose will repress the fermentation of xylose so that when the glucose is completely utilized, ethanol can no longer be produced. However, considering the high capability of mutant R-T1 and T-T2 of producing at most 3g/100ml ethanol, it is suggested that mutant strains were capable of employing their metabolism pathways to efficiently use xylose in the same manner to that of glucose utilization. Indeed, yeast genera of *Pichia* has been reported to possess pentose phosphate pathways, in addition to glycolysis, that serve as the main carbon utilization pathway when yeast cells are cultivated in a pentose sugar medium (Jeffries *et al.*, 2007; Agbogbo and Coward-Kelly, 2008).

In media with xylose as the main carbon source, the efficiency of ethanol production is lower than glucose—containing media. A previous study had reported that a protein transporter responsible for xylose uptake in *Pichia stipitis* (Sut1) had lower transport capacity than that *S. cerevisiae*'s xylose transporter (Gfx1) (Runquist *et al.*, 2010). Thus it is likely that the xylose transport rate may occur in a slow manner that leads to the lower xylose-dependent ethanol production as shown by both mutant and WT strains.

## Conclusion

From our study, it is suggested that the development of a stress tolerant yeast of *P. kudriavzevii* through mutagenesis may enhance yeast performance in ethanol production. Stress tolerant phenotypes of mutant strains *P. kudriavzevii* R-T1 and T-T2 may play an important role in mediating ethanol production in a significantly higher yield than its wild type and conventional industrial yeast, *S. cerevisiae*. The ability of mutants to utilize glucose and xylose suggests its future application in 2nd generation of bioethanol productions.

## Acknowledgement

The authors thank to Prof. Hiroshi Takagi of Nara Institute of Science and Technology, Japan for kindly provide *S. cerevisiae* isolate.

## Funding Information

This project was granted by Penelitian Terapan Unggulan Perguruan Tinggi, The Ministry of Research, Technology and Higher Education [1359/IT3.11/PN/2017] to RIA.

## Author's Contributions

**Rika Indri Astuti:** Had led the project, contributed in the experimental design, data analysis and manuscript writing.

**Sena Alifianti:** Had contributed in laboratory experiments and data analysis.

**Ratu Nabila:** Had contributed in laboratory experiments and data analysis.

**Nisa Rachmanis Mubarik:** Had contributed experimental design and data analysis.

**Anja Meryandini:** Had contributed experimental design and data analysis.

## Ethics

This article is authentic from authors works. The corresponding author ensures that all of the other authors have read and recognized the manuscript.

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