Furfural and 5-(Hydroxymethyl) furfural Tolerance *Candida* strains in Bioethanol Fermentation

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Abstract

The toxic fermentation inhibitors in lignocellulosic hydrolysates pose significant problems for the production of second-generation biofuels and biochemicals. Among these inhibitors, 5-(hydroxymethyl)furfural (HMF) and furfural are specifically well known. This study investigated the furfural and 5-HMF tolerance in *Candida* strains, which could be used for the development of advanced generation bioethanol processes. The 10 isolates of *Candida* were selected based on the cell growth and bioethanol production on YPD medium containing several concentrations of furfural and 5-HMF by using spectrophotometer and HPLC. *Candida parapsilosis* Y80 could grow and produce bioethanol in the medium that contains furfural and 5-HMF with a concentration of 60 mM and 40 mM, respectively. Based on the results, *C. parapsilosis* Y80 has potential activity in the development of bioethanol fermentation.

Keywords: Furfural, 5-Hydroxymethylfurfural, *Candida*, bioethanol

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Introduction

The utilization of raw materials from lignocellulosic biomass for bioethanol production requires a pre-treatment process (Ahmad *et al*., 2017; Chen *et al*., 2017; Kumar & Sharma 2017; Baruah *et al*., 2018). This step-change the biomass polymer structure to sugar monomer and enhanced the efficiency of hydrolysis by enzymes (Satari *et al*., 2019). Lignocellulose pretreatments produced various small compounds, including furan derivates, organic acids, and phenolic compounds (Jonsson *et al*., 2016; Sarks *et al*., 2018). These compounds include salt (Zacky *et al*., 2017), weak acids (acetic, formic, levulinic, coumaric, etc.), furyl compounds (furfural or hydroxymethylfurfural (HMF)) or phenolic compounds (vanillin or vanillic acid) (Zhang *et al*., 2013; Steinbach *et al*., 2017). The hydrothermal method (130 ^{o}C or above) generates between 6 – 90 mM acetic acid depending on the temperature chosen with similar concurrent increases in formic acid, furfural, and HMF (Greetham *et al*., 2014). The presence of inhibitory compounds has been shown to reduce fermentation efficiencies, for example, the presence of 0.5 mM furfural has been reported to reduce ethanol production and at 4 mM (Greetham *et al*., 2014). The presence of 20 mM acetic acid has been shown to induce ethanol production, however, higher concentrations can inhibit ethanol production. These compounds are strong inhibitors for enzymes and fermenting microorganisms. Furfural and 5-(hydroxymethyl)furfural (5-HMF) are considered as the major inhibitors because both are high in concentration and strong in inhibition strength to ethanol fermenting strains (Iwaki *et al*., 2013; Jöansson & Martin, 2014; Kahar *et al*., 2017). They are formed as hexose and pentose dehydrated products, respectively, and have been shown to inhibit cell growth, decrease ethanol productivity, induce DNA damage and inhibit some enzymes in glycolysis, thus posing serious challenges to the feasibility of ethanol production of lignocellulose (Modig *et al*., 2002; Allen *et al*., 2010).

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The development of stress-tolerant ethanologenic yeast is one of the significant challenges for cost-competitive bioethanol production (Kahar *et al.,* 2017). Many yeasts were reported could grow on the medium containing inhibitor by adaptation (Heer and Sauer, 2008; Liu *et al.,* 2004; Parawira and Tekere, 2011). Some genetic traits also proposed for the improvement of the yeast strain mainly through gene overexpression in the laboratory (Klein *et al.,* 1999). However, most of the studies investigated the strains tolerance in the medium containing single inhibitor or simple mixtures of furfural, 5-HMF and acetic acid (Gorsich *et al*., 2006; Park *et al*., 2011).

In the bioethanol industry, yeast strains play an important role in fermentation. Stronger bioethanol-producing yeasts are needed to overcome these process constraints and hence, there is a need to increase understanding of physiological responses to HMF and furfural to increase strain resistance. Some reports also showed that this strain was tolerant to furfural and 5-HMF (Iwaki *et al*., 2013; Ask *et al*., 2013; Ohta *et al*., 2016; Narayanan *et al*., 2017; Lia *et al*., 2017; Unrean *et al*., 2018; Mertensa *et al*., 2018).

However, the inhibitors-tolerant and bioethanol-producing non-*Saccharomyces* yeast strains was reported to be relatively less. For this reason, it is necessary to obtain information regarding non-*Saccharomyces* inhibitors tolerant strains for bioethanol fermentation. The purposes of this study were to screen furfural and 5-HMF tolerant *Candida* strains and to characterize the degradation process of these compounds.

Materials and Methods

Yeast strain and medium

In this study, we used 10 strains of the genus *Candida* yeast from 7 different species. The *Candida* yeasts were obtained from Indonesia Culture Collection (InaCC), Indonesian Institute of Sciences (LIPI).

Medium

For routine subculturing and maintenance, yeast strains were grown on Yeast Peptone Glucose (YPD) agar containing 10 g/L yeast extract (Difco), 20 g/L bacto peptone (Difco), 20 g/L glucose (Merck), and 15 g/L agar (Difco). For pre-culture, one single colony of strain was inoculated from the YPD plate into 12 mL YPD liquid medium in 100 mL Erlenmeyer flask. The culture was incubated overnight at 30 ^{o}C and 150 rpm.

Yeasts Screening in various concentrations of furfural and 5-HMF

The ten strains from the *Candida* genus were screened based on cell growth and bioethanol production in various concentrations of furfural and 5-HMF. The yeast strains were cultured in YPD liquid medium containing glucose 20 g/L to obtain the cell biomass as a pre-culture. The fermentation was conducted at 400 rpm with 30 ^{o}C overnight in deep well. The pre-culture of biomass yeasts were cultured in Yeast Nitrogen Base (YNB) without amino Acids and Ammonium Sulfate (Difco) medium including various concentration furfural (Nacalai Tesque, Japan) (0, 20, 40, 60, 80, and 100 mM) and 5-HMF (Nacalai Tesque, Japan) (0, 20, 40, 60, 80, and 100 mM)**.** The fermentation was conducted at 1400 rpm with 30 ^{o}C for 24 h in deep well. All experiments were performed in triplicate.

Fermentation of InaCCY80 strain in medium containing furfural and 5-HMF

InaCCY80 strain was selected as tolerant yeast in this study based on growth and bioethanol production in various medium containing furfural and 5-HMF. The purpose of this step was to obtain the information of InaCCY80 fermentation in medium containing furfural or 5-HMF. The yeast strain was cultured in YPD medium to obtain cell biomass as a pre-culture. The fermentation was conducted at 150 rpm with 30 ^{o}C for overnight in the flask. The main culture of InaCCY80 biomass was cultured in YNB medium containing glucose 20 g/L and 40 mM furfural, and glucose 20 g/L including 20 mM 5-HMF. The extended lag phase corresponded to the increase in the concentration of furfural, and the cells lost their survival ability in the medium containing 60 and 80 mM (Fig. 1A). According to these results, we decided to use the YNB medium containing 40 mM furfural for further analysis, because the InaCCY80 strain could grow and ferment the sugars under such stress conditions. The fermentations were conducted at 150 rpm with 30 ^{o}C for 30 h in

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the flask. All experiments were performed in triplicate.

Effect of furfural and 5-HMF mixtures on Fermentation of InaCCY80 strain

The purpose of this step is to get knowledge regarding the effect of cell growth and bioethanol production upon the stress challenge in the presence of furfural and 5-HMF mixtures. The previous results showed that the InaCCY80 strain was able to grow and produce ethanol in the medium containing furfural and 5-HMF at certain concentrations. However, the cell strain growth was carried out in a medium containing furfural and 5-HMF separately. For this reason, it was necessary to test the growth of the InaCCY80 strain in a medium containing the mixture of furfural and 5-HMF.

InaCCY80 yeast strain were grown in the YNB medium as a control, and the YNB medium containing a mixture of furfural and 5-HMF in different concentrations (Table 1). YNBi-1 medium contained furfural and 5-HMF with fewer concentration than in the YNBi-2 medium. InaCCY80 strain was cultured in the YPD medium to obtained cell biomass as a pre-culture. The fermentation was conducted overnight at 150 rpm with 30 ^{o}C in the flask. The pre-culture of InaCCY80 biomass was cultured in YNB medium containing glucose 50 g/L, furfural, and 5-HMF mixture with the variation of concentrations based on the previous results (Table 1). The fermentation was conducted at 150 rpm with 30 ^{o}C for 30 h in the flask. All experiments were performed in triplicate.

Table 1. Composition of furfural and 5-HMF compounds in different synthetic media

Medium	Furfural (mM)	5-HMF (mM)
YNB (control)	0	0
YNBi-1	12	2
YNBi-2	30	5

Fermentation product analysis

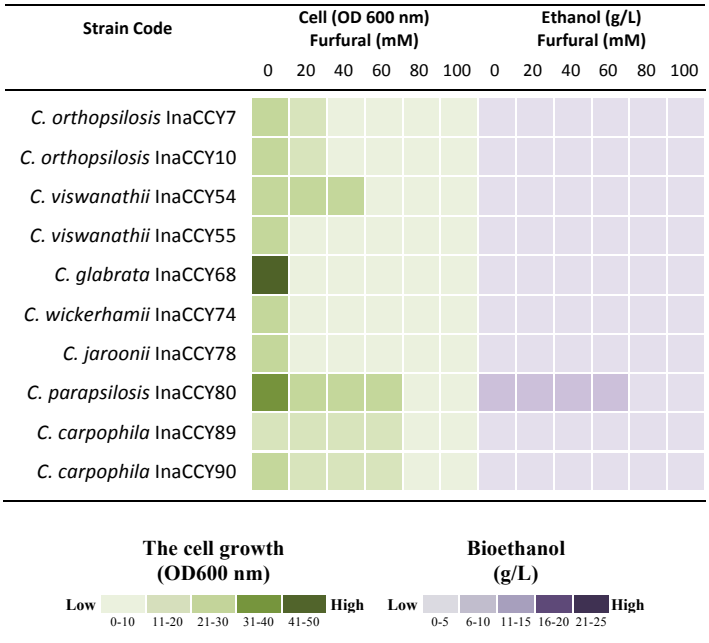
The culture cell growth was measured using the dry cell weight. Cells were freeze-dried prior to weighing using freeze dryer Alpha 1-2 LD plus (Germany). The fermentation products (glucose consumption and ethanol production) were analyzed using High-Performance Liquid Chromatography (HPLC) (Shimadzu LC-20AB, Japan). The analytes were detected using refractive index detector (RID) and Aminex HPX-87H column from Bio-Rad as a stationary phase, with the condition as follows, the flow rate of 0.6 mL/min, oven temperature for the column of 60 ºC, injection volume of 20µL, elution time of 30 minutes, and eluent of 5 mM H_2SO_4. The calculation of ethanol yield referred to the Nutawan method (Nutawan *et al*. 2010).

Results

Screening of BTCC yeasts in furfural and 5-HMF

The growth of ten strains in YNB medium containing furfural showed that the isolates were able to grow at various concentrations (Table 2). In general, this *Candida* strain produced bioethanol with a concentration of 0-5 g/L. However, only the strain InaCCY80 produced bioethanol in furfural to a concentration of 60 mM. The volume of bioethanol produced ranged from 6-10 g/L.

Table 2. The cell growth and ethanol production of several yeast strains after incubation in medium YNB containing furfural with different concentrations



The growth of ten strains in YNB medium containing 5-HMF showed that the isolates were able to grow at various concentrations

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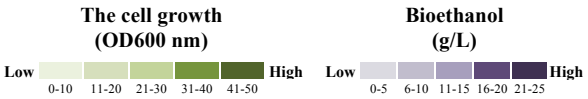
(Table 3). In general, this *Candida* strain produced bioethanol with concentration of 0-5 g/L. However, strain InaCCY80, InaCCY89, and InaCC90 still produced bioethanol in medium with the 5-HMF concentration up to 20 mM. InaCCY80, in particular, could produce bioethanol at 40 mM, ranged from 6-10 g/L.

Table 3. The cell growth and ethanol production of several yeast strains after incubation in medium YNB containing 5-HMF with different concentrations

Strain Code	Cell (OD 600 nm) 5-HMF (mM)				Ethanol (g/L) 5-HMF (mM)			
	0	20	40	60	0	20	40	60
C. orthopsilosis InaCCY7								
C. orthopsilosis InaCCY10								
C. viswanathii InaCCY54								
C. viswanathii InaCCY55								
C. glabrata InaCCY68								
C. wickerhamii InaCCY74								
C. jaroonii InaCCY78								
C. parapsilosis InaCCY80								
C. carpophila InaCCY89								
C. carpophila InaCCY90								



InaCCY80 strain was grown in YNB medium containing furfural in different concentrations (0, 40, 60, and 80 mM), or 5-HMF (0, 40, and 60 mM) to investigate the inhibitors effect on its growth and fermentation profiles.

Metabolites profile of *C. parapsilosis* InaCCY80 in furfural

The strain grown in YNB medium without furfural (0 mM) was used as control. The growth was stopped as the cells deceased. Cell survival was observed in the medium containing a comparatively low amount of furfural (60 and 80 mM) with a shorter lag phase compared to that in the full strength YNB medium.

Fig. 1B shows that glucose consumption was delayed as the furfural concentration increased. The rate of glucose consumption varied depending on the level of furfural in the medium. As shown in Fig. 1B, a higher concentration of furfural prolonged the lag phase during the fermentation process. On the medium containing 40 mM, the strain readily consumed the glucose when the fermentation was started. The glucose was rapidly used just after 9 h, and the remaining 7 g/L was consumed at 30 h. In the medium YNB containing 60 and 80 mM, the strain consumed the glucose around 4-6 g/L.

On the medium containing 40 mM, the lag phase was up to 9 h, and then the strain started to consume the glucose for cell growth, and after 24 hours, the remaining glucose was 7 g/L. On the medium YNB containing 60 and 80 mM, the strain grew lower than 40 mM furfural, and started to utilize glucose at 9 h, and assimilated the glucose of 13-17 g/L at 30 h. The ethanol production was growth-dependent as it increased just after the glucose consumption started. InaCCY80 strain could produce ethanol up to 3.8 g/L in the YNB medium containing 40 mM furfural. In another result, 0.8 and 1.6 g/L ethanol was produced at 60 and 80 mM of furfural (Fig. 1C).

From the profiles of cell growth and bioethanol production, the cells survived due to the succesful detoxification of 40 and 60 mM furfural (Fig. 1D). The concentration of furfural declined during the fermentation. In the YNB medium containing 60 and 80 mM furfural, the cells decreased as a result of incapability for survival, as shown from the inhibitor profiles that the detoxification of furfural failed, where furfural remained 72 and 92% of initial concentration at the end of incubation (Fig. 1D).

Metabolites profile of *C. parapsilosis* InaCCY80 in 5-HMF

The cell growth of the InaCCY80 strain in a medium containing 5-HMF was lower than in a medium without 5-HMF (Figure 2B). The lag phase of the InaCCY80 cell occurred at the 9 hour, but the growth toward the stationary phase was relatively low in the YNB medium containing 40 and 60 mM 5-HMF. Figure 2B shows that glucose consumption by the InaCCY80 strain containing 40 and 60 mM 5-HMF was lower than in the YNB medium without the presence of this compound. In a medium without 5-HMF, glucose was consumed by the InaCCY80 strain until it was remained at the 30 hour after fermentation. However, in a medium containing 5-HMF, at the 30 hour after incubation glucose remained

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up to 63.5% and 86% respectively at concentrations of 40 and 60 5-HMF.

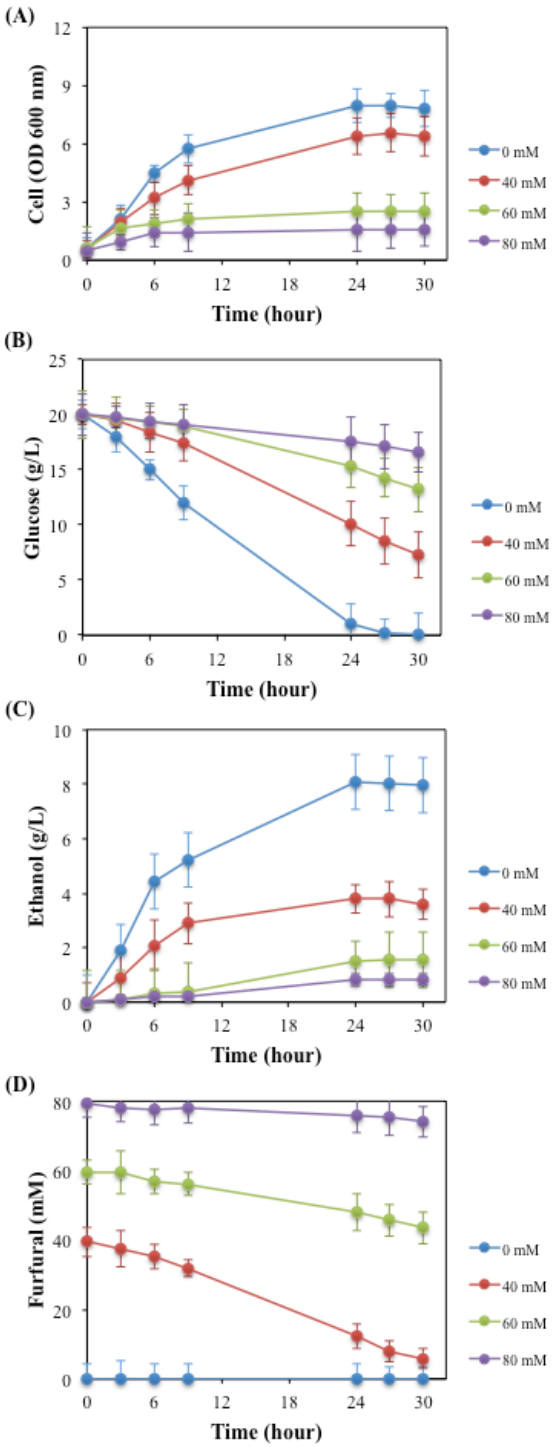

Figure 1. Metabolites profile (A) cell growth, (B) glucose consumption, (C) ethanol, and (D) furfural degradation of *C. parapsilosis* InaCCY80 in medium containing furfural.

Glucose consumption in medium containing 5-HMF had an impact on ethanol production by the InaCCY80 strain. In the medium conditions without 5-HMF, the InaCCY80 strain was capable to produce about 6 g/L ethanol. However, ethanol was produced around 2.6 and 1.2 g/L in the medium containing 40 and 60 mM 5-HMF (Figure 2C).

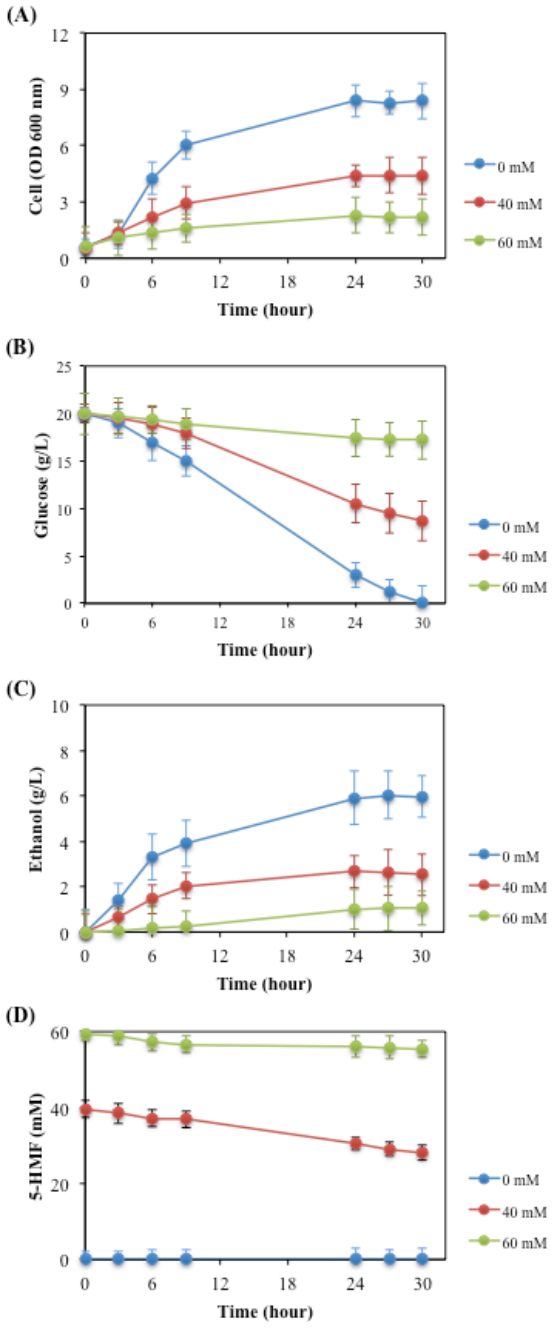

Figure 2. Metabolites profile (A) cell growth, (B) glucose consumption, (C) ethanol, and (D) 5-HMF degradation of *C. parapsilosis* InaCCY80 in medium containing 5-HMF.

Figure 2D shows that the degradation of 5-HMF by InaCC Y80 strain was relatively low

at a concentration of 40 mM, which was about 30% of the initial concentration. Even at the concentration of 60 mM, about 5% of 5-HMF was degraded. The higher the 5-HMF concentration in the medium, the lower its degradation ability by InaCCY80 strain.

Effect of Furfural and 5-HMF mixture *to C. parapsilosis* InaCCY80

Figure 3B shows the growth of the InaCCY80 strain in the YNBi-1 medium. In this medium, the InaCCY80 strain was able to grow, although it was relatively lower than its growth in the YNB medium without the presence of furfural and 5-HMF. This yeast strain consumed all glucose after 24 hours fermentation. However, the speed of consumption was relatively slower than in the medium without the two inhibitor compounds (YNB medium). Likewise, its ability to produce bioethanol was relatively lower when compared to medium without a mixture of furfural and 5-HMF. In this medium, bioethanol was produced around 14 g/L, while in medium without furfural and 5-HMF bioethanol was produced around 17 g/L. As for the furfural and 5-HMF compounds, they were consumed after 24-h incubation. These results indicated that the InaCCY80 strain was able to carry out its metabolism well in the presence of a mixture of 12 mM furfural compound and 2 mM 5-HMF.

Different results were shown when the InaCCY80 strain was grown in YNBi-2 media. The YNBi-2 medium contained 30 mM furfural and 5 mM 5-HMF. The content of inhibitor compounds in the YNBi-2 medium is higher than YNBi-1. Figure 3C shows that the InaCCY80 strain was able to grow relatively low compared to YNB and YNBi-1. This strain was only able to consume glucose by 31% after 24 hours of incubation. After that, glucose was relatively unconsumed by the InaCCY80 strain. This shows that the InaCCY80 strain in YNBi-2 was inhibited by a mixture of 30 mM furfural and 5 mM 5-HMF. This condition was followed by a decrease in bioethanol production compared to when the InaCCY80 strain was grown in the YNBi-1 medium. In the YNBi-2 medium, the highest bioethanol production was obtained at 8 g/L at 24 hours after incubation. This result was lower than when the InaCCY80 strain was grown in the YNB and YNBi-1 medium (Figures 3A and 3B). Furfural and 5-HMF degradation by InaCCY80 strains ranged from 20 to 40%. This shows that the higher furfural and 5-HMF concentrations, the metabolism of the InaCCY80 strain was more inhibited.

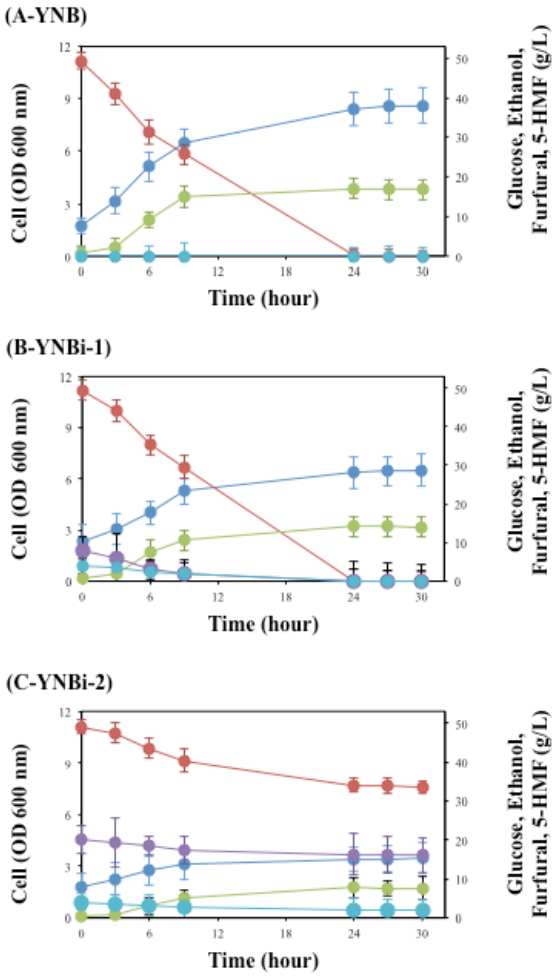

Figure 3. Metabolites change of *C. parapsilosis* InaCCY80 in medium containing 5-HMF. Cell growth (●); glucose (●); bioethanol (●); furfural (●) ;5-HMF (●).

Discussion

Screening of inhibitor-tolerant yeast was performed individually and then tested in furfural and 5-HMF mixtures. The aim of this strategy is to investigate the effects of individual or mixtures of furfural and 5-HMF to the cell growth and ethanol production. Ten strains of the genus *Candida* were tested for the tolerance in medium containing furfural and 5-HMF as inhibitors. In general, the *Candida* strains were able to grow and produced bioethanol with varying

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concentrations of furfural and 5-HMF. Yeast of *C. parapsillosis* InaCCY80 was selected as a potential strain. This strain has the capability to produce bioethanol at a relatively high concentrations of furfural and 5-HMF than other *Candida* strains tested.

Pandey *et al*. (2019) reported that *Candida* strain is one of the yeast groups that can tolerate inhibitor compounds besides *Saccharomyces*, *Kluyveromyces*, *Candida*, *Scheffersomyces*, *Ogatea* and *Wickerhamomyces*. In this study *C. parapsillosis* was more tolerant in furfural and 5-HMF than the other *Candida* strains.

Furfural is one of the main inhibitors of hemicellulose hydrolysate. This compound has the capability to inhibit the growth of microorganism cells (Taherzadeh *et al*., 2006) and enzyme activity in the glycolytic pathway (Jönsson *et al*., 2013). On the other hand, furfural can increase the toxic effect of acetic acid and phenols in furfural toward yeast (Almarsdottir *et al*., 2012). In *Saccharomyces cerevisiae*, furfural leads to the accumulation of ROS and cell organelle damage (Allen *et al*., 2013). Wang *et al*. (2013) also reported that *C. tropicalis* cell growth and fermentation was inhibited by the presence of furfural.

InaCC80 strain produced ethanol in medium containing furfural and 5-HMF. However, ethanol production in medium containing furfural was higher than in 5-HMF. This means that the InaCC80 strain is more tolerant of furfural compounds than 5-HMF. This is interesting because some strains are more intolerant to furfural compared to 5-HMF. The furfural and 5-HMF are known to cause specific biological effects. Furfural induces the accumulation of reactive oxygen species (ROS) in *S. cerevisiae* to cause cellular damage includes damage to mitochondria and vacuole membranes, the actin cytoskeleton and nuclear chromatin (Allen *et al*., 2010) also causes DNA damage, induce DNA mutations in many organisms, reduces growth rates, cell budding, ethanol, and biochemical enzyme activity (Palmqvist and Hahn-Hägerdal, 2000; Modig *et al*., 2002). Moreover, it affects energy metabolism while changing the TCA and glycolytic fluxes (Horvath *et al*., 2003). 5-HMF is less toxic than furfural, probably because it is hard to penetrate the yeast cell membrane even though in-vivo degradation. Interestingly, we found that the InaCCY80 strain appears capable of detoxifying 5-HMF as its level decreased during the fermentation. This fact indicated that 5-HMF could penetrate the membrane cell of the strain. This inhibitor was completely degraded after 24-h fermentation in the medium 0.2YNBi +0.8YNB. The 5-HMF directly inhibits the activities of key enzymes such as aldehyde dehydrogenase, alcohol dehydrogenase, and pyruvate dehydrogenase (Modig *et al*., 2002). It also generates reactive oxygen species (ROS) and induces cell apoptosis, as well as furfural.

Furfural induces the accumulation of ROS in *S. cerevisiae* to cause cellular damage includes damage to mitochondria and vacuole membranes, the actin cytoskeleton, and nuclear chromatin (Allen *et al*., 2010) also causes DNA damage, induce DNA mutations in many organisms. The inhibitory effect on various microorganisms including budding yeasts (Almeida *et al*., 2007; Delgenes *et al*., 1996) and bacteria (Zaldivar *et al*., 2000) such as reducing fermentation rate and/or stop growing and enter an extended lag phase (Branberg *et al*., 2004; Heer and Sauer, 2008). For some tolerance *S. cerevisiae* yeasts, they could convert furfural and 5-HMF to their less reactive alcohol derivatives using NAD(P)H-dependent reduction reactions mostly during the lag phase (Almeida *et al*., 2007).

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