Growth of thermotolerant *Pichia kudriavzevii* UniMAP 3-1 strain for ethanol production using xylose and glucose at different fermentation temperatures

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**Abstract.** The applications of thermotolerant microorganisms in the production of lignocellulosic bioethanol is the key factor for successful simultaneous saccharification and fermentation process. Thus, this study aimed to isolate a thermotolerant yeast strain that was able to convert both glucose and xylose into ethanol. An analysis based on D1/D2 region of the large-subunit ribosomal DNA identified the isolated strain namely as *Pichia kudriavzevii* UniMAP 3-1. The growth of this newly isolated yeast was tested with fermentation temperature at 30 °C and 40 °C on xylose and glucose. *P. kudriavzevii* UniMAP 3-1 was able to ferment xylose to ethanol at both 30 °C and 40 °C with a yield of 0.013 g/g and 0.019 g/g with concomitant xylitol yield of 0.24 g/g and 0.25 g/g, respectively. Fermentation of glucose to ethanol was also tested at 30 °C and 40 °C and the yields were 0.42 g/g and 0.41 g/g, respectively. The potential of this thermotolerant yeast to be used in high-temperature fermentation in both glucose and xylose are proven in this study.

1. **Introduction**

Hydrolysis of lignocelluloses liberating pentose and hexose sugars can be used in the fermentation of microbes in producing biofuels. Being the second most abundant sugar in lignocellulose, efficient D-xylose utilization is a crucial factor in the conversion of lignocellulose into fuel. Microorganisms that have tolerance to temperature normally serve as ideal workhorses in industrial practices. In lignocellulosic ethanol production, simultaneous saccharification and fermentation (SSF) or consolidated bioprocessing (CBP) has been proposed as alternative processes due to their simplicity. SSF & CPB require less equipments in comparison to the conventional processes. However, the major disadvantages of these processes lie on the requirement to have similar temperature for saccharification and fermentation. Thus, thermotolerant xylose-fermenting yeasts would therefore be highly desirable. Recently, many researchers have attempted to explore highly effective thermotolerant yeasts. Among them is the *Kluyveromycopsis marxianus*. This yeast species has remarkable thermotolerance and possess other advantageous characteristics [1]. The other emerging thermotolerant yeast species is the *Pichia*...
*kudriavzevii*. *P. kudriavzevii* was found to be able to produce ethanol at high temperature as well as to exhibit multi-stress tolerance including acid-, ethanol- and salt-tolerance [2]. The present study aimed at isolating yeasts that were capable of fermenting xylose at high temperature, from wastewater ponds of an ethanol-producing facility. The isolated yeast that was able to utilize both xylose and glucose as carbon sources were characterized and the effects of temperature on growth, sugar consumption and product formation were determined.

2. Materials and methods

2.1 Isolation of thermotolerant xylose-utilizing yeasts
Samples were taken from the wastewater pond of Fermpro Sdn Bhd located at Chuping, Perlis, Malaysia. Samples were enriched in YPX medium (1 % yeast extract, 2 % peptone and 2 % D-xylose, w/v) supplemented with 0.02 % chloramphenicol. The enriched samples were incubated at 40 °C for 3 to 10 days and subsequently spreaded on YPX agar for microbial isolation. Representative yeast colonies were selected, purified, kept on YPX agar at 4 °C and stored in YM broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose and 1.5 % agar, w/v) containing 10 % (w/v) glycerol at −80 °C.

2.2 Characterization of the thermotolerant xylose-utilizing isolate
Morphological characteristics were examined according to Kurtzman et al. [3]. The assimilation test was performed using the yeast identification kit API® 20C Aux (bioMérieux, Marcy l’Etoile, France) according to the manufacturer’s protocol. The kit evaluates the assimilation of 20 carbon sources by the yeast. The test strips were incubated at 30 °C for 48 - 72 h. Potential isolate was identified based on the analysis of the D1/D2 region of their large subunit (LSU) of rDNA sequences. The D1/D2 region was amplified using the primer set LR0R (5’- ACCCGCTGAACCTTAAGC-3’) and LR7 (5’- TACTACCACCAAGATCT-3’) and was sent to Macrogen Inc., Seoul, Korea for sequencing. The sequences obtained were compared with the sequences in the GenBank database (http://www.ncbi.nlm.nih.gov). Each sequence was aligned with related yeast sequences retrieved from GenBank using the multiple alignment program ClustalW available within the Molecular Evolutionary Genetics Analysis X (MEGA X) software [4]. A phylogenetic tree was constructed using the Neighbor-joining method with maximum likelihood correction based on the evolutionary distance. Phylogeny was tested by performing 1,000 bootstrap replication using the MEGA X software. The sequence obtained was deposited into the GenBank.

2.3 Fermentation conditions
Starter culture and inoculum were prepared according to the method described by Inokuma et al. [5]. Fermentation was done in 100 mL of YP medium (10 g/L yeast extract and 20 g/L peptone) supplemented with 40 g/L of D-xylose or 40 g/L of D-glucose as substrate. Ten g/L of wet cells was inoculated into 100 mL of YPX medium and was shaken on a rotary shaker at 150 rpm at 30 °C or 40 °C for 96 h. Three mL samples were extracted at 0, 3, 6, 9, 12, 24, 36, 48, 60, 72 and 96 h interval time. Cell growth was monitored by determination of biomass formation. All experiments were done in triplicates.

2.4 Analysis of substrates and products
The fermentation samples were taken at 0, 3, 6, 9, 12, 24, 36, 48, 60, 72 and 96 h and were centrifuged at 4,000 rpm for 5 minutes. The supernatant was transferred to a new tube and kept for further analysis of sugars, ethanol and xylitol concentrations. The analytes were determined using High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, Japan) with a refractive index detector. Aminex® HPX-87H (BioRad) column was used with oven temperature set to 45°C. The mobile phase used was 5 mM H₂SO₄ with a flow rate of 0.5 mL/min.
3. Results and discussion

3.1 Isolation of xylose-utilizing yeasts

New yeast strains with the ability to utilize xylose as well as other sugars at elevated temperature is highly desirable to be used in the industry. Enrichment cultures derived from the wastewater samples from an ethanol-producing facility were obtained in YPX media supplemented with 0.02% of chloramphenicol following incubation at 40 °C for 3-10 days. After incubation, several well-developed colonies were picked. The purified colonies were tested individually for their xylose as well as glucose-utilizing capabilities and the most efficient isolate was selected for further characterization.

3.2 Characterization of xylose-utilizing isolate

The isolate was characterized morphologically as well as molecularly. The UniMAP 3-1 strain exhibited dry, butyrous and light-cream coloured colonies. Light microscopy revealed that the strain has an elongate shaped with pseudohyphae formation. The D1/D2 region of large subunit (LSU) of this isolated strain was amplified and sent for sequencing. The nucleotide sequence of the D1/D2 region of the 26S rRNA gene of UniMAP 3-1 showed 99% identity of Pichia kudriavzevii or its synonym Issatchenkia orientalis. Based on the phylogenetic tree analysis, the UniMAP 3-1 strain was identified as P. kudriavzevii UniMAP 3-1 since it is in the same branch as P. kudriavzevii SLDY-056 (Figure 1). The partial sequence of D1/D2 region gene of P. kudriavzevii UniMAP 3-1 was submitted to the GenBank with accession number of KX538801.

Sugar assimilation ability was also tested for this strain using yeast identification kit API® 20C Aux (bioMérieux, Marcy l’Étoile, France) and is shown in Table 1. The isolated strain assimilated D-glucose, glycerol and N-acetyl-glucosamine, but not D-xylose. This result was verified by another study done by Kurtzman et al. and Gallardo et al. on other strains of P. kudriavzevii and I. orientalis, which also tested positive for D-glucose, glycerol and N-acetyl-glucosamine, but not D-xylose using API-ID32C kit (BioMerieux, Marcy-L’Étoile, France) as shown in Table 1 [3,6]. However, the genome sequence of P. kudriavzevii M12 has confirmed the existence of complete genes coding for the D-xylose metabolism and there were studies that emphasized the ability of P. kudriavzevii in utilizing D-xylose [7,8].
Table 1. Phenotypic characteristics of UniMAP 3-1, *P. kudriavzevii* and *I. orientalis*

| Characteristics | *P. kudriavzevii*<sup>a</sup> | *I. orientalis*<sup>b</sup> |
|-----------------|-----------------|-----------------|
| Assimilation of: |                  |                  |
| None            | -               | -               |
| D-Glucose       | +               | +               |
| Glycerol        | +               | +               |
| Calcium 2-Keto-Gluconate | - | - |
| L-Arabinose     | -               | -               |
| D-Xylose        | -               | -               |
| Adonitol        | -               | -               |
| Xylitol         | -               | -               |
| D-Galactose     | -               | -               |
| Inositol        | -               | -               |
| D-Sorbitol      | -               | -               |
| Methyl-aD-Glucopyranoside | - | - |
| N-Acetyl-Glucosamine | + | + |
| D-Cellobiose    | -               | -               |
| D-Lactose       | -               | -               |
| D-Maltose       | -               | -               |
| D-Saccharose    | -               | -               |
| D-Trehalose     | -               | -               |
| D-Melezitose    | -               | -               |
| D-Raffinose     | -               | -               |

<sup>a</sup>positive; - negative

<sup>b</sup>Data from Kurtzman et al. [3]

<sup>b</sup>Data from Gallardo et al. [6]

3.3 Growth on glucose and xylose as sole carbon source

In this study, the *P. kudriavzevii* UniMAP 3-1 strain was cultivated in YP medium containing individual sugar of 40 g/L glucose and 40 g/L xylose. Basically, fermentation of *P. kudriavzevii* UniMAP 3-1 was more efficient under the consumption of glucose compared to xylose fermentation. Figure 2(a) depicted the glucose consumption profiles and products formation for *P. kudriavzevii* UniMAP 3-1 at 30 ºC and 40 ºC. In the figure, *P. kudriavzevii* UniMAP 3-1 completely consumed glucose within 6 and 9 h at 30 ºC and 40 ºC, respectively. In comparison, the consumption of xylose by *P. kudriavzevii* UniMAP 3-1 strain was lagging for the first 12 hours of fermentation (Figure 2(b)). Furthermore, the ability of the strain in consuming xylose was reduced by 4.8 % at 40 ºC compared to its ability at 30 ºC.

The growth parameters during exponential growth of the isolate were calculated and tabulated in Table 2. The specific growth rate, $\mu_{\text{max}}$ of *P. kudriavzevii* UniMAP 3-1 in glucose at 30 ºC was more rapid with $\mu_{\text{max}}$ of 0.139 h<sup>-1</sup> compared to the growth at elevated temperature with $\mu_{\text{max}}$ of 0.095 h<sup>-1</sup>. However, the specific growth rate in xylose was higher during elevated temperature with the rate of 0.062 h<sup>-1</sup> compared to 0.039 h<sup>-1</sup> at temperature of 30 ºC.
Throughout experiments the biomass yield, $Y_{x/s}$ in xylose was observed to be higher than the biomass obtained in glucose fermentation (Table 2). It can be explained that xylose was utilized for growth, thus reflected on the amount of biomass obtained. In Crabtree-positive yeasts such as *S. cerevisiae*, low amount of biomass yield is expected when *S. cerevisiae* is cultivated on glucose during batch fermentation [9]. This is due to the repression of the respiratory genes by glucose and an overflow of the carbon flux at the pyruvate level and led to ethanol production instead of biomass formation [10,11].
Table 2. Growth kinetics and fermentation parameters of *P. kudriavzevii* UniMAP 3-1 cultivated in D-xylose and D-glucose at 30°C and 40°C.

| Type of sugar | Temp (°C) | Initial substrate conc (g/L) | Time for max. ethanol (h) | Temp (°C) | Initial substrate conc (g/L) | Time for max. ethanol (h) | Max. ethanol conc. (g/L) | Max. xylitol conc. (g/L) | μmax (h⁻¹) | Yx/s (g/g) | Yx/y/s (g/g) | Ys/s (g/g) |
|---------------|-----------|------------------------------|--------------------------|-----------|------------------------------|--------------------------|--------------------------|--------------------------|-------------|-------------|--------------|------------|
| Glucose      | 30        | 40                           | 6                        | 40        | 40                           | 9                        | 0.21                      | ND                       | 0.139       | 0.194       | NA           | 0.42       |
| Xylose       | 30        | 40                           | 60                       | 40        | 40                           | 60                       | 0.54                      | 2.08                     | 0.062       | 0.552       | 0.24         | 0.019      |

3.4 Production of ethanol and xylitol by *P. kudriavzevii* UniMAP 3-1

As depicted in Figure 2, *P. kudriavzevii* UniMAP 3-1 was able to ferment glucose into ethanol and ferment xylose into mainly xylitol and traces amount of ethanol. During glucose fermentation at 30°C, 16.85 g/L of ethanol was produced and the yield, *Yx/s*, obtained was equivalent to 0.42 g/g. The amount of ethanol produced during fermentation at 40°C was slightly lower with 16.55 g/L ethanol with *Yx/s* of 0.41 g/g. The yield of ethanol obtained was 82.4% and 80.3% of theoretical ethanol yield for 30°C and 40°C, respectively.

On the other hand, fermentation in xylose produced mainly xylitol and traces amount of ethanol. This phenomenon was observed due to the co-factor imbalance that occurs between the first two enzymes involved in the xylose metabolism. In yeast, xylose is first reduced by xylose reductase (XR) into xylitol followed by oxidation of xylitol to xylulose by xylitol dehydrogenase (XDH). The co-factor imbalance occurred due to the preference of XR to NADH co-factor while XDH prefers NAD, thus, resulted in accumulation of xylitol. The yield of xylitol produced at 30°C was 0.24 g/g while xylitol produced at 40°C was 0.25 g/g. The yield was 4% lower at normal temperature compared to higher temperature. Ethanol production was also higher at elevated temperature with concentration of 0.78 g/L compared to 0.54 g/L of ethanol produced at 30°C.

Literature has described many different species of thermotolerant yeasts that were able to convert sugars into ethanol. The most prominent thermotolerant yeast is the *Kluyveromyces marxianus*. This yeast was reported to be able to grow well at temperatures as high as 45-52°C and can ferment sugars efficiently at temperatures between 37-45°C. As reported by Arora et al., two thermotolerant strains identified as *K. marxianus* NIRE-K1 and NIRE-K3 were isolated. Both strains were able to produce ethanol from glucose and xylose at 45°C. In YP medium supplemented with 20 g/L glucose, both *K. marxianus* NIRE-K1 and NIRE-K3 were able to consume sugar completely in 16 h, producing maximum ethanol yields of 0.31 and 0.36 g/g, respectively. In medium supplemented with xylose (20 g/L), ethanol was produced with concomitant xylitol production. *K. marxianus* NIRE-K1 produced maximum ethanol and xylitol concentration of 0.3 g/L and 4.34 g/L, respectively after 24 h of fermentation. Similarly, with the same duration of fermentation, *K. marxianus* NIRE-K3 accumulated 0.08 g/L of ethanol and 0.8 g/L of xylitol [13].

Besides *K. marxianus*, *P. kudriavzevii* also reported to have thermotolerance ability. As mentioned by Koutinas et al., *P. kudriavzevii* KVMP10 was able to produce ethanol from various carbon sources with concentration of 10 g/L each, temperature of 42°C and pH 4.8. The carbon sources were glucose, sucrose, fructose, galactose and xylose, which produced 4.5, 4.9, 5.0, 3.5 and 1.9 g/L of ethanol, respectively [2]. Recently, Choi et al. isolated *P. kudriavzevii* MBY1358 from *munik* (a traditional Korean fermentation starter) that was able to yield 0.44 g/g of ethanol at 44°C, which is 1.14 times higher than the control strain, *P. kudriavzevii* KCTC17763 [14].
4. Conclusion
This study has successfully isolated a new yeast strain from a wastewater pond of an ethanol production facility. Molecular and phylogenetic tree analysis based on the D1/D2 region of the large subunit ribosomal DNA identified the isolated strain as *Pichia kudriavzevii* UniMAP 3-1. The isolate, *P. kudriavzevii* UniMAP 3-1 has demonstrated the ability to ferment both glucose and xylose at both normal and higher temperature. Fermentation of glucose at 30 °C and 40 °C had yielded 0.42 g/g and 0.41 g/g of ethanol, respectively while fermentation of xylose produced mainly xylitol with small amount of ethanol. The ethanol and xylitol yielded from xylose fermentation at 30°C were 0.013 g/g and 0.24 g/g, respectively, while at 40 °C, it yielded 0.019 g/g ethanol and 0.25 g/g xylitol. This thermotolerant *P. kudriavzevii* UniMAP 3-1 strain has the desirable traits to be explored as a potential ethanol or xylitol producer at elevated temperature in the future. Further studies on their physiology using other carbon sources such as lignocellulosic hydrolysates at elevated temperature would be very appealing to assess their biotechnological potential.

5. References
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Acknowledgements
This work was supported by the Fundamental Research Grant Scheme (FRGS/1/2014/SG05/UNIMAP/02/2), Ministry of Higher Education, Malaysia. One of the authors, K.H.A.R would like to acknowledge the Ministry of Higher Education for rewarding the scholarship for her PhD studies under the Skim Latihan Akademik IPTA (SLAI).