Exogenous L-proline Increases Stress Tolerance of Yeast *Pichia kudriavzevii* Against Inhibitors in Lignocellulose Hydrolysates and Enhances its Ethanol Production

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Abstract. Three ethanologenic yeast isolates (E, R and T) capable to use both pentose and hexose sugars as substrate for ethanol fermentation was isolated. Thus, suggesting its potential for 2nd generation of ethanol production by using lignocellulose hydrolysate. Based on ITS sequence, those three isolates were identified as yeast *Pichia kudriavzevii*. Prior its utilization as fermentation substrate, lignocellulose is subjected to complex hydrolysis reactions, which results certain toxic compounds (furfural and organic acid). Our results indicate that *P. kudriavzevii* isolates were sensitive toward 0.15% furfural and 0.375% acetic acid. Interestingly, supplementation of 1.5% L-proline in the medium significantly enhanced the survival rate of all *P. kudriavzevii* isolates against the particular toxic compounds. Moreover, supplementation of L-proline also enhanced the production of ethanol in glucose as substrate, in a dose dependent manner. Indeed, addition of 1.5% L-proline could increase the ethanol yield of isolate E, R and T for approximately 57%, 53%, and 62%, respectively, as compared to that 0.5% L-proline. The highest ethanol production was exhibited by isolate T (14g/100mL) in 1.5% L-proline treatment. Our data indicate, that L-proline supplementation may potentially be applied to enhance ethanol production by *P. kudriavzevii* isolates, particularly for its future application in lignocellulose-based ethanol productions.

Keywords: stress response, *Pichia kudriavzevii*, proline, lignocellulose inhibitors, bioethanol.

1. Introduction

Bioethanol is one of the most promising energy alternatives for fossil energy. Bioethanol is made biologically by fermentation of sugars derived from variety of sources. The development of bioethanol production plant needs to be accelerated to contribute a solution to overcome the fast growing need of fuel, especially for transportation and industrial sectors. The existing production of bioethanol in Indonesia is mainly from sugar and starch fermentations, commonly known as 1st generation of bioethanol production. However, recently, the employment of 1st generation of bioethanol production is argued, due to its potential conflict, as sugar and starch are common food sources in Indonesia. Thus, substrate alternatives are essential to improve bioethanol productions. The deployment of biomass resources mainly lignocellulose materials (woody biomass) for bioethanol productions is currently projected to overcome the problems caused by the 1st generation of bioethanol production,
widely known as 2\textsuperscript{nd} generation of bioethanol productions. The 2\textsuperscript{nd} generation bioethanol involves lignocellulosic feedstock by its saccharification, followed by microbial fermentation and product recovery [1].

Lignocellulose is a complex polymer containing of three types of polymers cellulose (35-50\%), hemicellulose (20-35\%) and lignin (12-20\%) [2]. Cellulose is composed by monomeric form of glucose, whereas hemicellulose is composed by glucose dan xylose. Prior fermentations, lignocellulose biomass must undergo pretreatment steps, including hydrolysis and saccharifications, which produce certain inhibitor products that may inhibit microbial growth yielding lower ethanol productions. Most of the inhibitors are produced during solubilization and degradation of hemicellulose and lignin. When acid based method is used, application of acid such as H\textsubscript{2}SO\textsubscript{4}, SO\textsubscript{2}, HCl and H\textsubscript{3}PO\textsubscript{4} will release inhibitory compounds such as aliphatic carboxylic acids, phenolic compounds, furans, which commonly occurred during hydrolysis of hemicelluloses to monosaccharides [3]. On the other hand, when mild alkaline (NaOH or NH\textsubscript{3}) is used to remove lignin and a minor part of hemicelluloses, inhibitory compounds including acetic acid, hydroxy acids, dicarboxylic acids, phenolic compounds will be produced. The inhibitory effect of inhibitory compounds to the microbial fermentations may vary. The presence of aliphatic carboxylic acids may inhibit microbial growth due to less efficient production of ATP [4]. In addition, the occurrence of phenolic compounds even at low level may cause significant growth defect to microbial growth [5].

To increase ethanol yield, certain strategy can be employed including the utilization of inhibitory compounds-resistant yeast to conduct the bioconversion of lignocellulose hydrolysate to ethanol. In yeast, stress tolerance mechanisms have been reported to involve amino acid, L-proline [6]. Intracellular L-proline accumulation significantly enhances survival rate of yeast \textit{S. cerevisiae} under certain stresses, including freeze drying, osmotic and high temperature stresses. In this study, we aimed to increase stress tolerance phenotype of non-conventional yeast \textit{Pichia kudriavzevii} under inhibitory compound-derived stress exposure. Yeast \textit{P. kudriavzevii} is gaining significant interest recently, since the capability of these yeast genera to use both pentose and hexose sugar compounds as substrate for ethanol fermentations. Such capability is important for 2\textsuperscript{nd} generation of bioethanol productions which absence in common ethanologenic yeast, \textit{S.cerevisiae}.

\section{2. Materials and methods}

\subsection{2.1. Isolates and medium}
Three selected yeast \textit{Pichia} spp isolates were used in this study. \textit{S. cerevisiae} was used as control for spot test assay. Yeast isolates were routinely maintained in Yeast Peptone Dextrose (YPD) medium with following ingredients 1\% yeast extract, 2\% glucosa, and 2\% bacto peptone. For L-proline supplementation analysis, yeast cells were cultivated in Synthetic Defined (SD) medium containing 0.17\% yeast nitrogen base and 2\% glucose with addition of various L-proline concentrations (0.5, 1, 1.5 mM). SD medium with 20\% glucose content was used as basal fermentation medium with addition of L-proline at various concentrations.

\subsection{2.2. Isolation of yeast}
Yeast colony was isolated by using Yeast Malt Extract Agar (YMEA) from rotten tropical fruit including watermelon, papaya and melon. Isolation was done according to previous study [7].

\subsection{2.3. Yeast identification based on ITS sequence}
Genomic DNA of each selected yeast isolates were isolated by using YeastStar genomic DNA kit according to manufacturer’s protocol. Genomic DNA was further used as template for ITS-gene amplification using specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 5'-TCTTCCGCTTAATTGATATGC-3'. PCR mix solutions were prepared as follows: 100ng/\mu l DNA template, 22.5 \mu l GoTaq \textit{Polymerase} and adjusted to 50 \mu l by nuclease free water. Amplification was done by using PCR machine with following conditions pre-denaturation at 94\degree C for 4 min,
denaturation at 94°C for 30 sec, annealing at 49°C for 30 sec, elongation at 68°C for 1 min, and post PCR at 72°C for 5 min. PCR was done in 35 cycles. Amplicon size was verified by agarose gel electrophoresis. The correct size of DNA amplicon was then purified and subjected to sequencing analysis. DNA sequence was further used for homology analysis using BLASTN approach against GenBank database.

2.4. Spot assays
Spot assay was done to confirm the capability of yeast isolates to confer stress conditions caused by major lignocellulose-derived inhibitory compounds, including furfural and acetic acid. Each yeast isolates were cultivated in YPD medium until mid log phase. Cultures were then adjusted to OD600:1 and further be serially diluted. About 3µl culture from each dilution was then spotted on top of YPD agar medium containing various concentration of furfural (0.05%, 0.1% and 0.15%) and acetic acid (0.125%, 0.25% and 0.375%). Each plate culture was then incubated in room temperature for 2-3 days.

2.5. L-proline supplementation
This assay was conducted to verify the effect of L-proline on stress tolerance phenotype of yeast isolates against lignocellulose-derived inhibitory compounds. Each yeast cells were cultured in SD medium with various L-proline content (0.5%, 1% and 1.5%) until mid log phase. Culture was then serially diluted and was used for spot test assay in YPD agar medium with high furfural (0.15%) and acetic acid (0.5%) stress conditions. Each plate culture was then incubated in room temperature for 2-3 days.

2.6. Ethanol content measurement
Each yeast isolates were cultured in batch fermentation by using SD medium containing 20% glucose with addition of various L-proline content (0.5, 1 and 1.5 mM). Ethanol content was measured by using piknometer at 6, 30, and 48 hours of incubations. Ethanol content was measured using formula as described previously [8].

3. Results

3.1. Isolation of yeast and its identification based on ITS sequence
Three isolates (isolate E, R and T) were used in this study as isolated from rotten tropical fruit. Further, we identified the isolates based on ITS sequence gene analysis. We observed that each of yeast isolates was identified as *P. kudriavzevii* (table 1). However, isolates E and R showed the least identity to the species *P. kudriavzevii* with only 85% and 93% identity. Thus, it is likely that those two isolates are novel yeast species. Indeed, advanced identification strategies are needed to evaluate the identity of these two isolates.

| Isolate | Homology           | Identity/Query cover | E-value | Accesion number  |
|---------|--------------------|----------------------|---------|-----------------|
| E       | *Pichia kudriavzevii* strain M312A | 85%/100% 2e-99 | KP675519.1 |
| R       | *Pichia kudriavzevii* strain Y4 | 93%/99% 0 | JF715195.1 |
| T       | *Pichia kudriavzevii* | 97%/95% 0 | JQ083432.1 |

Table 1. Homology analysis of yeast isolates based on ITS sequence
3.2. Stress tolerance phenotype against inhibitory compounds

All three yeast isolates showed severe growth defect in medium containing 0.15% furfural and 0.375% acetic acid (figure 1). It indicates that 0.15% furfural and 0.375% acetic acid are toxic for yeast *P. kudriavzevii*. Amongst all yeast tested, isolate R showed relatively better growth than that other yeast isolates in furfural-mediated stress conditions. However, all isolates exhibited similar growth phenotype in acetic acid stress conditions (figure 1). It is worth noting that all *P. kudriavzevii* isolates showed higher survival in furfural and acetic acid stress conditions than *S. cerevisiae*.

![Figure 1. Spot test assay of yeast *P. kudriavzevii* isolate E, R, and T and *S. cerevisiae* (S), in furfural and acetic acid-induced stress conditions. Each yeast cells were cultured in YPD medium until mid log phase. Each culture was then serially diluted and was spotted on top of YPD agar medium containing furfural (0.05, 0.1 and 0.15%) and acetic acid (0.125%, 0.25% and 0.375%). Plates were incubated for 3 days at 30°C.](image)

3.3. Effect of L-proline on yeast stress tolerance

To increase yeast cell survival following furfural and acetic acid-induced stress conditions, we treated yeast cells with various L-proline concentration prior stress exposure. Interestingly, addition 1.5% L-proline significantly enhanced the growth of all *P. kudriavzevii* isolates in toxic 0.15% furfural and acetic acid conditions (figure 2). It is worth noting that L-proline increased stress tolerance phenotype of yeast *P. kudriavzevii* against both furfural and acetic acid stress in a dose dependent manner. Addition of L-proline also enhanced cell survival rate of *S. cerevisiae* in furfural stress only, but not in acetic acid stress. Amongst isolates tested, *P. kudriavzevii* R showed markedly an increased growth under toxic 0.15% furfural and 0.3755 acetic acid conditions.
Figure 2. Spot test assay of yeast *P. kudriavzevii* isolate E, R, and T and *S. cerevisiae* (S), in furfural and acetic acid-induced stress conditions. Each yeast cells were cultured in YPD medium until mid log phase. Each culture was then serially diluted and was spotted on top of YPD agar medium containing 0.15% furfural and 0.375% acetic acid (0.125%, 0.125% and 0.375%). Plates were incubated for 3 days at 30°C.

3.4. Ethanol quantitation

L-proline addition significantly enhanced ethanol production of each *P. kudriavzevii* isolates tested, in a dose dependent manner (figure 3, figure 4 and figure 5). Indeed, addition of 1.5% L-proline could increase the ethanol yield of isolate E, R and T for approximately 57% (figure 3), 53% (figure 4), 62% (figure 5) compared to that 0.5% L-proline supplementation after 48 hours of fermentation. On the other hand, *S. cerevisiae* culture with 1.5% L-proline addition showed 37% higher ethanol yield than 0.5% L-proline addition. Suggesting that L-proline play significant role in the fermentation process of those *P. kudriavzevii* isolates than *S. cerevisiae*. Although isolates R showed higher stress tolerance against acetic acid an furfural stresses, nonetheless, isolate T produced the highest ethanol concentration (14.8 g/100mL) amongst yeast isolates within 48 hours of incubation (figure 5), whereas isolate E yielded the lowest ethanol content after 48 hours of fermentation (figure 3). It is worth noting that, production of ethanol by *S. cerevisiae* was lower than all *P. kudriavzevii* isolates.
Figure 3. Effect of L-proline on the production of ethanol of yeast *P. kudriavzevii* isolate E. *S. cerevisiae* was used as control. Fermentation was done in YPD medium containing 20% glucose as substrate for 48 hours. Ethanol content was calculated by using Piknometer. Experiment was done in triplicate.

Figure 4. Effect of L-proline on the production of ethanol of yeast *P. kudriavzevii* isolate R. *S. cerevisiae* was used as control. Fermentation was done in YPD medium containing 20% glucose as substrate for 48 hours. Ethanol content was calculated by using Piknometer. Experiment was done in triplicate.

Figure 5. Effect of L-proline on the production of ethanol of yeast *P. kudriavzevii* isolate T. *S. cerevisiae* was used as control. Fermentation was done in YPD medium containing 20% glucose as substrate for 48 hours. Ethanol content was calculated by using Piknometer. Experiment was done in triplicate.
4. Discussion

Yeast genera of *Pichia* is gaining world wide attention due its physiological properties which enable this yeast to produce ethanol or lactic acid by using pentose compounds as carbon source [9,10]. Such character mediates its advance utilization as fermentation agent in 2nd generation of bioethanol production using lignocellulose biomass which contains nearly 45% of pentoses sugar, which mostly derived from hemicelluloses [11]. Based on ITS sequence, all yeast isolates were identified as *P. kudriavzevii* isolates. Advanced identification methods are needed to distinct the emerged sub-species taxa between these three isolates. Yeast *P. kudriavzevii* is one of the potential ethanologenic yeasts as reported elsewhere [12-14].

As ethanologenic yeast for 2nd generation of bioethanol production, it is very important to employ lignocellulose hydrolysate inhibitors- resistant yeast as fermentation agent. In our study, all *P. kudriavzevii* isolates were more resistant to at least 0.1% furfural and 0.25% acetic acid stress treatment than *S. cerevisiae*. Similar result was formerly reported as *P. anomala* 29X was more resistant to various lignocellulose inhibitors compounds including furfural, benzoic acid, furanic acid and salicylic acid [15]. Other yeast isolates that has been shown to combat furfural (1-2 g/L) and acetic acid (1-2 g/L) stresses are *C. xylanilytica*, *C. tropicalis*, *Cryptococcus laurentii* [16]. Indeed, certain mechanisms might involve in furfural resistant phenotype, including the activity of NADH-dependent oxidoreductases to keep NADH/NADPH redox balance essential for furfural reduction [17].

Interestingly, our study showed that addition of exogenous L-proline significantly enhanced stress tolerant phenotype of all isolates tested to both furfural and acetic acid stresses. Both, furfural and acetic acid may mediate the development of radical molecule that further cause several damages inside yeast cells including the development of protein aggregates and mitochondrial defects [18,19]. L-proline has been reported to involve in stress tolerant mechanisms in yeast. In instance, L-proline is involved in maintaining stability of proteins and membrane structure following stress exposure. In addition, L-proline has also been reported to inhibit the development of protein aggregate and to reduce reactivity of radical molecules [20].

L-proline supplementation induced ethanol production of all isolate tested. Interestingly, production of ethanol by *Pichia* isolates was higher than *S. cerevisiae*. In this experiment, fermentation process was conducted in simple sugar (glucose) not in lignocellulose hydrolysate, thus it is possible that L-proline addition may be utilized by *Pichia* isolates as nitrogen source. Indeed, nitrogen source plays important role in ethanol fermentation. Addition of amino acid L-proline has been reported to induce cell growth of yeast *S.cerevisiae* [21]. To date, our study is the first report in respect to an enhancement of ethanol production via exogenous L-proline supplementation in yeast *P. kudriavzevii*. In yeast *S. cerevisiae*, it has been reported that intracellular accumulation of L-proline may increase stress-tolerant phenotype against high temperature stresses, ethanol, freeze drying, dehydration, high salinity [22,23]. Thus, various strategies have been developed to construct mutant yeast that accumulates intracellular L-proline. In instance, modulation over L-proline synthetetic pathway in yeast including deletion of *PUT1* gene and mutation over *PRO1* gene of genomic DNA of *S. cerevisiae* resulted L-proline accumulation phenotype which further enhance the performance of *S. cerevisiae* as fermentation agent [6, 24]. Indeed, several reports showed that L-proline-accumulating yeast are potential to be applied in various industrial sectors, including baking and alcohol fermentation [23,25]. However, to date, very limited reports available regarding the effect of L-proline towards non-conventional yeasts, such as *Pichia* spp., in mediating fermentation process.

Amongst *Pichia* isolates, the highest ethanol production was showed by isolate T in 1.5% L-proline treatment, for approximately 14g/100mL by using glucose as substrate. The particular result indicates that production of ethanol by isolate T is higher than previous study. In instance, furfural-resistant yeast *P. kudriavzevii* KP34ey, isolated from decomposed dung samples was capable to produce 2.3g/L ethanol from xylose [16]. The fact that isolate T was resistant to furfural and acetic acid stress in addition of L-proline, may suggest this isolate as potential fermentation agent in lignocellulose hydrolysate. However, further analysis regarding ethanol production by this particular isolate by using pentose sugar is needed.
5. Conclusion

Our study indicates that addition of exogenous L-proline man enhance stress tolerance phenotype of yeast *P. kudriavzevii* against lignocellulose inhibitors. Thus suggesting its potential contribution in lignocellulose-based fermentation for ethanol productions. Indeed, our data indicates that isolate T which capable to cope with furfural dan acetic acid stress produced ethanol higher than other isolates, including the common industrial yeast *S. cerevisiae* in glucose as fermentation substrate. It is worth noting that production of ethanol was increased in dose dependent manner of L-proline supplementation. Thus, L-proline supplementation may be one of the potential strategies to increase ethanol production by yeast *P. kudriavzevii* in industrial scale.

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